Morphine withdrawal activates hypothalamic-pituitary adrenal axis and heat shock protein 27 in the left ventricle: Role of extracellular signal-regulated kinase

E Martínez-Laorden, MA Hurle, MV Milanés, ML Laorden, P Almela

Department of Pharmacology, Faculty of Medicine, University of Murcia, Spain (E, MV, ML, P) and Department of Physiology and Pharmacology, Faculty of Medicine, University of Cantabria, Santander, Spain (MA)

Footnotes
Running Titled

Hypothalamic-pituitary adrenal axis and shock protein 27 during morphine withdrawal

Address to correspondence

M.L. Laorden
Departamento de Farmacología
Facultad de Medicina
Murcia. Spain
e-mail: laorden@um.es

Number of text pages: 34

Number of tables: 0

Number of figures: 8

Number of references: 51

Number of words in the Abstract: 247

Number of words in the Introduction: 667

Number of words in the Discussion: 1423

Abbreviations: ACTH, adrenocorticotropic hormone; CRF, corticotrophin releasing factor; COMT, catechol-O-methyl transferase; ERK, signal-regulated protein kinase; HPA, hypothalmo-pituitary-adrenocortical; HPLC, high-performance liquid chromatography; HR, heart rate; Hsps, heat shock proteins; MAP, mean arterial pressure; MB-COMT, membrane-catechol-O-methyl transferase; MK, mitogen-activated protein kinases; NMN, normetanephrine; PVN, paraventricular nucleus; S-COMT, soluble-COMT.

Section: Cellular and Molecular, Cardiovascular
ABSTRACT

The negative affective states of withdrawal involve recruitment of brain and peripheral stress circuitry [e.g. noradrenergic activity, induction of hypothalamo-pituitary-adrenocortical (HPA) axis and the expression and activation of heat shock proteins (Hsp)]. The present study investigated the role of extracellular signal-regulated protein kinase (ERK) and beta-adrenoceptor on the response of stress system to morphine withdrawal by means of SL327, a selective inhibitor of ERK activation, or propranolol (a beta-adrenoceptor antagonist) administration. Dependence on morphine was induced by a 7-day s.c. implantation of morphine pellets. Morphine withdrawal was precipitated on day 8 by injection of naloxone (2 mg/kg, s.c.). Plasma concentrations of adrenocorticotropic hormone (ACTH) and corticosterone were determined by radioimmunoassay (RIA); noradrenaline (NA) turnover in left ventricle by high-performance liquid chromatography (HPLC); catechol-O-methyl transferase (COMT) and Hsp27 expression and phosphorylation at serine82 (Ser82) were determined by quantitative blot immunolabeling. Morphine withdrawn rats showed an increase of NA turnover and COMT expression in parallel with an enhancement of ACTH and plasma corticosterone concentrations. In addition, we observed an enhancement of Hsp27 expression and phosphorylation. Pretreatment with SL327 or propranolol significantly reduced morphine withdrawal-induced increases of plasma ACTH and Hsp27 phosphorylation at Ser82 without any changes in plasma corticosterone levels. Present findings demonstrate that morphine withdrawal is capable of inducing the activation of HPA axis in parallel with an enhancement of Hsp27 expression and Hsp27 phosphorylation at Ser82 and suggest a role of beta-adrenoceptors and ERK pathways in mediating morphine withdrawal activation of HPA axis and cellular stress response.
INTRODUCTION

Stress is a scientific term describing any significant distressing situation which demands physiologic and/or behavioral readjustment or adaptation. Exposure to a stressful situation leads to the activation of two systems: the catecholaminergic system and hypothalamo-pituitary-adrenocortical (HPA) axis. Activation of the former results in enhanced circulating catecholamines levels that can damage the heart (Kas, 1987). Via the HPA axis, the level of peptides such as corticotrophin releasing factor (CRF) and adrenocorticotropic hormone (ACTH) and glucocorticoid rises.

Whereas in rodent morphine may exert stimulatory and inhibitory roles in the control of the HPA axis depending on the dose and the time course of its administration, it has a predominantly inhibitory role in humans, with consequent activation of the HPA axis after administration of naloxone/naltrexone (for review see Young et al., 2010). In rats, activation of the HPA axis has been observed with agonists of the three types of opioid receptors when given peripherally and centrally (Haracz et al., 1981; Pfeiffer et al., 1985; Iyengar et al., 1987) and it has been described that like stressors, morphine withdrawal increases heart rate (Almela et al., 2011) and activates HPA axis in rats, which results in neuronal activation of stress-related neurosecretory neurons in the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN), an increase in CRF transcription and boost of ACTH and corticosterone secretion (Cleck and Blendy, 2008, Laorden et al., 2002; Nuñez et al., 2009). Furthermore, the profound cellular stress induced by chronic morphine treatment and withdrawal is also evidenced by overexpression of heat shock proteins (Hsps) (Kas, 1987; Sharma and Ali, 2006). Hsps were identified primarily on the basis of their fast and typically protective response to cellular stressors. Hsps are rapidly induced at the transcriptional level following stress, but also undergo several post-translational modifications that alter...
their functional roles for being used as immediate response elements (Stetler et al., 2009). Hsp27, a member of the small-Hsp family, acts as an endogenous cytoprotective stress response protein, eliciting cardioprotection to ischemic injury, via its role as a molecular chaperone and in phosphorylation-dependent stabilization of actin (Peart et al., 2007). Hsp27 can be reversibly phosphorylated on three serine residues by the mitogen-activated protein kinases 2 and 3 (MK2/3), which are themselves activated by phosphorylation through either the p38 or the extracellular signal-regulated protein kinase (ERK) signalling pathway (Jantschitsch et al., 1988; Guay et al., 1997; Morrow and Tanway, 2003; Duverger et al., 2004; O’shaughnessy et al., 2007). ERK transduces a broad range of extracellular stimuli to yield diverse intracellular responses. The ERK signalling pathway could be important as a regulator of cardiac function (Michel et al., 2001) and may play an important role in the coupling of CRF neuron excitation with both gene induction as well as neurohormone secretion (Osterlund et al., 2011). In addition, it has been shown that Hsp27 and Hsp70 are upregulated by ACTH (Blake et al., 1991; 1993). However, the possible interaction between HPA axis/ERK and the activation of Hsp27 during morphine dependence and withdrawal has not been established.

