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IL-1α and TNFα Down-Regulate CRH Receptor-2 mRNA Expression in the Mouse Heart

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Two receptors (CRH receptor type 1 and CRH receptor type 2) have been identified for the stress-induced neuropeptide, CRH and related peptides, urocortin, and urocortin II. We previously found marked down-regulation of cardiac CRH receptor type 2 expression following administration of bacterial endotoxin, lipopolysaccharide, a model of systemic immune activation, and inflammation. We postulated that inflammatory cytokines may regulate CRH receptor type 2. We show that systemic IL-1α administration significantly down-regulates CRH receptor type 2 mRNA in mouse heart. In addition, TNFα treatment also reduces CRH receptor type 2 mRNA expression, although the effect was not as marked as with IL-1α. However, CRH receptor type 2 mRNA expression is not altered in adult mouse ventricular cardiomyocytes stimulated *in vitro* with TNFα or IL-1α. Thus, cytokine regulation may be indirect. Exogenous administration of corticosterone *in vivo* or acute restraint stress also reduces cardiac CRH receptor type 2 mRNA expression, but like cytokines, *in vitro* corticosterone treatment does not modulate expression in cardiomyocytes. Interestingly, treatment with urocortin significantly decreases CRH receptor type 2 mRNA in cultured cardiomyocytes. We speculate that *in vivo*, inflammatory mediators such as lipopolysaccharide and/or cytokines may increase urocortin, which in turn down-regulates CRH receptor type 2 expression in the heart. Because CRH and urocortin increase cardiac contractility and coronary blood flow, impaired CRH receptor type 2 function during systemic inflammation may ultimately diminish the adaptive cardiac response to adverse conditions. (*Endocrinology* 142: 3537–3545, 2001)

CRH is well known as a primary mediator of the mammalian stress response, acting through numerous central pathways to initiate an array of neuroendocrine, behavioral, and autonomic adaptive changes (1). Considerable evidence also suggests a role for CRH in regulating peripheral responses. Iv administration of CRH elicits a number of cardiovascular changes that include marked hypotension and vasodilation in selective vascular beds (2–4). Direct actions of CRH have been demonstrated in isolated working heart preparations where addition of CRH induces a sustained increase in coronary blood flow, a transient positive inotropic effect, and a rapid rise in the release of atrial natriuretic peptide (5). Urocortin (Ucn), a member of the CRH family which shares 45% homology to rat/human CRH and 63% homology to fish urotensin, also has pronounced effects on the cardiovascular system when given systemically that exceed those elicited by CRH (6). Ucn produces a marked, long-lasting (>30 min) reduction in mean arterial pressure in rats (6) and increases cardiac contractility and coronary blood flow in sheep (7). It is unknown as yet whether the recently discovered CRH-related peptide, urocortin II (Ucn-II), significantly influences cardiovascular function (8).

CRH receptor type 2 (CRH-R2), which bears 69% sequence identity with CRH receptor type 1 (CRH-R1) (9–12), is highly expressed in peripheral sites, including heart, skeletal muscle, gastrointestinal tract and arterioles, with lower levels of expression in limited brain regions (11, 13–15). This pattern of expression is distinct from CRH-R1, which is found predominantly in the pituitary and various brain regions including cerebral cortex, cerebellum, and brain stem (10, 16). We have recently shown that murine cardiovascular responses to systemic Ucn depend critically on CRH-R2. Ucn causes a pronounced decrease in mean arterial pressure in wild-type mice, whereas mice lacking functional CRH-R2 ($Crhr2^{-/-}$) do not show this hypotensive response to Ucn (17). In addition, we found that Ucn injection increases the velocity of left ventricular contraction in wild-type mice but again had no effect in $Crhr2^{-/-}$ mice. Thus, $Crhr2^{-/-}$ mice do not display measurable cardiovascular responses to systemic Ucn, providing strong evidence that CRH-R2 mediates Ucn-induced effects on cardiovascular function. CRH-R2 located on cardiac myocytes may mediate CRH/Ucn actions on the myocardium *in vivo* since neonatal cardiac myocytes express CRH-R2 and respond to CRH stimulation *in vitro* with increases in intracellular cAMP (18). Interestingly, CRH-R2 has approximately 40-fold greater affinity for Ucn than for CRH (6). This unique sensitivity to Ucn and the greater potency of Ucn in eliciting cardiovascular responses are in keeping with the proposal that a peripheral regulatory system comprised of Ucn and CRH-R2 may exist (6).

The role of a peripheral CRH/Ucn system that is stress-responsive remains unclear. Previous studies from our laboratory have shown that CRH-R2 mRNA levels in the heart are markedly down-regulated (~9-fold) following administration of bacterial endotoxin [lipopolysaccharide (LPS)] (19). Furthermore, we found that LPS had the opposite effect on CRH-R2 in skeletal muscle leading to increased (~3-fold)

Abbreviations: CRH-R1 or R2, CRH receptor type 1 or 2; HPA, hypothalamic-pituitary-adrenal; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; SON, supraoptic nucleus; Ucn, urocortin.

CRH-R2 expression. Thus, endotoxin has differential effects on CRH-R2 expression in the periphery—up-regulation in skeletal muscle and down-regulation in the heart. Together, these studies establish an important link between CRH-R2 and inflammation and suggest that a CRH/Ucn system in the periphery may be activated during immune challenge, which subsequently contributes to local cardiac and skeletal muscle responses.

