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Increase of tyrosine hydroxylase levels and activity during morphine withdrawal in the heart

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Abstract

Our previous studies have shown an enhanced activity of the noradrenergic pathways innervating the heart in rats withdrawn from morphine. However, the possible adaptive changes that can occur in these pathways during morphine dependence are not known. We studied the alterations in tyrosine hydroxylase (the rate-limiting enzyme in catecholamines biosynthesis) and tyrosine hydroxylase activity in the heart (right and left ventricle) during morphine withdrawal. In the same paradigm, we measured Fos expression as a marker of neuronal activation and the normetanephrine/noradrenaline ratio (an index of noradrenaline turnover). We evaluated the levels of tyrosine hydroxylase and Fos by quantitative Western blot analysis, and noradrenaline turnover using high-performance liquid chromatography (HPLC). Dependence on morphine was induced by a 7-day s.c. implantation of morphine pellets. Morphine withdrawal was precipitated on day 8 by an injection of naloxone (5 mg/kg s.c.). The results show a significant increase in tyrosine hydroxylase levels and activity in the right and left ventricle 30 or 90 min after naloxone precipitated withdrawal in parallel with an increase in noradrenaline turnover. Morphine withdrawal also induced an increase in the Fos expression, which indicates an activation of cardiac cellular activity. Our results suggest that an increase in tyrosine hydroxylase protein levels and tyrosine hydroxylase enzyme activity might contribute to the enhanced noradrenergic activity in the heart in response to morphine withdrawal.

Keywords: Fos; Morphine withdrawal; Heart; Tyrosine hydroxylase; Western blot

1. Introduction

The repeated use of opioid induces adaptive changes in the central and peripheral nervous system leading to the development of tolerance and dependence. The effects of opioid and the physical aspects of withdrawal have been associated with changes in noradrenergic transmission in the brain (Nestler, 1992; Maldonado, 1997; Milanés et al., 1998; Fuertes et al., 2000). In addition, previous studies from our laboratory demonstrated that morphine withdrawal increases the turnover of noradrenaline in the heart (Milanés et al., 2000; Rabadán et al., 1998), which suggests that catecholaminergic pathways are involved in the hyperactivity of the autonomic nervous system associated with morphine withdrawal.

The processes underlying opioid tolerance and dependence involve complex compensatory changes in many opioid and nonopioid neuronal circuits (Nestler and Aghajanian, 1997; Nye and Nestler, 1996). In the locus coeruleus and the ventral tegmental area, levels of tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of catecholamines, are up-regulated in response to chronic administration of morphine, suggesting that tyrosine hydroxylase could contribute to the complex effects that opioids exert on catecholaminergic transmission (Boundy et al., 1998).

On the other hand, morphine dependence exerts long-lasting effect on gene expression in the central nervous system (Nestler and Aghajanian, 1997; Nestler, 1992; Blendy and Maldonado, 1998). The transcription factor

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Fos has been shown to be altered in several brain areas following morphine withdrawal (Curran et al., 1996; Laorden et al., 2002) and has been widely used as an indicator of cellular activity (Morgan and Curran, 1991). Cardiac cells have excitation–depolarization characteristics similar to those of neuronal cells, suggesting that the immediately-early genes may be markers for monitoring cardiac activity. Thus the immediately-early genes such as c-*fos*, are rapidly induced in the cardiac cells in response to stimuli such as noradrenaline administration (Hannan et al., 1993), immobilization or emotional stress (Ueyama et al., 1996; Ueyama et al., 1999) or ischemia–reperfusion (Mizukami and Yoshida, 1997). Moreover, we previously reported that morphine withdrawal induces the expression of Fos protein in the heart (González-Cuello et al., 2003). Fos protein forms part of the activator protein-1 (AP-1) transcription factor, which binds to AP-1 elements in the promoter regions of specific genes (e.g., tyrosine hydroxylase gene) to affect transcription and expression of other delayed-response genes (Morgan and Curran, 1991; Gizang and Ziff, 1994). However, the neurotransmitter mechanisms initiating the expression of Fos in morphine withdrawal are generally not known, and the role of noradrenergic pathways in this response is unclear. Therefore, in the present study, we investigated whether tyrosine hydroxylase levels and noradrenaline turnover in the heart (right and left ventricle) were affected by naloxone-precipitated morphine withdrawal. We also studied the changes in Fos expression (as a marker of cellular activation) in the heart during morphine withdrawal. Furthermore, we examined the activity of tyrosine hydroxylase in the right and left ventricle, to clarify the mechanisms by which morphine dependence alters noradrenergic activity in the heart.

2. Materials and methods

Male Sprague–Dawley rats (220–240 g at the start of the experiments) were housed four to five per cage under a 12 h light/dark cycle (light: 8:00–20:00 h) in a room with controlled temperature (22±2 °C) and humidity (50±10%) and food and water available ad libitum. Animals were pre-handled for several days preceding the experiment to minimize stress, as previously described (Laorden et al., 2000). All surgical and experimental procedures were performed in accordance with European Communities Council Directive of 24 November 1986 (86/609/EEC) and the local Committee.