It is known that drugs of abuse induce cardiac morphological alterations and cardiac disorders (Cerretani et al., 2008; Dettmeyer et al., 2009) but the mechanism underlying the drug-induced myocardial damages still remains unclear. Therefore, the purpose of the present study was to establish some mechanisms implicated in the cardiac adaptive changes observed during morphine withdrawal, here we examined 1) the expression of catecol-O-methyltransferase (COMT), normetanephrine (NMN) [extraneuronal noradrenaline (NA) metabolite generated by COMT], and NMN/NA ratio, as index of NA turnover, in morphine dependent and withdrawn rats; 2) the interaction between HPA axis and ERK pathways, investigating the effects of SL327, a selective
inhibitor of ERK activation (Atkins et al., 1998), on the plasma concentrations of ACTH and corticosterone; 3) the expression and phosphorylation of Hsp27 in serine 82 (Ser82) in order to determine the magnitude and severity of cellular stress during chronic morphine treatment and withdrawal; and 4) the activation of Hsp27 induced by morphine withdrawal in morphine dependent rats treated with SL327 or propranolol (a beta-adrenoceptor antagonist).
MATERIALS AND METHODS

Animals and treatments

Male Sprague-Dawley rats (220-240 g at the beginning 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), humidity (50 ± 10%), food and water available ad libitum and prehandled for several days preceding the experiment to minimize stress, as previously described (Laorden et al., 2000). All surgical and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the local Committee.

Experimental procedure

Rats were rendered 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., 1997). On day 8, the animals pretreated with morphine or placebo pellets were injected with saline s.c. (control group) or naloxone (2 mg/kg s.c.) and decapitated 30, 60 and 90 min after the opioid antagonist administration. Another group of rats was injected with propranolol (3 mg/kg i.p) 20 min before naloxone administration. The chest was opened with a midsternal incision and the left ventricle was dissected and stored immediately at -80°C, so this study was performed in the left ventricle.

Rats weight gain was checked during the treatment to ensure that 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,
we determined the body weight before and after saline or naloxone injection to control and morphine dependent rats.

To determine the effect of inhibiting ERK phosphorylation on the morphine withdrawal-induced changes in HPA activation and in Hsp27 phosphorylation, Hsp27 phosphorylated at Ser82 immunoreactivity was determined by western-blots and plasma ACTH and corticosterone levels were determined by radioimmunoassay (RIA) in morphine dependent and control rats treated with SL327, a selective inhibitor of mitogen-activated extracellular protein kinase (MEK) (Atkins et al., 1998) or vehicle (DMSO), administered 1h before the injection of naloxone or saline. SL327 was dissolved in DMSO 100% and injected intraperitoneally at an injection volume of 1 ml/kg and at doses of 100 mg/kg. Previous studies (Almela et al., 2008; 2009) have demonstrated that this dose of SL327 induces inhibition of ERK phosphorylation.

Radioimmunoassay
Plasma ACTH and corticosterone concentrations were measured by commercially available kits for rats (125I-ACTH and 125I-corticosterone radioimmunoassay; MP Biomedicals, Solon, OH). The sensitivity of the assay was 5.7 pg/ml for ACTH and 7.7 ng/ml for corticosterone.

Determination of NA and its metabolite NMN in the left ventricle
NA and its metabolite NMN 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 in 1.5 ml perchloric acid (0.1 M). The homogenates were then centrifuged (8000 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 (6000 g, 4°C, 20 min) again through Ultra free MC 0.2 filter (Millipore). From each sample, 10 μl was injected into a 5-μm C18 reverse phase column
(Waters, Milford, MA, USA) through a Rheodyne syringe-loading injector 200 μl loop. Electrochemical detection was accomplished with a glass carbon electrode set at a potential of +0.65 V with respect to the Ag/AgCl reference electrode (Waters). The mobile phase consisted of a 95% (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). NA and NMN were simultaneously detected by the described HPLC method at an elution time of 4.25 and 7.32 min, respectively. NA and NMN were quantified by reference to calibration curves run at the beginning and at the end of each series of assays. Linear relationships were observed between the amount of standard injected and the peak height measured. The lower detection limit for both NA and NMN was 100 pg. The content of NA and NMN in the left ventricle was expressed as nanogram per gram of tissue weight.