Endotoxemia and systemic inflammation is marked by increased cytokine production by both immune and nonimmune cells in the periphery. Proinflammatory cytokines, such as TNFα, IL-1, and IL-6, modulate CRH pathways in the central nervous system (20, 21), raising the possibility that peripheral CRH pathways may be similarly regulated by these immune mediators. Moreover, these cytokines are known to have prominent effects on the cardiovascular system. Systemic administration of TNFα or IL-1, acting in part through nitric oxide pathways, induces vasodilation and hypotension—effects that mimic the cardiovascular sequelae of endotoxin exposure. Accordingly, neutralization of TNFα or antagonism of IL-1 receptors diminishes the lethal effects of endotoxin in mammals (22–25). Direct effects of these cytokines have been demonstrated wherein TNFα and IL-1 directly depress cardiomyocyte contractility *in vitro* (26). Thus, cytokines could potentially modulate CRH pathways directly in peripheral tissues. Alternatively, stimulation of CRH gene expression in the hypothalamic paraventricular nucleus by endotoxin and/or cytokines (*e.g.* IL-1 and IL-6) leads to activation of the hypothalamic-pituitary-adrenal (HPA) axis and an elevation in circulating glucocorticoids (20, 21). High levels of glucocorticoids have been shown to down-regulate CRH-R1 in the hypothalamus and the pituitary (27); thus it is conceivable that CRH-R2 in the periphery may be negatively regulated by glucocorticoids that increase during sepsis and inflammation.

To test whether cytokines regulate CRH-R2 expression in the heart, we injected mice ip with the inflammatory cytokines, TNFα and IL-1α. Here we report that recombinant TNFα and IL-1α markedly down-regulate CRH-R2 in the heart *in vivo*. However, we find that TNFα and IL-1α do not regulate CRH-R2 mRNA in isolated mouse cardiomyocytes *in vitro*, suggesting that the suppressive effects of these cytokines, *in vivo*, occur indirectly. In testing alternative routes of modulation, we find that both *in vivo* corticosterone treatment or exposure to restraint stress reduces CRH-R2 mRNA expression in the mouse heart. However, similar to cytokines, *in vitro* treatment of corticosterone does not modulate expression in cardiomyocytes. Interestingly, we find that the high affinity CRH-R2 ligand, Ucn, significantly down-regulates CRH-R2 in cardiomyocytes *in vitro*. Thus, we speculate that endotoxin and/or cytokines increase CRH or Ucn expression *in vivo*, which, in turn down-regulates CRH-R2 expression in the heart.

Materials and Methods

Animals and reagents

C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TNFα receptor double knockout ($p55^{-/-}$, $p75^{-/-}$) mice were obtained from Immunex Corp. (Seattle, WA). Mice were housed under specific pathogen-free conditions on a 12-h light, 12-h dark schedule and were given free access to pathogen-free laboratory chow and tap water. All experimental procedures met NIH guidelines with the approval of the Oregon Health Sciences University Institutional Animal Care and Use Committee.

Rat Ucn and CRH were purchased from Bachem (Torrance, CA). For *in vivo* studies, LPS (*Escherichia coli*, serotype 055:B5) was purchased from Sigma (St. Louis, MO). Recombinant mouse TNFα was purchased from Roche Molecular Biochemicals (Indianapolis, IN), and recombinant human IL-1α was a generous gift of Dr. Alvin Stern at Hoffman-LaRoche, Inc. (Nutley, NJ). For *in vitro* studies, recombinant mouse TNFα, IL-1α, and IL-6 were purchased from Endogen, Inc. (Woburn, MA). Corticosterone was purchased from Sigma.

In vivo regulation of CRH-R2 mRNA by IL-1α, TNFα, or corticosterone

Adult female C57Bl/6J mice (8–12 wk) were housed in pairs in shrouded cages 12 h before the start of the experiment and left overnight undisturbed to minimize stress, as previously described (19). In separate experiments, mice were injected ip with either recombinant human IL-1α (10 μg/mouse), recombinant mouse TNFα [2.5 μg/mouse (1×10^6 U)] or corticosterone (2.5 or 8.3 μg/mouse). Each experiment included time-matched groups of mice treated with vehicle alone (pyrogen-free saline for IL-1α and TNFα and pyrogen-free saline containing 11% ethanol for corticosterone). Animals were killed at the indicated times after injection (see *Results*), and hearts were removed from individual mice and flash frozen separately in liquid nitrogen.

In vivo regulation of CRH-R2 mRNA by LPS in TNFα-R-deficient mice

Mice homozygous for disruptions in the p55 and p75 forms of TNFα-receptor (backcrossed four generations onto C57Bl/6J) were intercrossed and the genotypes of offspring were confirmed by PCR of tail DNA (28). Male and female wild-type and TNFα-R ($p55^{-/-}$, $p75^{-/-}$) mice (7–12 wk) were housed as described above. Mice were killed at 1, 3, 9, or 24 h after ip injection of LPS (50 μg/mouse). Hearts were removed from individuals and flash frozen separately in liquid nitrogen. This experiment was performed twice.

In vivo regulation of CRH-R2 mRNA by restraint stress

Adult female C57Bl/6J mice (8–12 wk) were housed as described above. Unstressed animals (n = 6 mice) were killed in the morning, before the initiation of the restraint stress for other groups of mice. All other mice (n = 6 mice/group) were restrained for 90 min in ventilated 50 ml polypropylene tubes. Animals were killed immediately, 1.5, 3, or 6 h following the 90-min period of restraint. Hearts were removed and flash frozen separately in liquid nitrogen.

In vivo corticosterone response to LPS injection

Adult C57Bl/6J male and female mice were pair-housed in shrouded cages overnight as described above. At 0700 h, mice were injected with LPS (50 μg/mouse, ip), returned to home cages and killed at 1, 3, 9, or 24 h after injection. Trunk blood was collected in ice-cold EDTA-coated tubes and plasma was stored at −20 C until hormone assay. Plasma corticosterone levels were measured using a commercial RIA kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA).

RNase protection for CRH-R1 and CRH-R2

Quantitation of CRH-R2 mRNA was performed as previously described (18). Briefly, total RNA was purified from mouse tissues or isolated adult mouse ventricular cardiomyocytes using RNA STAT-60 according to manufacturer's protocol (Tel-Test, Inc., Friendswood, TX). Mouse CRH-R2 was detected with a 239 nt probe (mCRX-55) spanning TM3 and TM4 (corresponding to amino acids 204 through 283) (11). Mouse CRH-R1 was detected with a 169 nt probe spanning TM3 and TM4 (corresponding to amino acids 234–289). A 216 nt *Rsa*I fragment or 110 nt *Dde*I fragment of L3 cDNA, encoding a mouse ribosomal protein (29) was used as a control for equivalent loading of RNA. Under certain

hybridization conditions, a L3 doublet at ~110 nt is visible. ^{32}P-UTP probes for CRH-R2, CRH-R1, and mouse L3 were synthesized *in vitro* and hybridized to total RNA overnight. Reactions were digested with RNase A (1 μg/ml) and T_1 RNase (600 U/ml) and resolved on a 6% polyacrylamide gel. Gels were exposed to phosphorimage screens. Quantitation of CRH-R2 mRNA in heart and isolated ventricular cardiomyocytes was performed using the IP Lab Gel software package (Signal Analytics, Vienna, VA). All CRH-R2 band intensities were normalized to L3 band intensities from the same RNA sample.