Rats were rendered tolerant/dependent on morphine by s.c. implantation of morphine base pellets (75 mg): one on day 1, two on day 3 and three on day 5, under light ether anaesthesia. Control animals were implanted with placebo pellets containing lactose instead of morphine on the same time schedule. These procedures have repeatedly been shown to induce both tolerance and dependence as measured behaviourally and biochemically (Rabadán et al., 1998; Milanés and Laorden, 2000; Milanés et al., 2000). On day 8, the animals pretreated with morphine or placebo pellets were injected with saline s.c. or naloxone (5 mg/kg s.c.). Withdrawal signs were observed before and for 30 or 90 min after the administration of the opioid antagonist or saline. The weight gain of the rats was checked during treatment to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake (Berhow et al., 1995). In addition, body weight loss was determined as the difference between the weight determined immediately before saline or naloxone injection and a second determination made 30 and 90 min later.

2.1. Tissue preparation for Western blotting analyses

Ninety minutes (for Fos determination) and 30, 90 and 3 h (for tyrosine hydroxylase analysis) after administration of naloxone or saline, rats were killed by decapitation under light ether anaesthesia. The hearts were rapidly removed, and the right and left ventricles were dissected, fresh-frozen, and stored immediately at −80 °C until use.

2.2. Western blotting

Samples were placed in homogenization buffer [phosphate-buffered saline (PBS), 2% sodium dodecylsulfate (SDS) plus protease inhibitors, Boehringer Mannhein, Germany], homogenized for 50 s prior to centrifugation at 27 167×*g* for 20 min at 4 °C. The supernatant was boiled (5 min), and total protein concentrations were determined spectrophotometrically using the bicinchoninic acid method (Wiechelman et al., 1988). The optimal amount of protein to be loaded was determined in preliminary experiments by loading gels with increasing protein contents (25–100 ìg) from samples of each experimental group. Equal amounts of protein (50 ìg/lane) from each sample were loaded on a 10% sodium dodecylsulfate–polyacrilamide gel (SDS–PAGE), electrophoresed and transferred onto poly (vinylidene difluoride) (PVDF) membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratory, CA, USA). Similar loading and transfer was ascertained by cutting the lower portion of the blot and staining for total protein with Amido Black. Non-specific binding of antibodies was prevented by incubating membranes in 1% bovine serum albumin (BSA) in Tris-buffered saline Tween 20 (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20). The membranes were incubated overnight, at 4 °C, with polyclonal primary antibody c-*fos* (1:200 dilution; sc-7202, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with a monoclonal anti tyrosine hydroxylase antibody, which recognizes the nonphosphorylated form of the

enzyme, according to the manufacturer's instructions (1:4000 dilution; Diasorin, MN, USA) in Tris-buffered saline Tween 20 with BSA. After extensive washings with Tris-buffered saline Tween 20, the membranes were incubated for 1 h, at room temperature, with peroxidase-labeled secondary antibodies [anti-(rabbit sc-2004) for Santa Cruz; anti-(mouse AP308P) for tyrosine hydroxylase, Chemicon, CA, USA] at 1:5000 dilution. After washing, immunoreactiviy was detected with an enhanced chemiluminescence Western blot detection system (ECL, Amersham-Pharmacia-Biotechnology, Madrid, Spain) and visualised by Amersham Hyperfilm-ECL. After film scanning, the integrated optical density of the bands was estimated (Scion Image software, Scion, MD, USA), and normalised to the background values. Relative variations between the bands of the experimental samples and the control samples were calculated in the same image. Duplicate measurements in three or four different gels for each individual sample were performed. Measurements were in the linear range.

2.3. Measurement of tyrosine hydroxylase activity in the heart

Rats were killed by decapitation, 10, 30 or 90 min after saline or naloxone (5 mg/kg s.c.) injection to placebo- or morphine-pelleted animals. The heart was removed and the right and left ventricle were dissected, fresh-frozen and stored immediately at −80 °C until use. Samples were homogenized in PBS, 2 mM sodium orthovanadate and protease inhibitors and centrifuged (50 976×*g*, for 5 min at 4 °C). Supernatant aliquots (25 μl) were incubated at 37 °C in a final volume of 50 μl of a reaction mixture containing the following components: 0.2 M Tris/HCl (pH 7), 1 mM tetrahydrobiopterin, 10 mM β-mercaptoethanol, 0.02% catalase and 50 μM isotopically diluted L-[3,5^3-*H*]tyrosine (radioactive concentration 10 μ/Ci ml, specific activity 0.2 mCi/μmol; Amersham). For the blank reaction, samples were replaced with sodium orthovanadate. After a 4-h incubation, the reaction was stopped by addition of 1% trichloroacetic acid (TCA) and the radioactive organic compounds were separated from tritiated water by absorption onto activated charcoal (Jara et al., 1988). After centrifugation (12 000×*g*, 4 °C, 5 min), tritiated water was quantified in the supernatant by scintillation counting in a Wallac 1409 liquid scintillation counter. Tyrosine hydroxylase activity was determined as ^3H_2O produced/min/μg protein. The protein was determined according to the method described above.