**Western blot analysis**

Western blot analysis was performed for total Hsp27, Hsp27 phosphorylation, phosphorylated ERKs and COMT protein determination. Samples were placed in homogenization buffer [phosphate buffered saline, 2 % sodium dodecylsulfate (SDS) plus protease inhibitors (Roche, Germany) and phosphatase inhibitors Cocktail Set (Calbiochem, Germany)] and homogenized for 50 s prior to centrifugation at 6000 g for 20 min at 4°C. Total protein concentrations were determined spectrophotometrically using the bicinchoninic acid method (Wiechelman et al., 1988). The optimum amount of protein to be loaded was determined in preliminary experiments by loading gels with increasing protein contents (25 to 100 μg) from samples of each experimental group. Equal amounts of protein (50 μg/lane) from each sample were loaded on a 10% SDS-polyacrylamide gel (SDS-PAGE), electrophoresed, and transferred onto a poly vinylidene difluoride (PVDF) membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Lab.,
Non-specific binding of antibodies was mitigated by incubating membranes with 1% bovine serum albumin (BSA) in tris buffer saline tween (TBST: 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20). Blots were incubated overnight at room temperature (for total Hsp27 and Hsp27 phosphorylated at Ser82) or at 4°C (for phospho-ERK and COMT), with the following primary antibodies: polyclonal anti-total Hsp27 antibody (1:500; sc-1048, Santa Cruz Biotechnology, Santa Cruz, CA, USA); polyclonal anti-phospho Ser82 Hsp27 (1:400 dilution; ab39399, Abcam, UK), monoclonal anti-pERK1/2 (1:1000; sc-7383, Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal anti-COMT (1:5000; AB5873, Chemicon International, MA, USA) in TBST with BSA. After extensive washings with TBST, the membranes were incubated for 1h, at room temperature, with peroxidase-labeled secondary antibodies (anti-goat sc-2350 for total Hsp27; anti-rabbit sc-2004 for Hsp27 phosphorylated at Ser82; anti-mouse sc-2005 for phospho-ERK1/2 and for COMT, Santa Cruz) at 1:5000 dilution. After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, UK) and visualised by a Typhoon 9410 variable mode Imager (GE Healthcare). Antibodies were stripped from the blots by incubation with stripping buffer (glycine 25mM and SDS 1%, pH2), for 1h at 37°C. We used anti-β-actin (Cell Signalling, 45 kDa) or anti-total ERK (sc-154, Santa Cruz, 42 kDa) as our loading control for all the experiments. The ratio of total Hsp27/β-actin, phospho-Hsp27/β-actin, phospho-Hsp27/total-Hsp27, phospho-ERK1/totalERK, phospho-ERK2/totalERK and COMT/β-actin was plotted and analysed. Quantification of immunoreactivity corresponding to total Hsp27 (27 kDa), Hsp27 phosphorylated at Ser82 (28 kDa), phospho-ERK1/2 (44 and 42 kDa), soluble-COMT (S-COMT) and membrane-COMT (MB-COMT) (25 and 30 kDa, respectively) bands was carried out by densitometry (AlphaImager, Nucliber, Madrid). We measured the integrated optical density of bands. The software generates quantitative data of band intensities. The values are in units of
pixel gray levels and are proportional to the light intensity on that pixel during the exposure time of the image. The optical density was normalized to the background values. Relative variations between bands of experimental samples and control samples were calculated in the same image.

**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 dodecylsulphate, polyacrylamide gel and PVDF membrane were obtained from Bio-Rad Laboratory (Teknovas, Bilbao, Spain). SL327 (a-[Amino[(4-aminophenyl)thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile) was obtained from Abcam Biochemical (UK). NA bitartrate, NMN (used as an HPLC standard), naloxone HCl, propranolol and western blot reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA). Naloxone HCl was dissolved in sterile 0.9 % NaCl (saline) and administered in volumes of 0.1 mL/100g body weight.

**Data analysis**

Data are expressed as mean±SEM and analyzed using two-way or one-way (when required) ANOVA followed by a post hoc Newman Keuls test. Student’s t-test was used when comparison were restricted to two experimental group. Differences with a p-value < 0.05 were considered significant.
Results

The animals weight 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 (1.0 ± 0.09 g, n=30) than animals receiving placebo pellets (20.3 ± 2.5 g, n=43). Two-way ANOVA for weight loss at 30, 60 or 90 min showed a significant effect of naloxone injection (F1,13=11.80, p=0.0044; F1,31=24.83, p<0.0001; F1,12=7.96, p=0.0154, respectively), chronic morphine administration (F1,13=12.50, p=0.0037; F1,31=15.91, p=0.0004; F1,12=8.79, p=0.0118, respectively) and an interaction between morphine treatment and naloxone injection (F1,13=12.50, p=0.0037; F1,31=15.91, p=0.0004; F1,12=8.79, p=0.118, respectively). As shown in Figure 1 A,B,C, there was a significant enhancement in body weight loss after naloxone injection to morphine dependent rats, compared with control animals also receiving naloxone and with morphine dependent rats given saline. Acute naloxone injection had no effect in animals chronically treated with placebo, compared with the corresponding control group receiving saline.