Isolation of adult mouse ventricular cardiac myocytes

Cardiomyocytes were isolated from hearts of adult wild-type (C57Bl/6) or Crhr2$^{-/-}$ mice as described (30, 31). Briefly, mice were injected with heparin (200 U) and anesthetized with a mixture of ketamine (8 mg/mouse) and xylazine (2 mg/mouse). Hearts were removed with the aorta intact and connective tissue was removed. Hearts were mounted onto a modified Langerdorf apparatus and perfused with a 95% O_2/5% CO_2 saturated Ca^{2+} free modified Tyrode's solution for approximately 1 h. Hearts were subsequently perfused with Tyrode's solution containing 2 mg/ml collagenase type II (CLS2, Worthington Biochemicals, Freehold, NJ) until the tissue was soft. Ventricles were removed, teased apart and incubated in collagenase solution. Cardiomyocytes were allowed to pellet under gravity for 20 min and the supernantant (nonmyocytes) was aspirated off. Isolated cells were passed through a 70 μm mesh filter and plated onto laminin-coated Primaria (Becton Dickinson, Franklin Lakes, NJ) tissue culture plates in DMEM containing 10% FBS, sodium pyruvate, nonessential amino acids, holo-transferrin (5 μg/ml), insulin (10 μg/ml), cytosine-b-D-arabinofuranoside (3 μg/ml), 2× MEM vitamins and penicillin/streptomycin.

In vitro regulation of CRH-R2 mRNA in adult mouse ventricular cardiac myocytes

Cardiomyocytes were plated onto laminin-coated 60 mm Primaria dishes and cultured overnight at 37 C in a humidified 5% CO_2 incubated chamber. In separate experiments, graded doses of Ucn (0.1–100 nM), cytokines [TNFα (5 or 25 ng/ml), IL-1α (2 or 20 ng/ml), or IL-6 (5 or 25 ng/ml)] or corticosterone (5 μM) were added to the culture medium and cells were incubated for 9 h. Control wells received culture medium alone. Cells were lysed in RNA-STAT60 (Tel-Test, Inc., Friendswood, TX) and RNA was isolated as described above. Experiments were performed three times with duplicate wells assayed in each experiment.

To verify that our model of isolated myocyte cultures is responsive to cytokines, RT-PCR was used to measure cytokine-stimulated induction of inducible nitric oxide synthase (iNOS), an enzyme whose gene expression is regulated by LPS or cytokine exposure in rat and mouse cardiomyocytes (32, 33). Total RNA was collected from adult mouse cardiomyocytes following 9 h incubation with media alone or media containing IL-1α (20 ng/ml). Genomic DNA was removed with DNase treatment for 3 h at 37 C before reverse transcription at 42 C. A portion of each sample was processed without the addition of reverse transcriptase to verify the absence of contaminating DNA. PCR amplification of iNOS cDNA was allowed to progress for 33 cycles (determined in pilot studies to be in the linear range of amplification for iNOS; 1 min 94 C, 1 min 58 C, 1 min 72 C). RNA from a mouse macrophage cell line (J774.2), known to express iNOS upon stimulation with LPS was used as a positive control.

Stimulation of cAMP production in adult mouse ventricular cardiac myocytes

Cardiomyocytes were plated into laminin coated 24-well Primaria tissue culture plates and cultured overnight at 37 C (5% CO_2). Cells were washed and incubated in serum-free DMEM for 90 min followed by 30 min incubation in serum-free DMEM containing 1 mM IBMX (Sigma, St. Louis, MO). Myocytes were stimulated with graded concentrations of Ucn or CRH (10^{-6} to10^{-12} M) for 25 min in DMEM (1 mM IBMX). Medium was removed and cells were immediately lysed in extraction medium (95% EtOH/20 mM HCl) and incubated overnight at −20 C. cAMP was quantitated using a commercially available cAMP RIA kit (Biomed Tech, Inc., Stoughton, MA). Experiments were performed twice with triplicate wells assayed in each experiment.

Harvesting of individual cardiomyocytes and amplification of CRH receptor mRNAs by nested RT-PCR

Freshly isolated adult mouse ventricular cardiomyocytes in perfusion medium were individually harvested using a small-bore (6 μm diameter) glass pipette maneuvered into position by a hydraulic micromanipulator apparatus (Narishige, Japan). Cardiac myocytes were captured by applying negative pressure to the pipette and immediately placed in 15 μl of 1× reverse transcriptase buffer (Life Technologies, Inc.) containing 1% NP-40 (Sigma). Cells were stored at −80 C until needed. To remove genomic DNA, samples were treated with DNase overnight at 37 C. RT was carried out at 42 C using random hexamers (Amersham Pharmacia Biotech, Piscataway, NJ). A portion of each sample was processed without the addition of reverse transcriptase to verify the absence of contaminating DNA. The integrity of the cDNA was tested using nested primers for the L3 housekeeping gene. The samples, which were then used in subsequent PCRs, showed an amplified 395-bp band following the second round. The first round of PCR amplification for CRH-R1 and CRH-R2 used specific primer pairs that resulted in the following fragment sizes: CRH-R1, 157 bp; CRH-R2, 190 bp. These reactions (1 μl) were used for the second round of amplification, which used nested primers located internally to those primers used in the first round of amplification. The size of the products generated with these internal primer pairs were 60 bp and 68 bp, respectively. All PCR amplifications were allowed to progress for 45 cycles (1 min 94 C, 1 min 58 C, 2 min 72 C). RNA from HEK 293 cells transfected with cDNAs for mouse CRH-R1 or CRH-R2β were used as positive controls.