2.4. Estimation of noradrenaline and its metabolite normetanephrine in the right and left ventricle

Rats were decapitated 30 or 90 min after saline s.c. or naloxone (5 mg/kg s.c.), the chest was opened with a midsternal incision and the right and left ventricles were dissected and stored immediately at −80 °C. Noradrenaline and its metabolite normetanephrine were determined by high-performance liquid chromatography (HPLC) with electrochemical detection. Each tissue was weighed, placed in a dry-cooled propylene vial and homogenized with a Polytron-type homogenizer (setting 4 for 50 s) in 1.5 ml perchloric acid (0.1 M). The homogenates were then centrifuged (50 976×*g*, 4 °C, 15 min), the supernatant layer was removed into a 1-ml syringe and filtered through a 0.45 μm filter (Millipore, Bedford, USA) and centrifuged (27 167×*g*, 4 °C, 20 min) again through Ultrafree MC 0.2 (Millipore). From each sample, 10 μl was injected into a 5-μm C_{18} reverse phase column (Waters, Milford, MA, USA) through a Rheodyne (Rheodyne, Cotati, CA, USA) syringe-loading injector 200 μl loop. Electrochemical detection was accomplished with a glass carbon electrode set at a potential of +0.65 with respect to the Ag/AgCl reference electrode (Waters). The mobile phase consisted of a 95:5 (v/v) mixture of water and methanol with sodium acetate (50 mM), citric acid (20 mM), L-octyl-sodium sulfonate (3.75 mM), di-*n*-butylamine (1 mM) and EDTA (0.135 mM), adjusted to pH 4.3. The flow rate was 0.9 ml/min, and chromatographic data were analysed with Millenium 2010 Chromatography Manager Equipment (Millipore). Noradrenaline and normetanephrine were simultaneously detected by the described HPLC method at an elution time of 4.25 and 7.32 min, respectively. Noradrenaline and normetanephrine were quantified by reference to calibration curves run at the beginning and the end of each series of assays. Linear relationships were observed between the amount of standard injected and the peak height measured. The content of noradrenaline and normetanephrine in the right and left ventricle was expressed as n/g wet weight of tissue.

2.5. Drugs and chemicals

Pellets of morphine base (Alcaliber Laboratories, Madrid, Spain) or lactose were prepared by the Department of Pharmacy and Pharmaceutic Technology (School of Pharmacy, Granada, Spain); sodium dodecylsulfate, polyacrylamide gel and PVDF membrane were obtained from Bio-Rad Laboratory (Teknovas, Bilbao, Spain). Noradrenaline bitartrate, normetanephrine (used as an HPLC standard), naloxone HCl, Western blot and tyrosine hydroxylase activity reagents were purchased from Sigma (St. Louis, MO, USA). Naloxone HCl was dissolved in sterile 0.9% NaCl (saline). All drugs were administered in volumes of 0.10 ml/100 g body weight.

2.6. Statistical analysis

The mean±S.E.M. was expressed for values obtained from a minimum of four rats per group. Data were analysed by analysis of variance (ANOVA) followed by the Newman–Keul's post hoc test. Body weight gain and loss in naive and morphine-dependent rats was analysed by

unpaired Student's *t*-test. Differences with a *P*-value less than 0.05 were considered significant.

3. Results

Before performing the immunodetection assays, we assessed the efficacy of chronic treatment with morphine by pellets implantation, which has been previously shown to induce tolerance and dependence to the effects of morphine (González-Cuello et al., 2003; Martínez et al., 2003; Laorden et al., 2002). For this purpose, the weight of the animals was recorded on the days of pellets implantation and on the day of killing (day 8), before receiving any injections. Rats treated with morphine showed a significantly lower ($P<0.01$; *t*-test) body weight gain (20.88±2.21 g, $n=45$) than animals receiving placebo pellets (37.45±3.45 g, $n=78$). Administration of naloxone to control rats resulted in no significant changes in body weight when measured 30 (2.66±0.34 g, $n=14$) or 90 (2.38±0.50 g, $n=18$) min after drug injection, as compared to control rats receiving saline (1.66±0.38 g, $n=13$; 3.50±0.61 g, $n=17$ respectively; *t*-test). However, chronic morphine-treated animals showed a significant weight loss ($P<0.001$; *t*-test) 30 (17.21±0.32 g, $n=15$) or 90 min (16.48±0.40 g, $n=20$) after naloxone injection when compared with placebo-pelleted group also receiving naloxone. All morphine-dependent animals receiving naloxone demonstrated behaviours characteristic of opioid withdrawal, including jumping, wet-dog shakes, teeth chattering, ptosis, chromodiacryorrhea and irritability.

3.1. Effects of morphine withdrawal on Fos expression in the heart

Fos immunoreactivity in the right and left ventricle was examined by Western blot analysis 90 min after s.c. injection of saline or naloxone (5 mg/kg) to control rats and to animals considered dependent on morphine. The delay of 90 min after drug injection was chosen because it is well known that the peak effect of stimulated Fos is 90–120 min (Morgan and Curran, 1991)0. As shown in Fig. 1, Fos was detected in a band located at ≈62 kDa. In animals treated with placebo pellets for 7 days, the level of Fos 90 min after naloxone injection was similar to that of the control group receiving saline instead of naloxone. However, in animals rendered dependent on morphine by chronic administration of the opioid for 7 days, significant increases in the level of Fos were observed after naloxone administration in the right ($P<0.01$) and left ($P<0.001$) ventricle, compared with placebo-pelleted rats injected with naloxone.

