Morphine Withdrawal Activates Hypothalamic-Pituitary-Adrenal Axis and Heat Shock Protein 27 in the Left Ventricle: The Role of Extracellular Signal-Regulated Kinase

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ABSTRACT

The negative affective states of withdrawal involve the recruitment of brain and peripheral stress circuitry, i.e., noradrenergic activity, induction of the hypothalamic-pituitary-adrenocortical (HPA) axis, and the expression and activation of heat shock proteins (Hsps). The present study investigated the role of extracellular signal-regulated protein kinase (ERK) and β-adrenoceptor on the response of stress systems to morphine withdrawal by the administration of [amino[(4-aminophenyl)thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile (SL327), a selective inhibitor of ERK activation, or propranolol (a β-adrenoceptor antagonist). Dependence on morphine was induced by a 7-day subcutaneous implantation of morphine pellets. Morphine withdrawal was precipitated on day 8 by the injection of naloxone (2 mg/kg s.c.). Plasma concentrations of adrenocorticotropic hormone and corticosterone were determined by radioimmunoassay; noradrenaline (NA) turnover in left ventricle was determined by high-performance liquid chromatography; and catechol-O-methyl transferase (COMT) and Hsp27 expression and phosphorylation at 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 Morphine-withdrawn rats showed an increase of NA turnover and COMT expression in parallel with an enhancement of adrenocorticotropic and plasma corticosterone concentrations.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 adrenocorticotropic and Hsp27 phosphorylation at Ser82 without any changes in plasma corticosterone levels. The present findings demonstrate that morphine withdrawal is capable of inducing the activation of Hsp27 in parallel with an enhancement of Hsp27 expression and Hsp27 phosphorylation at Ser82 and suggest a role for β-adrenoceptors and ERK pathways in mediating morphine-withdrawal activation of the HPA axis and cellular stress response.

Introduction

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

Whereas in rodents morphine may exert stimulatory and inhibitory roles on 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 the administration of naloxone/naltrexone (for review see Vuong 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 the HPA axis in rats, which results in the neuronal activation of extracellular signal-regulated kinase (ERK) and heat shock protein 27 (Hsp27).
of stress-related neurosecretory neurons in the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN), an increase in CRF transcription, and boosts of adrenocorticotropic and corticosterone secretion (Laorden et al., 2002; Cleck and Blendy, 2008; Núñez et al., 2009). Furthermore, the profound cellular stress induced by chronic morphine treatment and withdrawal is also evidenced by the 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 after stress, but also undergo several post-translational modifications that alter their functional roles for use 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 the phosphorylation-dependent stabilization of actin (Pear et al., 2007). Hsp27 can be reversibly phosphorylated on three serine residues by mitogen-activated protein kinases 2 and 3, which are themselves activated by phosphorylation through either the p38 or the extracellular signal-regulated protein kinase (ERK) signaling pathway (Guay et al., 1997; Jantschitsch et al., 1998; 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 signaling 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 and neurohormone secretion (Osterlund et al., 2011). In addition, it has been shown that Hsp27 and Hsp70 are up-regulated by morphone and corticosterone levels were determined by radioimmunoassay in morphine-dependent and control rats treated with SL327, a selective inhibitor of mitogen-activated extracellular protein kinase (Atkins et al., 1998) or vehicle (dimethyl sulfoxide) administered 1 h before the injection of naloxone or saline. SL327 was dissolved in 100% dimethyl sulfoxide and injected intraperitoneally at an injection volume of 1 ml/kg and doses of 100 mg/kg. Previous studies (Almeida et al., 2008, 2009) have demonstrated that this dose of SL327 induces the inhibition of ERK phosphorylation.