Primers

Nested primers. L3 housekeeping gene first round: sense (5′-GATGTCTCACAGGAAATTCTCAGC-3′), antisense (5′-TAATCTCTGTTCGGTGATGGTAGC-3′); second round sense (5′-TGTGGGAATTCTGGGATATGTTGAGACCC-3′), antisense (5′-TTGTCAAGCTTATGACATCAATCATCTCATCCTGCC-3′). CRH-R1 first round: sense (5′-ATCCTCATGACCAAACTCCG-3′), antisense (5′-TGAAGACAACCCTGGAGACC-3′); second round: sense (5′-TACAGGAAGGCTGTGAAGGC-3′), antisense (5′-ACATGTAGGTGATGCCCAGG-3′). CRH-R2 first round: sense (5′-CTACACCTACTGCAACACGACC-3′), antisense (5′-TTCGCAGTGTGAGTAGTTGACC-3′); second round: sense (5′-ACCCGGAGCCCTAGTAGAGA-3′), antisense (5′-TTCCGGGTCGTGTTGTACTT-3′).

iNOS primers for RT-PCR. Sense (5′-GTCAACTGCAAGAGAACGGAGAAC-3′), antisense (5′-GAGCTCCTCCAGAGGGTAGG-3′).

Statistical analysis

Data were analyzed using ANOVA grouped on time or dose. Follow up analyses were performed using Newman-Keuls posthoc test. Differences in CRH-R2 mRNA levels between wild-type and TNFα-R (p55$^{-/-}$, p75$^{-/-}$) mice were examined using pooled RNA for each time point per genotype. Thus, statistical analysis could not be performed due to the lack of variance in this experiment. CRH-R2 mRNA expression was examined in individual wild-type and TNFα-R (p55$^{-/-}$, p75$^{-/-}$) mice at the 9 h timepoint and analyzed using Student's *t* test. Differences were considered statistically significant when $P < 0.05$.

Results

TNFα and IL-1α regulate CRH-R2 mRNA in the heart in vivo

We have previously shown that injection of LPS causes a time-dependent down-regulation of CRH-R2 mRNA in the heart (19). We investigated here whether administration of the inflammatory cytokines, IL-1α and TNFα also modulate expression of CRH-R2 in heart. Mice were injected with IL-1α (10 μg) or pyrogen-free saline (vehicle) and killed at various times (1, 3, 6, 9 h) post injection. IL-1α administration in-

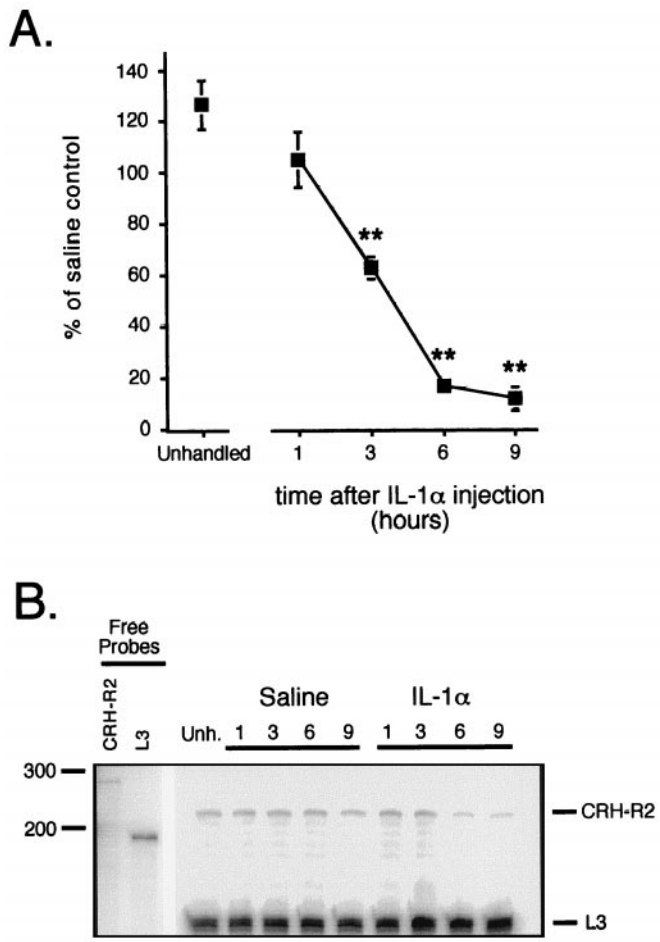

FIG. 1. IL-1α dramatically down-regulates CRH-R2 mRNA in mouse heart *in vivo*. A, IL-1α (10 μg) or vehicle (saline) was injected ip into C57Bl/6 mice (n = 6). CRH-R2 mRNA was quantitated by RNase protection and normalized to L3 mRNA, a ribosomal gene used to control for RNA loading. Points represent mean CRH-R2 mRNA levels expressed as percent of time-matched, saline-injected controls ± SEM. **, $P < 0.001$ using one-way ANOVA with Newman-Keuls posthoc analysis in time-matched, vehicle *vs.* treatment groups. B, Representative RNase protection autoradiograph of heart RNA (μg) following ip injection of saline or IL-1α. Unh lane contains RNA from unhandled animals. CRH-R2 probe protects a 239 nt fragment; L3 probe protects a 110 nt fragment.

duced a marked time-dependent decrease in CRH-R2 mRNA levels in the heart (Fig. 1, A and B) with marked suppression (12% and 5% of control values) by 6 h and 9 h, respectively, ($P < 0.001$). CRH-R2 mRNA levels in the heart also decreased significantly over time following treatment with TNFα (2.5 μg) (Fig. 2), although the reduction was not as dramatic as observed following IL-1α treatment. By 9 h post injection, CRH-R2 mRNA levels in the heart dropped to 50% of control levels ($P < 0.05$). Thus, cytokines modulate cardiac CRH pathways supporting the link between cardiac CRH responses and immune activation.