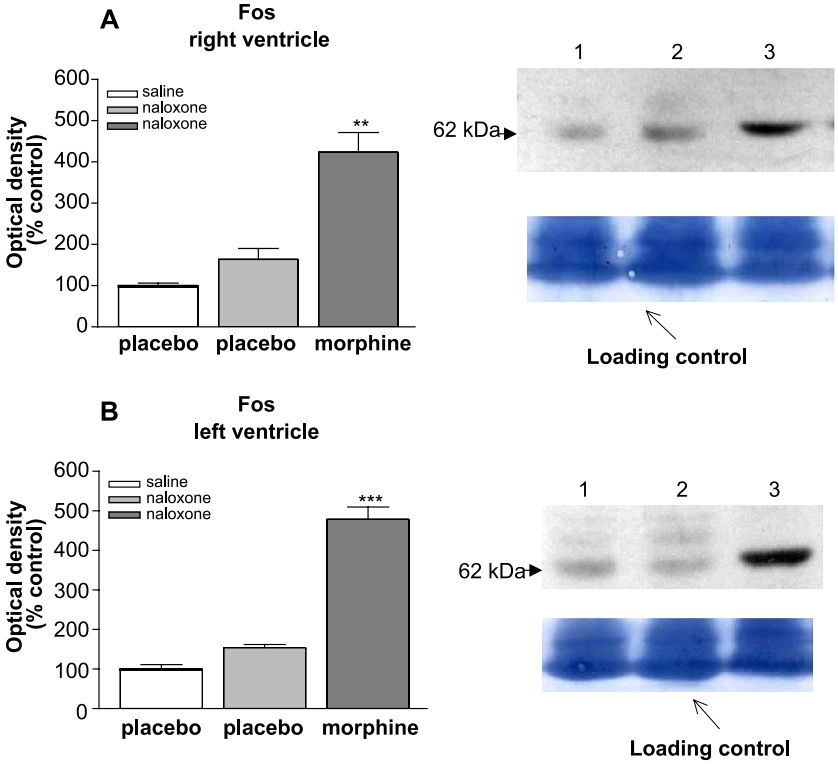

Fig. 1. Western blotting analysis of Fos immunoreactivity levels in the right (A) and left ventricle (B) in different experimental groups. Control animals were implanted with placebo pellets for 7 days. Opioid dependence was induced by s.c. implantation of morphine pellets for 7 days, and opioid withdrawal was precipitated by administration of naloxone (5 mg/kg s.c.) on day 8. The immunoreactivity corresponding to Fos is expressed as a percentage of that in the control group (placebo plus saline; defined as 100% value). Data are means±S.E.M., $n=3$–5 per group. **$P<0.01$, ***$P<0.001$ versus placebo+naloxone. Representative bands from autoradiograms at known apparent molecular weight for Fos are shown (1: placebo+saline; 2: placebo+naloxone; 3: morphine+naloxone). Similar loading and transfer was ascertained by cutting the lower portion of the blot and stained for total proteins with Amido Black.

3.2. Effects of morphine dependence on tyrosine hydroxylase levels in the heart

We studied tyrosine hydroxylase protein levels by Western blot analysis at different time points. As shown in Fig. 2C, tyrosine hydroxylase protein was not consistently altered in the right ventricle at 180 min after naloxone-induced morphine withdrawal compared with placebo-pelleted rats receiving naloxone. Similar results were obtained in the left ventricle at the same time point (Fig. 3C). When tyrosine hydroxylase immunoreactivity was determined by Western blot analysis at 30 or 90 min after morphine withdrawal, there was a significant increase in tyrosine hydroxylase level in the right ($P<0.05$, Fig. 2A,B) and in the left ($P<0.01$, Fig. 3A; $P<0.001$, Fig. 3B) compared with the control group receiving naloxone.

3.3. Effects of morphine dependence on tyrosine hydroxylase activity in the heart

In a parallel set of experiments, tyrosine hydroxylase activity was measured in the right and left ventricle. Morphine withdrawal was associated with an increase of the rate limiting step of catecholamines synthesis at different points after withdrawal. The tyrosine hydroxylase activity was measured 10, 30 or 90 min after naloxone-precipitated morphine withdrawal. As shown in Fig. 4A,D at 10 min after morphine withdrawal, there were not changes in the enzyme activity in the right or left ventricle. However, 30 or 90 min after naloxone-induced withdrawal, there was an increase ($P<0.001$) in the tyrosine hydroxylase activity in the right and left ventricle when compared with placebo group injected