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

Determination of NA and Its Metabolite MNM in the Left Ventricle. NA and its metabolite MNM 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 (Kinetica, Littau-Lucerne, Switzerland) in 1.5 ml of perchloric acid (0.1 M). The homogenates were then centrifuged (8000g; 4°C; 15 min), and the supernatant layer was removed into a 1-ml syringe and filtered through a 0.45-μm filter (Millipore Corporation, Billerica, MA) and centrifuged (6000g; 4°C; 20 min) again through an Ultrafree MC 0.2 filter (Millipore Corporation). From each sample, 10 μl was injected into a 5-μm C18 reverse-phase column (Waters, Milford, MA) through a Rheodyne syringe-loading injector 200-μl loop (IDEX Health and Science, Oak Harbor, WA). 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%/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 analyzed with Millenium 2010 Chromatography Manager Equipment (Millipore Corporation). NA and NMN were simultaneously detected by the described HPLC method at elution times of 4.25 and 7.32 min, respectively. NA and NMN were quantified by reference to calibration curves run at the beginning and end of each series of assays. Linear relationships were observed between the amount of standard injected and the peak height measured. The lowest 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% SDS plus protease inhibitors (Roche Diagnostics, Mannheim, Germany), and phosphatase inhibitors Cocktail Set (Calbiochem, Darmstadt, Germany)] and homogenized for 50 s before centrifugation at 6000 rpm. The optimum amount of protein to be loaded was determined by spectrophotometrically using the bicinchoninic acid method (Wichelman et al., 1988). The optimum amount of protein to be loaded was determined in preliminary experiments by loading gels with increasing protein contents (25–100 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, electrophoresed, and transferred onto a polyvinylidene difluoride membrane by using a Mini Trans-Blot Electroblotting Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Nonspecific binding of antibodies was mitigated by incubating membranes with 1% bovine serum albumin in Tris buffer saline (TBST; 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween 20). Blots were incubated overnight at room temperature (for total Hsp27 and Hsp27 phosphorylated at Ser82) or 4°C (for phospho-ERK and COMT) with the following primary antibodies: polyclonal antitotal Hsp27 antibody (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal antiphospho Ser82 Hsp27 (1:400 dilution; Abcam plc, Cambridge, UK), monoclonal anti-pERK1/2 (1:1000; Santa Cruz Biotechnology, Inc.), or monoclonal anti-COMT (1:5000; Millipore Bioscience Research Reagents, Temecula, CA) in TBST with bovine serum albumin. After extensive washings with TBST, the membranes were incubated for 1 h at room temperature with peroxidase-labeled secondary antibodies (anti-goat for total Hsp27; anti-rabbit for Hsp27 phosphorylated at Ser82; anti-mouse for phospho-ERK1/2 and COMT; Santa Cruz Biotechnology, Inc.) at 1:5000 dilution. After washing, immunoreactivity was detected by using an enhanced chemiluminescent/chemifluorescent Western blot detection system (ECL Plus; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). Antibodies were stripped from the blots by incubation with stripping buffer (25 mM glycine and 1% SDS, pH 2) for 1 h at 37°C. We used anti-β actin (Cell Signaling Technology, Danvers, MA; 45 kDa) or antitotal ERK (Santa Cruz Biotechnology, Inc.; 42 kDa) as our loading control for all experiments. The ratio of total Hsp27/β-actin, phospho-Hsp27/β-actin, phospho-Hsp27/total Hsp27, phospho-ERK1/total ERK, phospho-ERK2/total ERK, and COMT/β-actin was plotted and analyzed. 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, Spain). 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 Pharmaceutical Technology, School of Pharmacy, University of Granada (Granada, Spain). Sodium docetyl sulphate, polyacrylamide gel, and polyvinylidene difluoride membrane were obtained from Teknovas (Bilbao, Spain). SL327 was obtained from Abcam plc. NA bitartrate, NMN (used as an HPLC standard), naloxone HCl, propranolol, and Western blot reagents were purchased from Sigma (St. Louis, MO). Naloxone HCl was dissolved in sterile 0.9% NaCl (saline) and administered in volumes of 0.1 ml/100 g body weight.

**Data Analysis.** Data are expressed as mean ± S.E.M. and were analyzed by 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 was restricted to two experimental groups. Differences with a p value <0.05 were considered significant.