While TNFα administration caused a significant reduction in CRH-R2 mRNA, the effect was not as marked as with IL-1α. To examine whether the modest influence of TNFα on CRH-R2 regulation corresponded to a dose physiologically relevant to endogenous TNFα release, we administered LPS (50 μg/

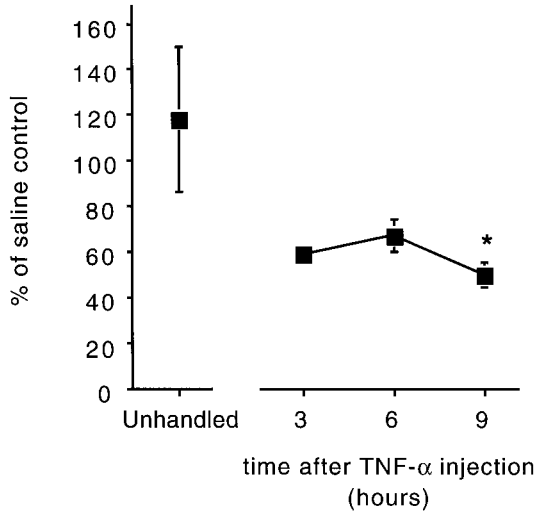

FIG. 2. TNFα down-regulates CRH-R2 mRNA in mouse heart *in vivo*. TNFα (2.5 μg) or vehicle (saline) was injected ip into C57Bl/6 mice (n = 4). CRH-R2 mRNA was quantitated by RNase protection and normalized to L3 mRNA, a ribosomal gene used to control for RNA loading. Points represent mean CRH-R2 mRNA levels expressed as percent of time-matched, saline-injected controls ± SEM. *, $P < 0.05$, using one-way ANOVA with Newman-Keuls posthoc analysis in time-matched, vehicle *vs.* treatment groups.

mouse, ip) to wild-type and TNFα receptor double knockout ($p55^{-/-}$, $p75^{-/-}$) mice (27). Systemic LPS injection caused a decrease in CRH-R2 mRNA levels in the hearts of wild-type and TNFα-R ($p55^{-/-}$, $p75^{-/-}$) mice compared with time-matched, vehicle-injected controls (Fig. 3A). This decrease reached 34% of controls in wild-type mice by 9 h after injection of endotoxin and had not yet returned to basal levels by 24 h. However, the decrease in CRH-R2 mRNA in cardiac tissue of TNFα-R ($p55^{-/-}$, $p75^{-/-}$) mice at 9 h was less pronounced (65% of control) compared with wild-type mice ($P < 0.001$) (Fig. 3B). These results, and those showing 50% reduction in CRH-R2 mRNA expression following systemic treatment with TNFα (Fig. 2), indicate that TNFα modestly regulates CRH-R2 mRNA expression in the heart *in vivo* in comparison to IL-1α and points to a partial role for this cytokine in mediating LPS-induced down-regulation of this receptor.

Corticosterone and restraint stress down-regulate cardiac CRH-R2

Inflammation and cytokines are potent activators of the HPA axis (20) resulting in elevated levels of glucocorticoids. In our studies, LPS treatment (50 μg/mouse, ip) significantly increases circulating corticosterone levels 1 h post injection (saline control 33.2 ± 3.4 *vs.* LPS treated 618 ± 76.8, $P < 0.001$) that remain elevated 9 h post injection (saline control 239.2 ± 58.1 *vs.* LPS treated 518.1 ± 72.8, $P < 0.01$). Thus, we investigated whether corticosterone has an effect similar to those of LPS and cytokines on cardiac CRH-R2 expression levels. Mice were injected with corticosterone (2.5 μg or 8.3 μg) or vehicle (pyrogen-free saline containing 11% ethanol) and killed 4 or 6 h post injection. Analysis of heart RNA (n = 6/group) revealed that cardiac CRH-R2 mRNA levels declined modestly, reaching ~76% of vehicle-injected control mice 4 h following injection of either a low or high dose of corticosterone ($P < 0.01$) (Table 1). Thus,