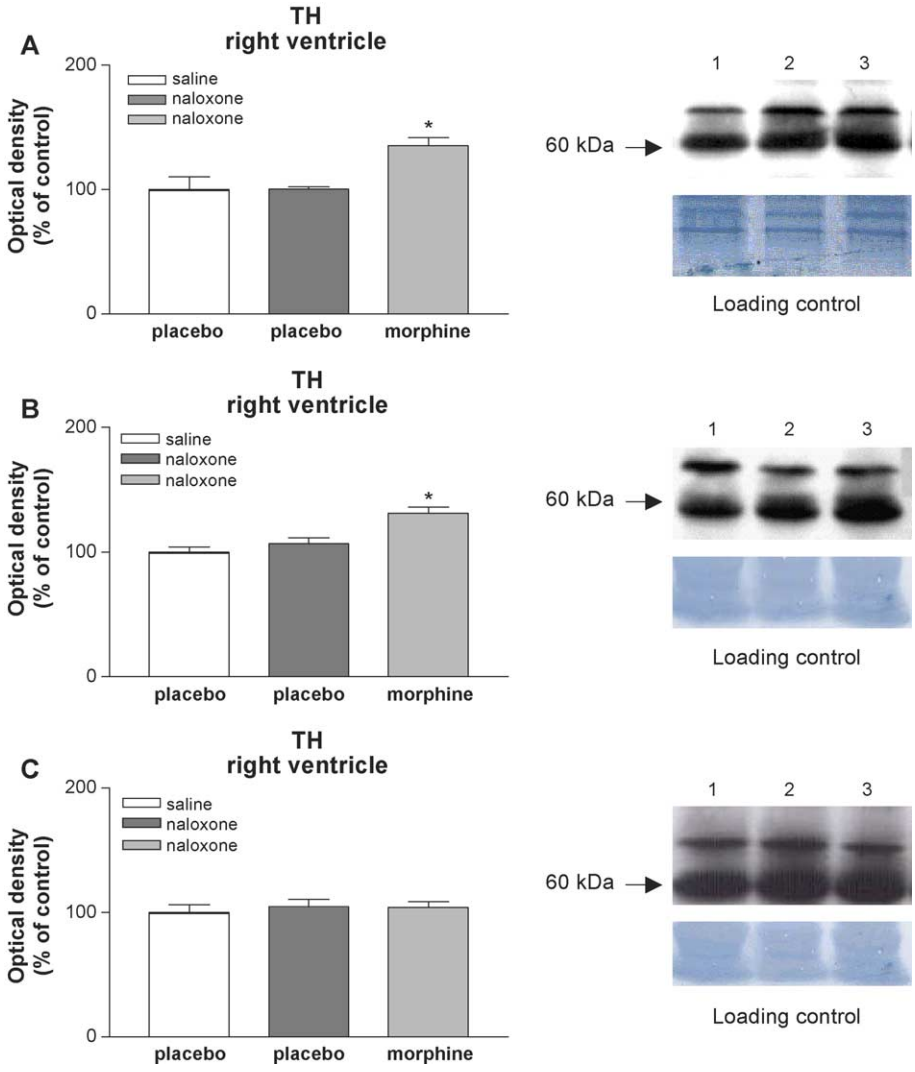

Fig. 2. Western blotting analysis of tyrosine hydroxylase (TH) immunoreactivity levels in the right ventricle in different experimental groups. Control animals were implanted with placebo pellets for 7 days. Opioid dependence was induced by s.c. implantation of morphine pellets for 7 days, and opioid withdrawal was precipitated by administration of naloxone (5 mg/kg s.c.) on day 8. Animals were decapitated 30 min (A), 90 min (B) or 180 min (C) after saline or naloxone injection. The immunoreactivity corresponding to TH is expressed as a percentage of that in the control group (placebo plus saline; defined as 100% value). Data are means±S.E.M., n=3–5 per group. *$P<0.05$ versus placebo+naloxone. Representative bands from autoradiograms at known apparent molecular weight for TH are shown (1: placebo+saline; 2: placebo+naloxone; 3: morphine+naloxone). Similar loading and transfer was ascertained by cutting the lower portion of the blot and stained for total proteins with Amido Black.