**Results.**

The animals' weights were recorded on the days of pellet implantation and 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; F1,13 = 24.83, p < 0.0001; F1,12 = 7.96, p = 0.0154, respectively), chronic morphine administration (F1,13 = 12.50, p = 0.0037; F1,13 = 15.91, p = 0.0004; F1,13 = 8.79, p = 0.0118, respectively), and an interaction between morphine treatment and naloxone injection (F1,13 = 12.50, p = 0.0037; F1,13 = 15.91, p = 0.0004; F1,12 = 8.79, p = 0.118, respectively). As shown in Fig. 1, A to 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 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 (F1,12 = 23.21, p < 0.0001). As shown in Fig. 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 pre-treated 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 (F1,20 = 8.82; p = 0.0076) with main effect of morphine treatment (F1,20 = 8.86; p = 0.0075) and naloxone injection (F1,20 = 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 compared with morphine-depen-
dent rats receiving saline instead of naloxone or naive rats injected with naloxone (Fig. 2, A-C). In parallel to the enhancement of NMN, an extraneuronal NA metabolite produced by COMT, we observed an increase in 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 Fig. 2, D and E, morphine-dependent rats 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 subcutaneous injection of saline or naloxone to control (placebo) rats and animals dependent on morphine (Fig. 3). Two-way ANOVA for phospho-ERK1 and phospho-ERK2 expression at the left ventricle showed a significant effect of naloxone injection ($F_{1,19} = 3.85, p = 0.0645; F_{1,19} = 15.52, p = 0.0009$, respectively) and morphine treatment ($F_{1,19} = 20.12, p = 0.0003; F_{1,19} = 7.04, p = 0.0157$, respectively). Our study shows that chronic morphine treatment did not induce significant changes in phospho-ERK1 or phospho-ERK2 levels after saline injection (Fig. 3, A and 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.

As in our previous studies (Almela et al., 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 Fig. 3, C and D, phosphorylation of ERK1/2 was significantly ($p < 0.001$) decreased in the presence of SL327 in both control and morphine-withdrawn animals.

**Influence of Morphine Withdrawal on HPA Axis Activation.** One-way ANOVA revealed significant differences in plasma adrenocorticotropic levels ($F_{3,18} = 8.330; p = 0.0003$) and plasma corticosterone levels ($F_{5,18} = 24.72; p < 0.0001$) in rats pretreated with vehicle, propranolol, or SL327. As shown in Fig. 4, naloxone-precipitated morphine withdrawal evoked a dramatic increase ($p < 0.001$) of both corticosterone and adrenocorticotropic 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 examined the effects of propranolol (3 mg/kg i.p.) on plasma adrenocorticotropic and corticosterone concentrations. As seen in Fig. 4A,
the administration of propranolol 20 min before naloxone to morphine-dependent rats significantly \((p < 0.05)\) decreased plasma adrenocorticotropic and corticosterone concentrations compared with the group treated with vehicle. However, the \(\beta\)-adrenoceptor antagonist did not prevent the enhancement of corticosterone secretion observed after naloxone-induced withdrawal in morphine-dependent rats (Fig. 4B).

Because previous studies (Núñez et al., 2007, 2009) have demonstrated an enhancement of ERK1/2 after naloxone precipitated withdrawal in the PVN in the present study, we investigated whether a causal link exists between ERK1/2 and HPA axis activation during morphine withdrawal. We measured plasma adrenocorticotropic and corticosterone concentrations in morphine-dependent animals 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 adrenocorticotropic compared with rats receiving vehicle instead of SL327, whereas plasma adrenocorticotropic in morphine-dependent rats injected with SL327 + naloxone was significantly \((p < 0.05)\) higher than in the morphine + vehicle + saline or placebo + SL327 + naloxone groups. Similarly to propranolol, SL327 did not modify the increase of corticosterone levels observed during morphine withdrawal (Fig. 4).

Expression of Hsp27 and Phospho-Hsp27 in Chronic Morphine Treatment and Withdrawal. We examined Hsp27 expression and phospho-Hsp27 at Ser82, 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 \((30 \text{ min}, F_{1,16} = 14.19, p = 0.0017; 60 \text{ min}, F_{1,16} = 24.07, p = 0.0001; 90 \text{ min}, F_{1,16} = 27.28, p < 0.0001)\). Post hoc tests revealed that chronic treatment with morphine induced an increase \((30 \text{ min}, p < 0.05; 60 \text{ min}, p < 0.01; 90 \text{ min}, p < 0.05)\) of Hsp27 expression in the left ventricle compared with the corresponding control group (placebo + saline) (Fig. 5). 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 in morphine-dependent rats versus the control group chronically treated with placebo and injected with naloxone (Fig. 5).