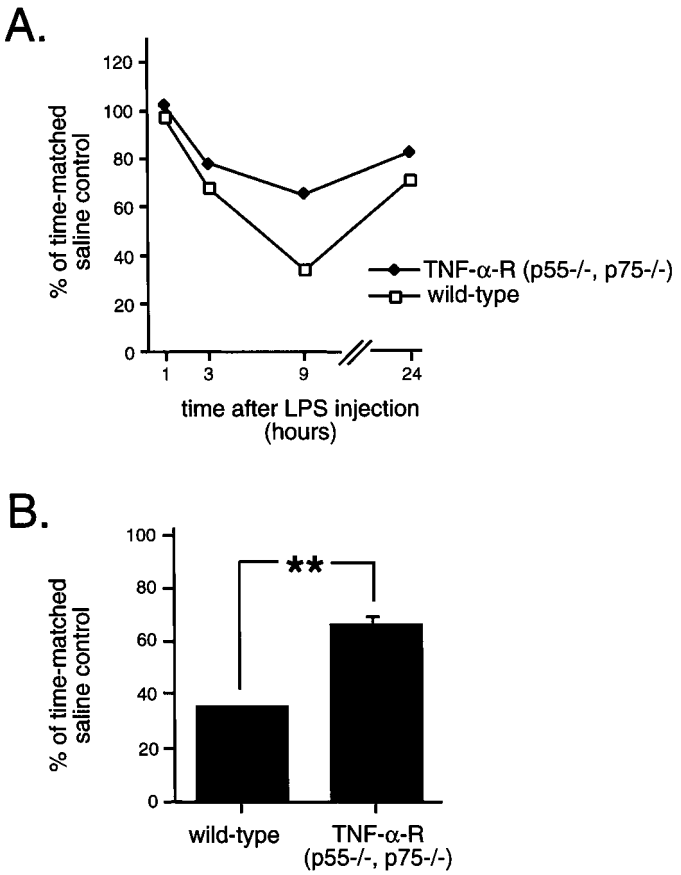

FIG. 3. Regulation of CRH-R2 mRNA in wild-type and TNFα receptor double knockout ($p55^{-/-}$, $p75^{-/-}$) mice by endotoxin. A, Endotoxin (50 μg/mouse) or vehicle (saline) was injected ip into wild-type (C57Bl/6) and TNFα-R ($p55^{-/-}$, $p75^{-/-}$) mice. Mice were killed at indicated times. CRH-R2 mRNA was quantitated by RNase protection and normalized to L3 mRNA, a ribosomal gene used to control for RNA loading. Points represent the CRH-R2 mRNA levels in RNA pooled from wild-type (n = 3) or TNFα-R ($p55^{-/-}$, $p75^{-/-}$) mice (n = 6), and are expressed as percent of time-matched saline-injected controls. Statistical analysis was not performed due to the use of pooled RNA samples. B, RNase protection analysis of individual heart RNA samples from endotoxin-treated animals at the 9 h time point. The *error bar* in wild-type mice is too small to be visible on the graph. $^{**}P < 0.001$, difference between wild-type and TNFα-R ($p55^{-/-}$, $p75^{-/-}$) mice, determined by *t* test. The experiment was performed twice yielding similar results.

peripheral CRH-R2 is regulated by glucocorticoids, similar to central CRH-R1. These results suggest that increased levels of corticosterone following endotoxin and/or cytokine administration could in part, contribute to the down-regulation of CRH-R2 mRNA in the mouse heart.

It is possible that stress-responsive hormones other than adrenal glucocorticoids regulate CRH-R2 mRNA in the heart. To begin to investigate this possibility, we subjected mice to 90 min restraint stress and compared CRH-R2 mRNA levels in the hearts of mice immediately, 1.5, 3, or 6 h following restraint to those of unstressed controls. Analysis of heart RNA (n = 6/group) shows that CRH-R2 mRNA levels were reduced (66% of unstressed controls, $P < 0.01$) in hearts of mice immediately following the termination of the 90 min restraint period. CRH-R2 mRNA levels were further lowered 1.5 and 3 h post stress (45% and 36% of controls respectively, $P < 0.01$) but begin to recover 6 h post stress (Table 2). Thus, it appears that glucocorticoids or other hormones induced by a cognitive stress (restraint) may play a role in mediating the suppressive effects of endotoxin, TNFα or IL-1α on expression of CRH-R2 in the mouse heart.

TABLE 1. *In vivo* corticosterone treatment significantly down-regulates CRH-R2 mRNA expression in heart tissue

Corticosterone (n = 6/group)	Time after injection	
	4 h	6 h
2.5 μg	78.8 ± 4.4[a]	110.7 ± 5.4
8.3 μg	76.2 ± 3.9[a]	100.8 ± 6.3

Data are mean values ± SEM of CRH-R2 mRNA levels expressed as a percent of time-matched, vehicle-injected controls.

[a] $P < 0.05$ treatment group *vs.* vehicle-injected controls using ANOVA.

Expression and pharmacology of CRH-R2 in adult mouse ventricular cardiomyocytes

We have shown previously that AT-1 atrial cardiomyocyte tumor cells express CRH-R2 and respond to CRH with increased cAMP production (18). However, transformed AT-1 cardiomyocyte responses may differ from those of primary adult cardiac myocytes; hence we tested the use of adult mouse cardiomyocytes as a suitable *in vitro* model system by examining the expression and pharmacology of CRH-R2. Cultures of highly enriched primary mouse ventricular cardiomyocytes express CRH-R2 mRNA at levels similar to those found in whole mouse heart (Fig. 4A). Furthermore, we do not find evidence of CRH-R1 expression. Although these primary cultures are enriched for cardiomyocytes, they are not completely devoid of nonmyocyte cells (90–95% cardiomyocytes); thus, we examined individually isolated cardiac myocytes for expression of CRH-R1 and CRH-R2. Cardiomyocytes were readily identified microscopically based on their characteristic striated, rod-shaped appearance in culture and were individually harvested using a small bore glass pipette (Fig. 4B). Using nested RT-PCR, we find that cardiac myocytes express CRH-R2, but not CRH-R1 (Fig. 4C). The product amplified with CRH-R2 specific primers was sequenced and found to be identical to mouse CRH-R2. Stimulation of cultured cardiomyocytes with Ucn or CRH causes a dose-dependent increase in intracellular cAMP (Fig. 4D). The EC_{50} values obtained for Ucn ($EC_{50} = 3.3 \pm 0.5 \times 10^{-10}$ M) and CRH ($EC_{50} = 3.4 \pm 1.1 \times 10^{-8}$ M) are similar to those reported previously for cells transfected with CRH-R2β (6). In addition, cAMP responses were not elevated in cardiomyocytes collected from $Crhr2^{-/-}$ mice, demonstrating that CRH/Ucn stimulation of cAMP is dependent on the presence of CRH-R2. Thus, cultured mouse cardiomyoctyes represent an appropriate *in vitro* model system to examine the regulation of CRH-R2 in the heart.

CRH-R2 expression in isolated cardiomyocytes is not regulated by IL-1α, TNFα, IL-6, or corticosterone

We tested whether cytokines and exogenous corticosterone that modulate cardiac CRH-R2 mRNA *in vivo* directly regulate CRH-R2 mRNA *in vitro*. Isolated cardiomyocytes were treated for 9 h with either TNFα (5 or 25 ng/ml), IL-1α

TABLE 2. CRH-R2 mRNA expression is significantly reduced during restraint stress

Restraint stress (n = 6/group)	Time after 90 min restraint stress			
	Immediate	1.5 h	3 h	6 h
	66.4 ± 6.1[a]	45.1 ± 5.7[a]	36.3 ± 10.0[a]	56.9 ± 3.9[a]

Data are mean values ± SEM of CRH-R2 mRNA levels expressed as a percent of unstressed control mice.

[a] $P < 0.01$ stressed group *vs.* unstressed controls using ANOVA with Newman-Keuls post-hoc analysis.

(2 or 20 ng/ml) or IL-6 (5 or 25 ng/ml) at doses previously shown to regulate cardiomyocyte function *in vitro* (26, 34, 35). We found that treatment with these cytokines failed to significantly alter CRH-R2 mRNA in these cells (Fig. 5). However, iNOS mRNA expression is significantly increased (2-fold) from basal levels in our cardiomyocyte cultures following 9 h incubation with IL-1α (data not shown). These results suggest that the *in vivo* effects of TNFα and IL-1α on CRH-R2 levels in the heart are indirect.