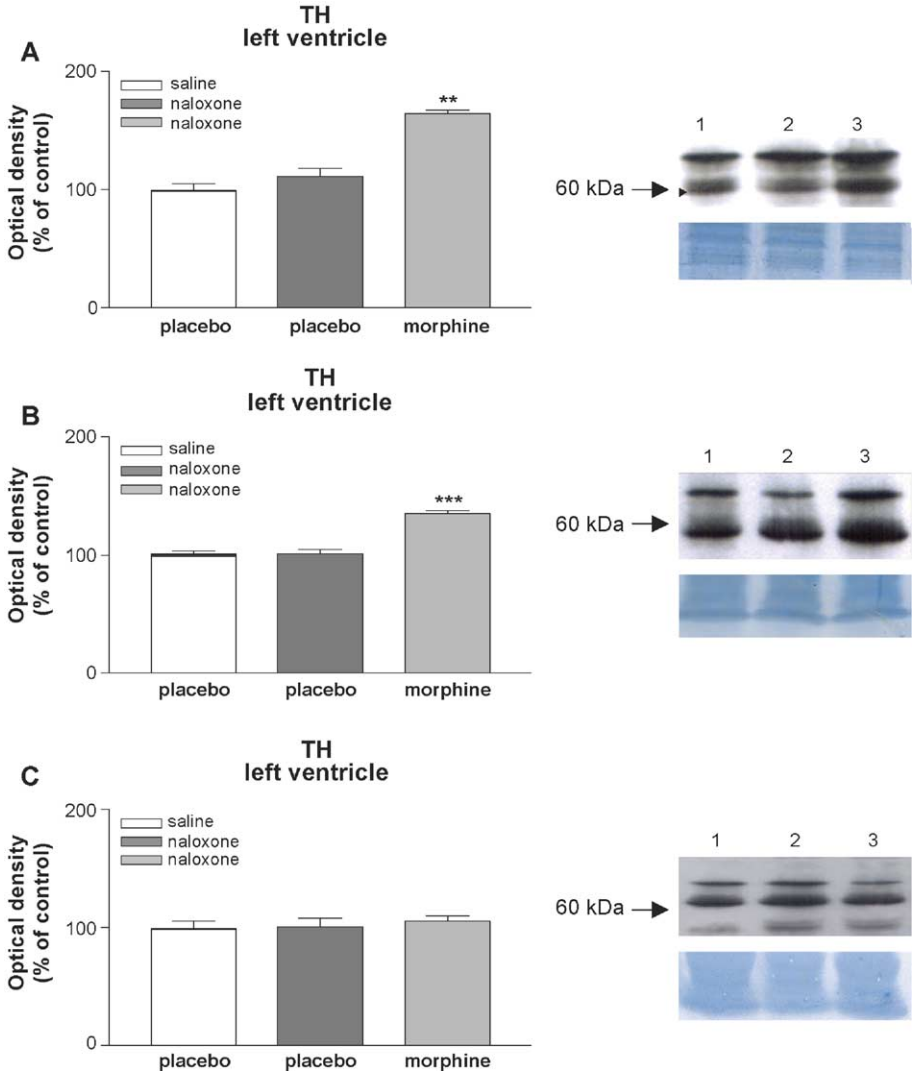

Fig. 3. Western blotting analysis of tyrosine hydroxylase (TH) immunoreactivity levels in the left ventricle in different experimental groups. Control animals were implanted with placebo pellets for 7 days. Opioid dependence was induced by s.c. implantation of morphine pellets for 7 days, and opioid withdrawal was precipitated by administration of naloxone (5 mg/kg s.c.) on day 8. Animals were decapitated 30 min (A), 90 min (B) or 180 min (C) after saline or naloxone injection. The immunoreactivity corresponding to TH is expressed as a percentage of that in the control group (placebo plus saline; defined as 100% value). Data are means±S.E.M., n=3–5 per group. **P<0.01, ***P<0.001 versus placebo+naloxone. Representative bands from autoradiograms at known apparent molecular weight for TH are shown (1: placebo+saline; 2: placebo+naloxone; 3: morphine+naloxone). Similar loading and transfer was ascertained by cutting the lower portion of the blot and stained for total proteins with Amido Black.

with naloxone (Fig. 4B,C,E,F). In contrast, administration of naloxone to placebo-pelleted rats did not change the enzyme activity compared with control rats injected with saline.

3.4. Effects of morphine withdrawal on noradrenaline turnover

Concentrations of noradrenaline, normetanephrine, as well as noradrenaline turnover (as estimated by normetanephrine/noradrenaline ratio) were estimated in the right and left ventricle for rats dependent on morphine. The noradrenaline turnover was not changed in control rats 30 or 90 min after naloxone administration when compared with control rats injected with saline in the right or left ventricle. However, noradrenaline turnover was increased in the right (P<0.01) and left ventricle 30 (P<0.01) or 90 (P<0.001) min after naloxone administration to morphine-dependent rats when compared to control rats injected with naloxone (Fig. 5).

4. Discussion

The present study shows that morphine withdrawal induced an increase in tyrosine hydroxylase protein levels and tyrosine hydroxylase enzyme activity in parallel with an enhanced noradrenergic activity in the heart. We also show an increase in the Fos expression after naloxone admin-