In animals pretreated with propranolol or SL327, one-way ANOVA revealed significant differences in body weight loss (F5,38=23.21, p<0.0001). As shown in Figure 1D, naloxone injection to rats acutely treated with SL327 or propranolol induced a significant (p<0.001, one-way ANOVA) weight loss, similar to that described in the group chronically pretreated with vehicle plus morphine.

NA turnover in the left ventricle

Two-way ANOVA for NA showed a significant effect of naloxone injection (F1,20=11.96, p=0.0025) and chronic morphine administration (F1,20=6.08, p=0.0229) and no interaction between morphine treatment and naloxone injection (F1,20=2.06, p=0.1669). Two-way ANOVA for NMN revealed a significant effect of naloxone injection (F1,20=7.65, p=0.0119).
Results for two way ANOVA for NMN/NA ratio showed an interaction between morphine treatment and naloxone injection ($F_{1,20}=8.82$, $p=0.0076$) with main effect of morphine treatment ($F_{1,20}=8.86$, $p=0.0075$) and naloxone injection ($F_{1,20}=79.61$, $p<0.0001$). Post hoc tests revealed that 60 min after naloxone administration to morphine dependent rats there was an increase ($p<0.001$) of NMN content and NMN/NA ratio (as index of NA turnover) concomitantly with a decrease in NA content when compared with morphine dependent rats receiving saline instead of naloxone or naïve rats injected with naloxone (Figure 2A,B,C). In parallel to the enhancement of NMN, extraneuronal NA metabolite produced by COMT, we observed an increase in the MB-COMT and S-COMT in the left ventricle after naloxone-induced withdrawal. Two-way ANOVA for MB-COMT showed a significant effect of naloxone injection ($F_{1,11}=12.62$, $p=0.0045$). ANOVA for S-COMT revealed a significant effect of naloxone injection ($F_{1,9}=9.36$, $p=0.0136$) and morphine treatment ($F_{1,11}=19.06$, $p=0.0018$). As shown in Figure 2D,E, rats dependent of morphine and injected with naloxone showed a significantly ($p<0.05$) higher expression of S-COMT versus the morphine dependent group injected with saline and the placebo group receiving naloxone whereas the increase of MB-COMT expression after morphine withdrawal was only significant ($p<0.05$) versus rats dependent on morphine and injected with saline.

**ERK1/2 activation after chronic morphine treatment and withdrawal**

The influence of morphine dependence and withdrawal on phosphorylated ERK1/2 was examined in the left ventricle 60 min after s.c. injection of saline or naloxone to control (placebo) rats and to animals dependent on morphine (Figure 3). Two-way ANOVA for phospho-ERK1 and phospho-ERK2 expression at left ventricle showed a significant effect of naloxone injection ($F_{1,19}=3.85$, $p=0.0645$ and $F_{1,19}=15.52$, $p=0.0009$, respectively) and morphine treatment ($F_{1,19}=20.12$, $p=0.0003$ and $F_{1,19}=7.04$, $p=0.0157$, respectively). Our study shows that chronic morphine treatment did not induce significant changes in phospho-ERK1 or phospho-ERK2 levels after saline injection (Figure 3A,B). Naloxone
treatment had no effect on animals chronically treated with placebo. However, rats dependent on morphine and given naloxone showed a significant ($p<0.01$) elevation of phospho-ERK1 and phospho-ERK2 60 min after the administration of the opioid antagonist versus the control group injected with naloxone or the dependent group injected with saline.

According to previous studies from our laboratory (Almela 2008, 2009) demonstrating that SL327 (100 mg/kg, i.p.) reduced basal expression of phospho-ERK1/2 immunoreactivity in the heart, rats were pretreated with SL327 (100 mg/kg, i.p.). As shown in Figure 3 C,D, phosphorylation of ERK1/2 was significantly ($p<0.001$) decreased in the presence of SL327 in both controls and morphine withdrawn animals.

**Influence of morphine withdrawal on HPA axis activation**

One-way ANOVA revealed significant differences in plasma ACTH levels ($F_{5,18}=8.330$, $p=0.0003$) and plasma corticosterone levels ($F_{5,18}=24.72$, $p<0.0001$) in rats pretreated with vehicle, propranolol or SL327. As shown in Figure 4 A,B, naloxone-precipitated morphine withdrawal evoked a dramatic increase ($p<0.001$) of both corticosterone and ACTH secretion compared with morphine dependent rats treated with saline instead of naloxone or placebo rats injected with the opioid antagonist.

To evaluate the role for NA in the regulation of CRF release and pituitary-adrenal function we have evaluated the effects of propranolol (3 mg/kg, i.p.) on plasma ACTH and corticosterone concentrations. As it can be seen in Figure 4A, administration of propranolol 20 min before naloxone to morphine dependent rats significantly ($P<0.05$) decreased plasma ACTH concentrations compared with the group treated with vehicle instead of propranolol. However, the beta-adrenoceptor antagonist did not prevent the enhancement of corticosterone secretion observed after naloxone-induced withdrawal in morphine dependent rats (Figure 4B).
Since previous studies (Nuñez et al., 2007, 2009) from our laboratory have demonstrated an enhancement of ERK 1/2 after naloxone precipitated withdrawal in the PVN in the present study we have investigated if a causal link exists between ERK1/2 and HPA axis activation during morphine withdrawal. We measured plasma ACTH and corticosterone concentrations in animals made dependent on morphine and treated with SL327 (100 mg/kg, i.p.) 60 min before naloxone administration. SL327 significantly ($p<0.01$) reduced morphine withdrawal-induced increases in plasma ACTH compared with rats receiving vehicle instead of SL327 whereas plasma ACTH in morphine dependent rats injected with SL327+naloxone was significantly ($p<0.05$) higher than in morphine+vehicle+saline or placebo+SL327+naloxone groups. Similarly to propranolol SL327 did not modify the increase of corticosterone levels observed during morphine withdrawal (Figure 4 A,B).