To examine the possible role of direct glucocorticoid modulation, we treated cardiomyocytes with corticosterone (5 μM). This dose of corticosterone has been shown to regulate several other genes (*e.g.* adrenomedullin, atrial natriuretic peptide, Na-K-ATPase genes) in cardiac myocytes and is 20-fold higher than that shown to down-regulate CRH-R1 mRNA in rat pituitary cells (36). CRH-R2 mRNA levels remained unchanged following incubation with corticosterone for 9 h (Fig. 5).

CRH-R2 expression in isolated cardiomyoctyes is down-regulated by Ucn

We tested the effect of Ucn, which recently has been reported to be up-regulated in the periphery by LPS (37). Treatment of cardiomyocytes *in vitro* with graded doses of Ucn (0.1–100 nM) for 9 h significantly decreased the level of CRH-R2 mRNA (59% of controls incubated with medium alone, $P < 0.05$) (Fig. 6). Thus, Ucn is capable of regulating CRH-R2 expression suggesting that LPS and/or inflammatory cytokines may regulate cardiac CRH-R2 mRNA indirectly by inducing and sustaining Ucn or CRH release into the heart, which subsequently leads to ligand-induced down-regulation of CRH-R2.

Discussion

Inflammation is a powerful physiologic stress that affects a number of organ systems leading to marked changes in energy metabolism, immune function and cardiovascular homeostasis (38). Sepsis, a potent inducer of systemic inflammation, is characterized by vascular dysfunction and refractory hypotension, a progressive depression of cardiac function and reduced organ perfusion (39, 40). Depression of cardiac function in later stages of sepsis may be due, in part, to TNFα and IL-1, which have been found to be the primary cardiodepressive factors in plasma of septic individuals (26). Although diminished cardiac function occurs following prolonged inflammation, early stages of sepsis are marked by increased cardiac function (40), which may be a compensatory response to hypotension resulting from the effects of inflammatory mediators on vascular endothelium. Increased cardiac function early in sepsis is likely to be a vital adaptation by the host that enhances survival against pathogens. Moreover, decreased cardiac function following prolonged inflammation may result from declining inotropic support due to changes such as receptor sensitivity and relative availability of inotropic mediators. Thus, mechanisms that lead to increased positive inotropic support for the heart may be particularly important during sepsis and endotoxemia. Given that Ucn increases cardiac contractility via its actions on CRH-R2 (17), it is plausible that this pathway may play a role in maintaining inotropic support for the heart. In addition, we (19) and others (37) have found that endotoxin modulates this peptide:receptor system, thereby establishing an important link between CRH-R2 and inflammation.

Our findings reported here add strength to the model that CRH-R2 responses are linked to inflammatory stimuli. We show for the first time that *in vivo* administration of IL-1α dramatically down-regulates CRH-R2 mRNA in the mouse heart. In addition, CRH-R2 mRNA expression is also significantly reduced following systemic administration of TNFα in wild-type mice, albeit the effect is less marked compared with IL-1α. The modest regulation by exogenous TNF appears to reflect endogenous release, as LPS-induced down-regulation of cardiac CRH-R2 is partially but not completely prevented in mice lacking TNFα receptors (p55 and p75 forms) compared with wild-type controls. Thus, TNFα does not regulate cardiac CRH-R2 expression as profoundly as IL-1. Modulation is likely complex, requiring the actions of multiple cytokines on various cell types. For example, TNFα may be involved in stimulating release of IL-1 from circulating macrophages which in turn influences cardiac CRH-R2.

We paired our *in vivo* experiments with *in vitro* analysis using primary cultures of isolated adult mouse ventricular cardiomyocytes to examine direct regulation of CRH-R2 by cytokines. Surprisingly, low or high levels of TNFα or IL-1α did not significantly regulate CRH-R2 mRNA in isolated cardiomyocytes. Moreover, IL-6, which is induced by TNFα and IL-1α, did not alter CRH-R2 mRNA expression in cardiomyocytes *in vitro*. These findings suggest that cytokines do not act directly on cardiomyocytes to modulate CRH-R2 expression. Cytokine regulation of CRH-R2 expression was recently shown in a transformed line of rat aortic smooth muscle cells (41), raising the possibility that modulation of CRH-R2 may be cell type specific. Thus, our observation of cytokine modulation *in vivo* could be due to direct effects of cytokines on nonmyocyte cells in the heart. Alternatively, as discussed above, it is plausible that several cytokines act synergistically or in conjunction with other hormones such as glucocorticoids to influence cardiac CRH pathways. Cytokines are potent stimulators of the HPA axis (42), and elevations in circulating glucocorticoids have been shown to regulate CRH-R1 and CRH-R2 expression in the central nervous system (27, 43–45) and more recently in the periphery (41, 46). We find that *in vivo* administration of corticosterone significantly reduced cardiac CRH-R2 mRNA expression 4 h post injection. In addition, restraint stress resulted in a pronounced decrease in CRH-R2 mRNA levels. Thus, activation of the HPA axis may play a role in cytokine or LPS-induced regulation of CRH-R2 *in vivo*. However, we find that corti-