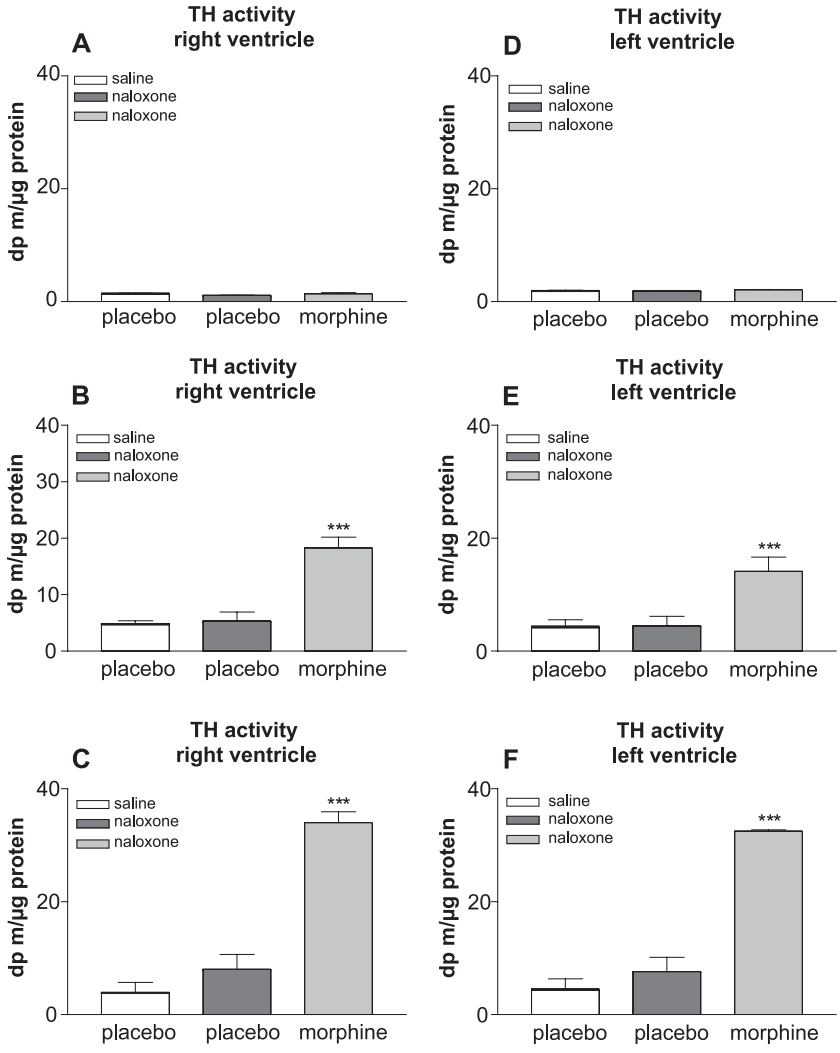

Fig. 4. Tyrosine hydroxylase (TH) activity in the right and left ventricle obtained from control rats and from rats dependent on morphine. Rats were killed 10 (A, D), 30 (B, E) or 90 (C, F) min after administration of saline or naloxone (5 mg/kg s.c.) on day 8. Values correspond to the mean±S.E.M. ***P<0.001 versus placebo group receiving naloxone.

istration to morphine-dependent rats, which indicates and activation of cardiac cellular activity.

The regulation of cellular events through altered expression of protein signalled by chronic activation of opioid receptors is critical for understanding tolerance and dependence to opioids (Williams et al., 2001). As a first step in studying the effects of morphine dependence on the heart, we examined the expression of Fos protein in the right and left ventricle. Western blot analysis revealed low signals in the control rats, whereas Fos expression was strongly expressed after naloxone administration to morphine dependent rats. In agreement with these findings, a recent study from our laboratory demonstrated Fos-immunoreactivity (as revealed by immunohistochemistry) in cardiomyocytes. The Fos expression was seen in morphine-dependent rats but not in U-50,488H (a selective κ-opioid receptor agonist)-dependent rats, indicating the involvement of μ-opioid receptor) (González-Cuello et al., 2003). As Fos protein functions as a transcription factor, which binds to the AP-1 sites of various target genes, its induction may modify the expression of other genes and has been implicated in a diverse range of cellular processes (Angel and Karin, 1991). Elevation of Fos in the heart observed in our study could contribute to the expression of target genes within the heart. Genes encoding neurotransmitter synthesizing enzymes that contain AP-1 sites in the promoter regions (e.g., tyrosine hydroxylase) could be putative targets of c-*fos* mediated gene expression in the heart induced by morphine withdrawal.

The present study also demonstrated that the administration of naloxone to morphine-dependent rats resulted in an increase in the normetanephrine/noradrenaline ratio in the right and left ventricle 30 or 90 min after the injection of the opioid antagonist and confirms previous result obtained in left atria (Rabadán et al., 1997), right atria (Rabadán, Milanés et al., 1998), right ventricle (Milanés et al., 2000) and left ventricle (González-Cuello et al., 2003) 30 min after

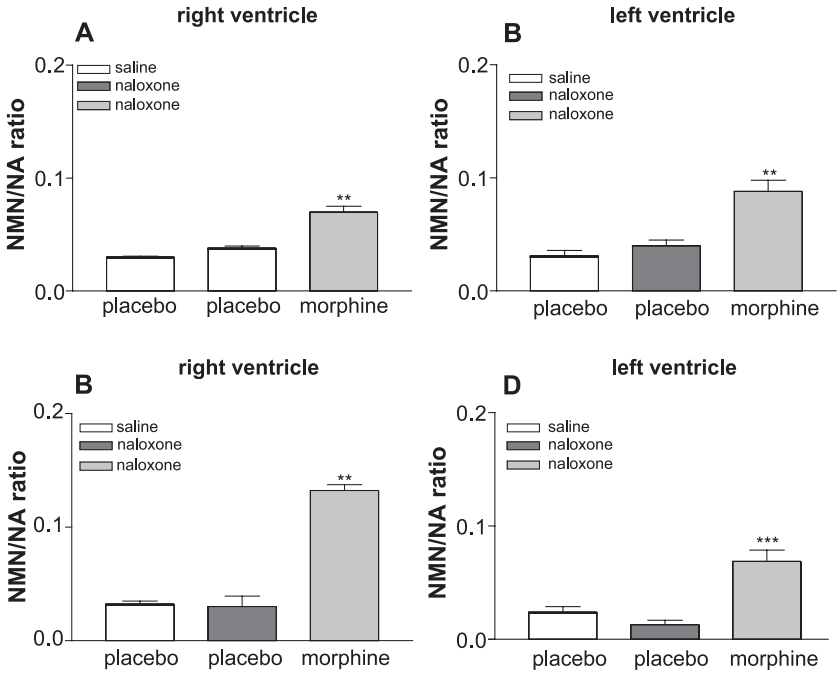

Fig. 5. Normetanephrine (NMN)/noradrenaline (NA) ratio in the right and left ventricle obtained from control rats and from rats dependent on morphine. Rats were killed 30 (A, B) or 90 (C, D) min after administration of saline or naloxone (5 mg/kg s.c.) on day 8. Values correspond to the mean±S.E.M. $^{**}P<0.01$, $^{***}P<0.001$ versus placebo group receiving naloxone.