**Expression of Hsp27 and phospho-Hsp27 in chronic morphine treatment and withdrawal**

We examined Hsp27 expression and phospho-Hsp27 at Ser 82, which is highly expressed in the heart (Kato et al., 1992) to determine the magnitude and severity of cellular stress during chronic morphine treatment and withdrawal. Two-way ANOVA for Hsp27 expression revealed a main effect of morphine treatment (30 min: $F_{1,16}=14.19$, $p=0.0017$; 60 min: $F_{1,18}=24.07$, $p=0.0001$; 90 min: $F_{1,16}=27.28$, $p<0.0001$). Post hoc revealed that chronic treatment with morphine induced an increase (30 min: $p<0.05$; 60 min: $p<0.01$; 90 min: $p<0.05$) of Hsp27 expression in the left ventricle compared with the corresponding control group (placebo+saline) (Figure 5 A,B,C). Acute naloxone treatment had no effect on animals chronically treated with placebo (control). However, Hsp27 expression was increased ($p<0.05$) 30, 60 and 90 min after naloxone injection to rats dependent on morphine versus the control group chronically treated with placebo and injected with naloxone (Figure 5 A,B,C).
We also studied the phosphorylation of Hsp27 at Ser82 in the left ventricle at different time points. Two-way ANOVA results for Hsp27 phosphorylated in Ser82 revealed a significant effect of naloxone injection (30 min: F_{1,18}=20.50, p=0.0003; 60 min: F_{1,18}=4.41, p=0.050; 90 min: F_{1,18}=13.16, p=0.0019), morphine treatment (30: F_{1,18}=15.05, p=0.0011; 60 min: F_{1,18}=5.53, p=0.0302) and significant interaction between morphine treatment and naloxone injection (30: F_{1,18}=16.82, p=0.0007; 90 min: F_{1,18}=11.63, p=0.0031). As shown in Figure 6 A,B,C, after saline injection to morphine dependent rats, there were not changes in the levels of phospho-Ser82-Hsp27. However, rats dependent on morphine and given naloxone showed significant (p<0.01) increases in phospho-Ser82-Hsp27 after the opioid antagonist injection compared with the corresponding placebo group receiving naloxone and with the morphine dependent animals receiving saline (30 min: p<0.01; 60 min: p<0.01; 90 min: p<0.05) (Figure 6 A,B,C). The ratio phospho-Hsp27/total-Hsp27 is represented in Figure 7. Two-way ANOVA results for pHsp27/Hsp27 ratio revealed a significant effect of naloxone injection (30 min: F_{1,16}=14.96, p=0.0015; 60 min: F_{1,18}=10.13, p=0.0052) and interaction between morphine treatment and naloxone injection (30min: F_{1,16}=8.95, p=0.0086). Post hoc test showed that morphine dependent rats treated with saline presented a decrease in this ratio versus the dependent group treated with naloxone (30 min: p<0.001; 60 min: p<0.01; 90 min: p<0.05) or the placebo group receiving saline (30 min: p<0.01; 60 min: p<0.05; 90 min: p<0.05).

We have evaluated the expression of Hsp27 and its phosphorylation at Ser82 in rats treated with propranolol, or SL327. Two-way ANOVA for Hsp27 in rats pretreated with propranolol revealed a main effect of morphine treatment (F_{1,18}=11.77, p=0.0030). As observed in Figure 8 A,B, morphine dependent rats treated with propranolol before naloxone showed an increased expression of Hsp27 (p<0.05) similar to that obtained in the group treated with vehicle instead of propranolol. Two-way ANOVA for Hsp27 phosphorylated at Ser82 in animals preteated with propranolol revealed that propranolol
injection and the interaction between morphine pretreatment and acute propranolol injection had a significant effect on Hsp27 phosphorylation (injection: $F_{1,18}=23.11$, $p=0.0001$; interaction: $F_{1,18}=19.81$, $p=0.0003$). As shown in Figure 8B, propranolol antagonized the increased expression of Hsp27 phosphorylation at Ser82 observed after naloxone-induced morphine withdrawal ($p<0.01$). Two-way ANOVA results for Hsp27 expression in rats pretreated with SL327 revealed a significant interaction between morphine treatment and acute SL327 injection ($F_{1,18}=7.82$, $p=0.0119$) while ANOVA for Hsp27 phosphorylation revealed that morphine treatment, SL327 injection, and the interaction between morphine treatment and acute SL327 injection had a significant effect on Hsp27 phosphorylation (morphine treatment: $F_{1,18}=78.80$, $p<0.0001$; injection: $F_{1,18}=131.61$, $p<0.0001$; interaction: $F_{1,18}=79.43$, $p<0.0001$). Post hoc analysis showed that the pretreatment with SL327 did not modify the increase of Hsp27 expression observed after naloxone administration to morphine dependent rats (Figure 8C). In contrast, the administration of SL327 before naloxone significantly ($p<0.001$) reduced the activation (phosphorylation at Ser82) of Hsp27 in morphine withdrawn rats (Figure 8D).
Discussion