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 \((30 \text{ min}, F_{1,18} = 20.50, p = 0.0003; 60 \text{ min}, F_{1,18} = 4.41, p = 0.050; 90 \text{ min}, F_{1,18} = 13.16, p = 0.001)\).
injection (30 min, p = 0.0019) and morphine treatment (30 min, \( F_{1.18} = 15.05, p = 0.0011; 60 \text{ min}, F_{1.18} = 5.53, p = 0.0302 \)) and significant interaction between morphine treatment and naloxone injection (30 min, \( F_{1.18} = 16.82, p = 0.0007; 90 \text{ min}, F_{1.18} = 11.63, p = 0.0031 \)). As shown in Fig. 6, after saline injection in morphine-dependent rats there were no changes in the levels of phospho-Ser82-Hsp27. However, morphine-dependent rats 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 the morphine-dependent animals receiving saline (30 min, \( p < 0.01; 60 \text{ min}, p < 0.01; 90 \text{ min}, p < 0.05 \)) (Fig. 6). The ratio of phospho-Hsp27/total-Hsp27 is presented in Fig. 7. Two-way ANOVA results for the phospho-Hsp27/Hsp27 ratio revealed a significant effect of naloxone injection (30 min, \( F_{1.18} = 14.96, p = 0.0015; 60 \text{ min}, F_{1.18} = 10.13, p = 0.0052 \)) and interaction between morphine treatment and naloxone injection (30 min, \( F_{1.18} = 8.95, p = 0.0086 \)). Post hoc tests showed that morphine-dependent rats treated with saline presented a decrease in this ratio versus the morphine-dependent group treated with naloxone (30 min, \( p < 0.001; 60 \text{ min}, p < 0.01; 90 \text{ min}, p < 0.05 \)) or the placebo group receiving saline (30 min, \( p < 0.01; 60 \text{ min}, p < 0.05; 90 \text{ min}, p < 0.05 \)).

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 \( (F_{1.18} = 11.77, p = 0.0030) \) similar to that obtained in the group treated with vehicle instead of propranolol. Two-way ANOVA for Hsp27 phosphorylated at Ser82 in animals pretreated with propranolol revealed that propranolol injection and the interaction between morphine pretreatment and acute propranolol injection had a significant effect on Hsp27 phosphorylation \( (F_{1.18} = 23.11, p = 0.0001; \text{interaction}, F_{1.18} = 19.81, p = 0.0003) \). As shown in Fig. 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), whereas 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 pretreatment with SL327 did not modify the increase of Hsp27 expression observed after naloxone administration to morphine-dependent rats (Fig. 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 (Fig. 8D).

**Discussion**

The principal findings of the present study are: 1) naloxone-induced withdrawal increases plasma adrenocorticotropin 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 adrenocorticotropin concentrations and Hsp27 activation (phosphorylation) are significantly attenuated.

Previous studies have demonstrated that chronic $\mu$-opioid receptor stimulation decreases muscle sympathetic nerve activity (Kienbaum et al., 2001, 2002), NA plasma concentration (Kienbaum et al., 2001), dopamine turnover in the heart (Rabadán et al., 1997), and mean arterial pressure and heart rate (Almela et al., 2011). However, $\mu$-opioid receptor blockade by naloxone in patients with chronic opioid abuse or