naloxone administration to morphine-dependent rats. Importantly, the effects were dependent on adrenoceptor activation, which indicates that the catecholaminergic hyperactivity in the heart during morphine withdrawal is mediated via stimulation of noradrenergic pathway (Milanés and Laorden, 2000). Together, all these data suggest that morphine withdrawal may activate noradrenergic neurons that innervate the heart. In addition, our previous studies demonstrated a marked increase in sympathetic activity in the heart after naloxone-methiodide and *N*-methyl levallorphan administration to morphine dependent rats (Milanés et al., 2001). Because quaternary compounds do not readily cross the blood–brain barrier (Milne et al., 1990), these data suggest that the changes in the heart observed during morphine withdrawal are mediated by peripheral mechanisms.

Although a number of studies have demonstrated an increase in noradrenaline turnover during morphine withdrawal the element that mediates the hyperactivity of heart catecholaminergic neurons has not yet been determined. The present data show that administration of naloxone to morphine-dependent rats resulted in an increase in the expression levels of tyrosine hydroxylase, as measured in the right and left ventricle 30 or 90 min after injection of the opioid antagonist. No significant changes in the level of tyrosine hydroxylase were observed 180 min after the induction of the abstinence syndrome. It is well known that the central means for controlling the synthesis of catecholamines is through regulation of the enzyme in terms of both level and activity. There is now evidence that tyrosine hydroxylase protein and activity levels can be regulated by two different mechanisms: short-term regulation changes of enzyme activity (e.g., enzymatic phosphorylation) and medium- to long-term of gene expression (transcriptional regulation) (Kumer and Vrana, 1996). It has proposed tyrosine that drugs that perturb catecholaminergic function can induce changes in tyrosine hydroxylase mRNA and protein expression. Thus, morphine and cocaine enhancement tyrosine hydroxylase immunoreactivity in dopaminergic brain reward regions. An AP-1 sequence (the Fos/Jun binding site) has been identified in tyrosine hydroxylase gene (Gizang and Ziff, 1994). Because both cocaine and morphine induced the expression of Fos, it has been hypothesized that this transcription factor augments tyrosine hydroxylase transcription by binding to AP-1 sites (Kumer and Vrana, 1996). Additionally, it has been proposed that glucocorticoids and cAMP can regulate the levels of tyrosine hydroxylase mRNA. Thus, tyrosine hydroxylase protein and mRNA were shown to be increased by glucocorticoids (Stachowiak et al., 1988) and cAMP analogue (Kumer and Vrana, 1996). Moreover, a glucocorticoid regulatory element (GRE) and the cAMP-responsive element (CRE) binding protein (CREB) are postulated to exist in the tyrosine hydroxylase gene (Kumer and Vrana, 1996). Previous results from our laboratory have demonstrated that morphine withdrawal produces a marked increased in cAMP levels in the right and left ventricle in parallel with an increase in corticosterone release (Rabadán et al., 1998; Fuertes et al., 2000; Milanés et al., 2000). According to all these data, the present results might suggest that morphine withdrawal could increase tyrosine hydroxylase expression in the heart through Fos

expression, and increased corticosterone release. As the tyrosine hydroxylase antibody only recognizes the non-phosphorylated form of the enzyme, we interpret the increase in tyrosine hydroxylase levels to reflect an increase in the synthesis of tyrosine hydroxylase in the neuronal soma from neurons localized within cardiac ganglia. Consistent with our results, the presence of catecholaminergic neurons within cardiac ganglia has been demonstrated (Singh et al., 1999; Horackova and Armour, 1995). In addition, intrinsic cardiac neurons can function independently of central neuronal input, as a "little brain" in the heart (Armour, 1991; Thompson et al., 2000). The activity generated by intrinsic cardiac neurons can be modified by different substances applied locally (Armour et al., 1993; Huang et al., 1993).

In parallel with the increase in tyrosine hydroxylase levels, we showed an increase in tyrosine hydroxylase activity 30 or 90 min after withdrawal. An increase in catecholamine synthesis and release is closely associated with the activation of the enzyme, which catalyses the conversion of tyrosine to DOPA. Tyrosine hydroxylase activity is acutely regulated by changes in the phosphorylation state of serine residues and increases in phosphorylation have been shown to accelerate enzymatic activity (Lew et al., 1998). All these data support the idea that the increase in noradrenaline turnover in the heart observed during morphine withdrawal could be exerted by stimulation of tyrosine hydroxylase levels and activity in the noradrenergic pathways innervating the heart. In conclusion, our results suggest that, among the adaptive changes produced by chronic opioid treatment, increases in tyrosine hydroxylase protein levels and tyrosine hydroxylase enzyme activity could contribute to the enhanced noradrenergic activity in the heart in response to morphine withdrawal.

Acknowledgments

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