The principal findings of the present study are as follows: 1) Naloxone-induced withdrawal increases plasma ACTH and corticosterone concentrations in parallel with an enhancement in the expression of MB-COMT and S-COMT, which could be responsible for the enhancement of NMN and NA turnover; 2) The severity of cellular stress during chronic morphine treatment and withdrawal is also evidenced by the expression and activation of Hsp27 and 3) Propranolol and SL327 do not block corticosterone release and Hsp27 expression whereas plasma ACTH concentrations and Hsp27 activation (phosphorylation) are significantly attenuated.

Previous studies have demonstrated that chronic μ-opioid receptor stimulation decreases muscle sympathetic nerve activity (Kienbaum et al., 2001, 2002), NA plasma concentration (Kiembau et al., 2001), dopamine turnover in the heart (Rabadán et al, 1997) and mean arterial pressure (MAP) and heart rate (HR) (Almela et al., 2011). However, μ-opioid receptor blockade by naloxone in patients with chronic opioid abuse or in morphine dependent rats unmasks these effects, resulting in markedly increased muscle sympathetic nerve activity, plasma NA concentrations (Peart and Gross, 2006), NA and dopamine turnover (Almela et al., 2008), total tyrosine hydroxylase expression (Almela et al., 2009) and an enhancement in MAP and HR (Almela et al., 2011), two objective and accurate measurable signs of opioid withdrawal in human. According to these data, present results demonstrated that withdrawal caused a decrease in myocardial NA levels and increases in myocardial NMN (extraneuronal NA metabolite generated by COMT), in parallel with an increased expression of two isoforms of COMT: MB-COMT and S-COMT, suggesting that both forms of COMT are implicated in the degradation of NA.
Several areas of the central nervous system are involved in the integration between behavioral and cardiovascular response associated with morphine withdrawal. Thus, naloxone-induced morphine withdrawal produces adaptive changes in the heart (Almela et al., 2008; Peart and Gross, 2006; Rabadán et al., 1997) as well as activation of the HPA axis (Cleck and Blendy, 200; Laorden et al., 2002; Nuñez et al., 2009). According to previous reports, present findings demonstrated that naloxone administration to morphine dependent rats significantly elevated plasma ACTH and corticosterone concentrations. Enhanced responsiveness of the HPA axis after morphine withdrawal has been associated with activation of noradrenergic neurons in the nucleus of the solitary tract (NTS-A₂) that project to the hypothalamic PVN suggesting that one of the neuronal mechanisms that underlie morphine withdrawal-induced activation of the HPA axis may be dependent on activation of noradrenergic pathways innervating the PVN (Fuertes et al., 2000; Laorden et al., 2000, 2002). Adrenergic receptors expressed within the PVN include the alpha-2, alpha-1 and beta subtypes (Leibowitz et al., 1982). Since previous studies from our laboratory (Laorden et al., 2000) have demonstrated that the hyperactivity of the HPA axis during morphine withdrawal is mediated via a stimulatory noradrenergic pathway, we have investigated the role of beta-adrenoceptor in the HPA axis hyperactivity after morphine withdrawal. We found that propranolol significantly reduces the ability of morphine withdrawal to release ACTH, but this drug was not capable of modifying corticosterone secretion. We also measured plasma corticosterone and ACTH concentrations in animals made dependent on morphine and pretreated with SL327 to evaluate whether a causal link exists between ERK activation and HPA axis hyperactivation during morphine withdrawal. Similarly, to propranolol, SL327 did not block corticosterone release that is produced as a consequence of morphine withdrawal. However, ACTH concentrations were found to be decreased in animals pretreated with SL327. Although the presence of pituitary ACTH is clearly essential for adrenocortical
function, ACTH-independent mechanisms seem to have an important role in fine-tuning and modulating the highly sensitive adrenal stress system to adapt its response appropriately to physiological needs. In recent years, numerous studies have been published indicating that a large number of neuropeptides, neurotransmitters, opioids, growth factors, cytokines, adipokines and even bacterial ligands are capable of modulating adrenal glucocorticoid release independently of pituitary ACTH (for review see Bornstein et al., 2008). Adrenocortical cells express a great variety of receptor for these factors, thus enabling potential direct actions on corticoids release. Lesions of upstream stress regulatory pathways in the brain lead to dissociation between ACTH and corticosterone, suggesting that central nervous system pathways are capable of regulating HPA axis function at both the pituitary and adrenal level. For example, lesions of the anterior bed nucleus of the stria terminalis are able to attenuate corticosterone secretion without an attendant change in circulating ACTH (Choi et al., 2007). In addition, in rats, sucrose or saccharine consumption causes a decrease in corticosterone secretion without an accompanying alteration in ACTH, indicating that adrenal secretion can be decreased directly by central activation of reward pathways in the brain (Ulrich-Lai et al., 2007). Altogether these results suggesting that neurochemical and/or neurohormonal and neural stimulatory factors other than ACTH might be responsible for adrenal hyperactivity observed during morphine withdrawal, the separation of ACTH and corticosteroid secretion could have clinical relevance on endocrine stress regulation.