Figure 4. Plasma adrenocorticotropin (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 ± S.E.M. (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 min (A), 60 min (B), and 90 min (C) 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 ± S.E.M. (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.
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 mean arterial pressure and heart rate (Almela et al., 2011), two objective and accurate measurable signs of opioid withdrawal in humans. The 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 (Rabadán et al., 1997; Peart and Gross, 2006; Almela et al., 2008) and activation of the HPA axis (Laorden et al., 2002; Cleck and Blendy, 2008; Núñez et al., 2009). The present findings demonstrated that naloxone administration to morphine-dependent rats significantly elevated plasma adrenocorticotropic 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 that project to the hypothalamic PVN, suggesting that one of the neuronal mechanisms that underlie morphine withdrawal-induced activation of the HPA axis may depend on the activation of noradrenergic pathways innervating the PVN (Fuertes et al., 2000; Laorden et al., 2000, 2002). Adrenergic receptors expressed within the PVN include the α2, α1, and β subtypes (Leibowitz et al., 1982). Because our previous studies (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 β-adrenoceptor in HPA axis hyperactivity after morphine withdrawal. We found that propranolol significantly reduces the ability of morphine withdrawal to release adrenocorticotropic, but this drug was not capable of modifying corticosterone secretion. We also measured plasma corticosterone and adrenocorticotropic concentrations in morphine-dependent animals pretreated with SL327 to evaluate whether a causal link exists between ERK activation and HPA axis hyperactivation during morphine withdrawal. Like propranolol, SL327 did not block corticosterone release that was produced as a consequence of morphine withdrawal. However, adrenocorticotropic concentrations were decreased in animals pretreated with SL327. Although the presence of pituitary adrenocorticotropic is clearly essential for adrenocortical function, adrenocorticotropic-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. 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 adrenocorticotropic (for review see Bornstein et al., 2008). Adrenocortical cells express a great variety of receptor for these factors, thus enabling potential direct actions on corticoid release. Lesions of upstream stress regulatory pathways in the brain lead to dissociation between adrenocorticotropic and corticosterone, suggesting that central nervous system pathways are capable of regulating HPA axis function at both the pituitary and adrenal levels. For example, lesions of the anterior bed nucleus of the stria terminalis are able to attenuate corticosterone secretion without an attendant change in circulating adrenocorticotropic (Choi et al.,
In addition, in rats sucrrose or saccharine consumption causes a decrease in corticosterone secretion without an accompanying alteration in adrenocorticotropin, indicating that adrenal secretion can be decreased directly by the central activation of reward pathways in the brain (Ulrich-Lai et al., 2007). Altogether, these results suggest that neurochemical and/or neurohormonal and neural stimulatory factors other than adrenocorticotropin might be responsible for adrenal hyperactivity observed during morphine withdrawal, so the separation of adrenocorticotropin and corticosteroid secretion could have clinical relevance on endocrine stress regulation.

Because the repeated exposure to morphine and its withdrawal induces profound and severe stress reactions that are also evidenced by overexpression of Hsp27 (Almela et al., 2011; Houshyar et al., 2001a, 2001b; Sharma, 2004; Sharma et al., 2004) we have evaluated Hsp27 expression and activation in the left ventricle. Like previous data (Almela et al., 2011), the present investigation shows that chronic morphine treatment and its withdrawal are associated with an increase of Hsp27 expression. However, chronic morphine treatment did not modulate 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 induce profound cellular stress that could produce myocardial damages (Dettmeyer et al., 2009).

The present data demonstrate 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 conformation, which is thought to require unphosphorylated Hsp27 (Ammon-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 antiapoptotic molecule preventing cell death (Mehlen et al., 1997); or 4) exerting protective effects by regulating actin cytoskeleton reorganization (Robinson et al., 2010). We found that treatment with SL327 decreases the activation of both Hsp27 and the HPA axis, suggesting that ERK activation triggers Hsp27 phosphorylation at Ser82 and adrenocorticotropin plasma concentrations. Finally, we examined whether β-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 β-adrenergic blockage in the heart attenuated the protective response to cellular stressors. According to these data, it is known that the administration of the β-adrenoceptor blocker alprenolol, during the triggering preconditioning phase of ischemia, significantly attenuated cardioprotection (Lochner et al., 1999). Because 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 β-adrenoceptor blockers abolish the endogenous protective mechanism that the heart has at its disposal in basal conditions. Because propranolol decreases adrenocorticotropin 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 adrenocorticotropin concentrations in parallel with a decrease in Hsp27 activation, suggesting that Hsp27 phosphorylation at Ser82 is up-regulated by adrenocorticotropin. 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 adrenocorticotropin in that reduction. In adrenocortical tumors that secrete high quantities of cortisol (and thus lead to a reduction of adrenocorticotropin production), depleted expression of Hsp27 and Hsp70 has been observed (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 and the role that these pathways play in mediating HPA axis hyperactivity. The existence of a possible link between adrenocorticotropin 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 the development of potential therapies designed to address the adverse consequences of the withdrawal.

Authorship Contributions
Participated in research design: Hurle, Milanes, Laorden, and Almela.
Conducted experiments: Martinez-Laorden and Almela.
Contributed new reagents or analytic tools: Martinez-Laorden, Hurle, and Almela.
Performed data analysis: Hurle, Milanes, Laorden, and Almela.
Wrote or contributed to the writing of the manuscript: Milanes and Laorden.

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