Since the repeated exposure to morphine and its withdrawal induces profound and severe stress reactions that are also evidenced by overexpression of (Almela et al., 2011; Houshyar et al., 2001a, 2001b; Sharma, 2004; Sharma et al., 2004 Hsp27) we have evaluated Hsp27 expression and activation in the left ventricle. According to previous data (Almela et al., 2011), present investigation shows that chronic morphine treatment and its withdrawal are associated with an increase of Hsp27 expression. However, chronic
morphine treatment did not modify Hsp27 phosphorylation, whereas naloxone administration to morphine dependent rats induced an enhancement of Hsp27 phosphorylation at Ser82. Altogether, these results support the idea that morphine dependence and withdrawal induces profound cellular stress that could produce myocardial damages (Dettmeyer et al., 2009).

Present data demonstrated an increase in phospho-ERK1 and phospho-ERK2 with a parallel increase in phospho-Hsp27 within the heart of rats withdrawn from morphine. Hsp27 responds to stress by 1) acting as a chaperone, refolding proteins to their native confirmation, which is thought to require unphosphorylated Hsp27 (Amon-Treiber et al., 2004; Peart and Gross, 2006); 2) preventing oxidative damage, which is also believed to require the non-phosphorylated form (Rogalla et al., 1999); 3) acting as an anti-apoptotic molecule preventing cell death (Mehelen et al., 1997); or 4) Hsp27 reacts to stress, exerting protective effects by regulating actin cytoskeleton reorganization (Robin et al., 2010). We found that treatment with SL327 decreases the activation of both Hsp27 and HPA axis suggesting that ERK activation triggers Hsp27 phosphorylation at Ser82 and ACTH plasma concentrations. Lastly, we examined if beta-adrenoceptors are involved in mediating the influence of noradrenergic pathways after naloxone-induced withdrawal. We found that propranolol reduces Hsp27 phosphorylation after naloxone-induced withdrawal suggesting that beta-adrenergic blockade in the heart attenuated the protective response to cellular stressors. According to these data, it is known that the administration of the beta-adrenoreceptor blocker, alprenolol during the triggering preconditioning phase of ischemia significantly attenuated cardioprotection (Lochner et al., 1999). Since Hsp27 plays a pivotal role in the protection of the cell, the fact that propranolol decreases Hsp27 phosphorylation during morphine withdrawal below the control value, suggests that during stress situations beta-adrenoreceptor blockers abolish the endogenous protective mechanism that the heart has at its disposal in basal conditions. Because propranolol
decreases ACTH and morphine withdrawal increases circulating levels of NA (Peart et al., 2007) and NA turnover in the heart, it is possible that changes in peripheral catecholamines represent an important mechanism in the stimulatory effect of naloxone-induced withdrawal on the HPA axis. Together, these results demonstrated a reduction on plasma ACTH concentrations in parallel with a decrease in Hsp27 activation suggesting that Hsp27 phosphorylation at Ser82 is upregulated by ACTH. According to these results, it has been demonstrated in the adrenal cortex that stress induces an increased expression of Hsp70 (Blake et al., 1993; Udelsman et al., 1994) which was abolished in hypophysectomized rats (Blake et al., 1991; 1993), suggesting a role for ACTH in that reduction. In adrenocortical tumors which secrete high quantities of cortisol (and thus lead to a reduction of ACTH production), it has been observed a depleted expression of Hsp27 and Hsp70 (Pignatelli et al., 2003).

In conclusion, we present novel information regarding the ability of NA and ERK pathways to induce Hsp27 phosphorylation at Ser82 in the left ventricle, as well as the role that these pathways play in mediating HPA axis hyperactivity. The existence of a possible link between ACTH and Hsp27 remains to be determined. Nevertheless, we consider that these findings are important for our overall understanding of the mechanisms mediating morphine withdrawal, and also for the development of potential therapies designed to address the adverse consequences of the withdrawal.
Authorship Contributions

Participated in research designs: Almela, Hurle, Milanés, Laorden
Conducted experiments: Martinez-Laorden, Almela
Contributed analytic tools: Martinez-Laorden, Almela, Hurle
Performed data analysis: Almela, Hurle, Milanés, Laorden
Wrote or contributed to the writing of the manuscript: Laorden, Milanés
REFERENCES


Footnotes

This work was supported by the Ministerio de Ciencia e Innovación [Grants 2009-07178 and 2010-17907] Spain and Red de Trastornos Adictivos [RTA; RD06/0001/1006].
Figure legends

Figure 1. Body weight after naloxone-precipitated withdrawal. Animals received s.c. implantation of placebo or morphine (75 mg) pellets for 7 days. On day 8, rats were injected with saline or naloxone (2 mg/kg, s.c.) and were decapitated 30 (A), 60 (B) and 90 (C) min later. Other groups of animals were pretreated with propranolol (pro, 3 mg/kg, i.p.), SL327 (SL, 100 mg/kg, i.p.), or vehicle (veh) and decapitated 60 min after naloxone (nx) injection (D). Data are the mean±SEM (n=4-15). ***p<0.001, versus the morphine dependent group receiving saline instead of naloxone; +++p<0.01 versus the control group injected with naloxone; ####p<0.001 versus placebo+pro+nx; ####$p<0.001 versus placebo+SL+nx.

Figure 2. Noradrenaline (NA) and normetanephrine (NMN) concentrations and NMN/NA ratio in the left ventricle 60 min after saline or naloxone administration to placebo or morphine dependent rats (A, B, C). Western-blotting analysis of membrane-COMT (MB-COMT) and soluble-COMT (S-COMT) (D, E). The immunoreactivity corresponding to MB-COMT or S-COMT is expressed as a percentage of that in the control group defined as 100% value. Placebo (P), saline (s), naloxone (nx) and morphine (M). Data are the mean±SEM (n=3-7). *p<0.05, **p<0.01, ***p<0.001, versus morphine+saline; +p<0.05, +++p<0.001 versus placebo+naloxone.

Figure 3. Western-blotting analysis of phospho(p)-ERK1 (A) and phospho (p)-ERK2 (B) in the left ventricle from placebo (pla, P)- or morphine (mor, M)-pretreated rats 60 min after saline (s) or naloxone (nx) administration in presence or vehicle (veh, v) or SL327 (SL, 100 mg/kg, i.p.) (C,D). The immunoreactivity corresponding to phospho-ERK1 or phospho-ERK2 is expressed as a percentage of that in the control group defined as 100% value. Data are the mean±SEM (n=5-6). **p<0.01 versus the dependent group injected
with saline instead of naloxone; ++p<0.01 versus the placebo group injected with naloxone; &&p<0.001 versus veh+nx; ###p<0.001 versus SL+nx.

**Figure 4.** Plasma adrenocorticotropic hormone (ACTH) (A) and corticosterone (B) concentrations 60 min after saline or naloxone (nx) administration to placebo or morphine dependent rats pretreated with vehicle (veh), propranolol (pro, 3 mg/kg, i.p.) or SL327 (SL, 100 mg/kg, i.p.). Data are the mean±SEM (n=3-6). *p<0.05, **p<0.001, versus the morphine dependent group receiving saline instead of naloxone; +++p<0.001 versus the control group injected with naloxone; ###p<0.01, ####p<0.001 versus placebo+pro+nx; $P<0.05, $$p<0.001 versus placebo+SL+nx; &p<0.05, &&p<0.01 versus morphine+veh+nx.

**Figure 5.** Western-blotting analysis of Hsp27 in the left ventricle 30 (A), 60 (B) and 90 (C) min after saline (s) or naloxone (nx) administration to placebo (P)- or morphine (M)-pretreated rats. The immunoreactivity corresponding to Hsp27 is expressed as a percentage of that in the control group defined as 100% value. Data are the mean±SEM (n=5-6). +P<0.05 versus the placebo group injected with naloxone; &p<0.05, &&p<0.01 versus the placebo group receiving saline.

**Figure 6.** Western-blotting analysis of Hsp27 phospho (p)-Ser82 in the left ventricle 30 (A), 60 (B) and 90 (C) min after saline (s) or naloxone (nx) administration to placebo (P)- or morphine (M)-pretreated rats. The immunoreactivity corresponding to Hsp27 phospho-Ser82 is expressed as a percentage of that in the control group defined as 100% value. Data are the mean±SEM (n=5-6). *p<0.05, **p<0.01 versus morphine+saline; ++p<0.01 versus the placebo group injected with naloxone.

**Figure 7.** Western-blotting analysis of phospho(p)-Hsp27/total (t)-Hsp27 ratio in the left ventricle 30 (A), 60 (B) and 90 (C) min after saline or naloxone administration to placebo.
or morphine pretreated rats. Data are the mean±SEM (n=5-6). *p<0.05, **p<0.01, ***p<0.001 versus morphine+naloxone; &p<0.05, &&p<0.01 versus placebo+saline

Figure 8. Western-blotting analysis of Hsp27 (A,C) and phospho(p)-Hsp27 (B,D) in the left ventricle 60 min after saline (s) or naloxone (nx) administration to placebo (P)- or morphine (M)- rats pretreated with vehicle (veh), propranolol (pro, 3 mg/kg, i.p.) or SL327 (SL, 100 mg/ kg, i.p.). The immunoreactivity corresponding to Hsp27 or phospho-Hsp27 is expressed as a percentage of that in the control group defined as 100% value. Data are the mean±SEM (n=5-6). +p<0.05, ++p<0.01 versus the placebo group injected with veh+nx; #p<0.05 versus placebo+pro+nx; &&p<0.01 versus morphine+veh+nx
Fig. 1
Fig. 2
Fig. 3
Figure 4
Fig. 5

A

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio Hsp27/β-Actin (% versus control)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio Hsp27/β-Actin (% versus control)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio Hsp27/β-Actin (% versus control)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hsp27

β-actin
Fig. 6
Figure 7
**Figure 8**

A and B: Graph showing the ratio of Hsp27/β-Actin (%versus control) for placebo and morphine groups with pro+nx and veh+nx conditions.

C and D: Graph showing the ratio of pHsp27/β-Actin (%versus control) for placebo and morphine groups with SL+nx and veh+nx conditions.

**Legend:**
- veh+nx
- pro+nx
- M+veh+nx
- M+pro+nx
- SL+nx

**Images:**
- Western blots for Hsp27 and β-actin proteins under different conditions.