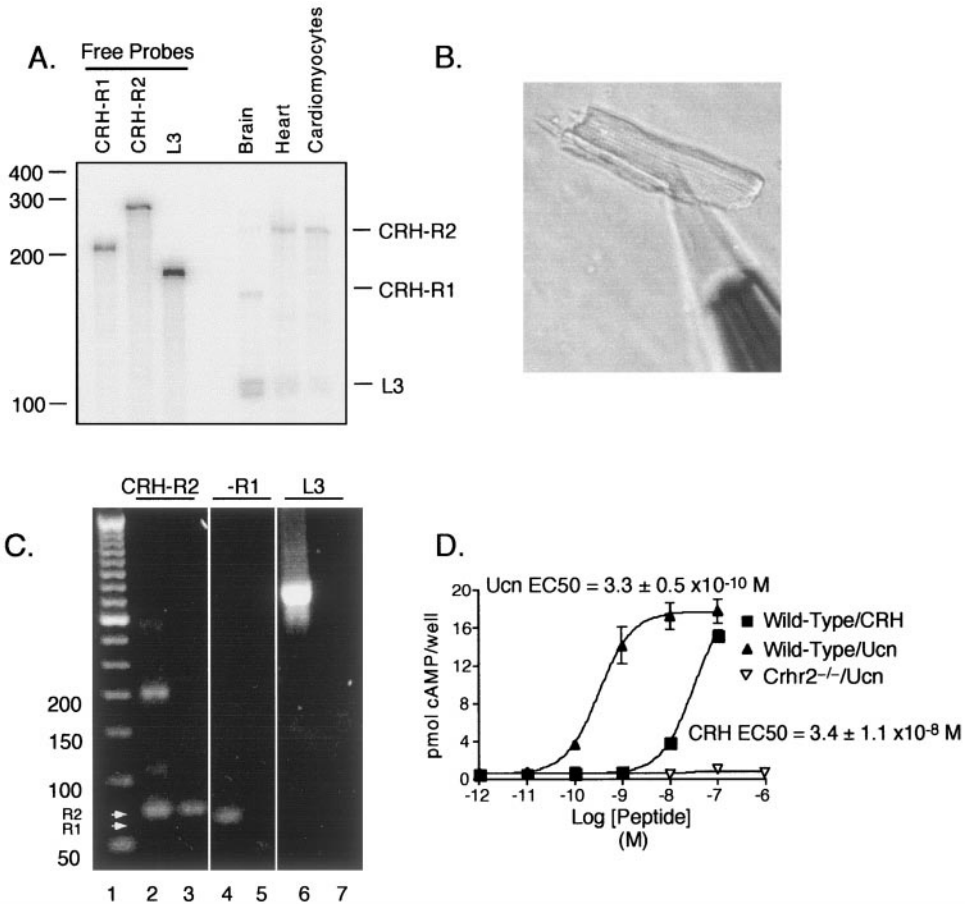

FIG. 4. CRH-R2 expression and coupling to elevations in cAMP in adult mouse ventricular cardiac myocytes. A, RNase protection analysis of CRH receptor expression in cultures of adult mouse ventricular cardiomyocytes. CRH-R2 mRNA is highly expressed in adult mouse cardiomyocytes and whole heart, while CRH-R1 mRNA is undetectable. B, Harvesting of individual cardiomyocytes with a 6 μm-bore glass pipette; note striated, rod-shaped appearance characteristic of cardiac myocytes. C, RT-PCR analysis of CRH receptor expression from four ventricular cardiomyocytes that were individually isolated and pooled for RT-PCR. CRH-R2 (68 bp) is evident following two rounds of PCR amplification. Lane 1, 50 bp ladder; lane 2, CRH-R2 transfectants (HEK 293 cells) amplified with CRH-R2 primers (11); lane 3, cardiomyocytes amplified with CRH-R2 primers; lane 4, CRH-R1 transfectants (HEK 293 cells) amplified with CRH-R1 primers; lane 5, cardiomyocytes amplified with CRH-R1 primers; lane 6, cardiomyocytes PCR amplified with L3 primers with prior reverse transcriptase treatment; lane 7, cardiomyocytes PCR amplified with L3 primers without prior reverse transcriptase treatment. D, Ucn and CRH stimulate increases in cAMP accumulation in adult mouse ventricular cardiomyocytes from wild-type mice, but not Crhr2$^{-/-}$ mice. *Points* are the mean ± SEM of triplicate wells/experiment and representative of duplicate experiments.

costerone does not alter CRH-R2 mRNA expression in mouse cardiomyocytes *in vitro*. These data are consistent with a recent report that showed LPS-induced down-regulation of CRH-R2 in rat heart was not completely reversed following adrenalectomy (41). Taken together, it appears that like cytokines, other mediators present *in vivo* may be necessary for glucocorticoid regulation of CRH-R2.

We demonstrate that Ucn, a high affinity agonist for CRH-R2, is capable of down-regulating CRH-R2 mRNA in primary cultures of cardiomyocytes in a dose-dependent manner. This is similar to CRH-induced down-regulation of CRH-R1 mRNA in rat anterior pituitary cells (36, 47) and Ucn-induced down-regulation of CRH-R2 mRNA in transformed smooth muscle cells (41). Moreover, a recent study reports modest down-regulation of cardiac CRH-R2 mRNA expression following a bolus injection of Ucn (46). Ligand-induced down-regulation has also been documented for a number of other G protein-coupled receptors (48–50) and often involves decreased mRNA stability (48, 49, 51). Down-regulation of CRH-R2 mRNA in cardiomyocytes by Ucn may have important implications for the control of heart function during stress and suggests that decreases in cardiac CRH-R2 mRNA during inflammation may be due to prolonged elevation of Ucn or CRH levels within the myocardium. *In vivo* administration of Ucn lends further support to this view (46), although paracrine sources of Ucn likely regulate cardiovascular function which may not be simulated accurately with bolus iv administration. We (Heldwein, K., and M. Stenzel-Poore, unpublished) and others (52) have found that Ucn and CRH mRNA are expressed in mouse heart; however little is known thus far about the regulation of these cardiac transcripts. Heat shock (42 C, 3 h) has been shown to induce Ucn mRNA expression in cultured neonatal rat cardiomyocytes, although in this study the increase was minor (~1.8-fold over control) and seen 18 h after the cells were returned to 37 C (53).

Inflammation has been shown to regulate Ucn expression in rat thymus and spleen *in vivo* (37). Thymic Ucn mRNA levels were increased 2-fold following systemic endotoxin administration and decreased to the same degree in the spleen. Interestingly, this up-regulation of Ucn mRNA in the thymus was adrenal dependent, and ACTH or corticosterone also increased thymic Ucn mRNA. In contrast, Ucn mRNA levels in the spleen were unaltered by ACTH injection. These data indicate that splenic Ucn expression may be regulated directly by endotoxin or inflammatory mediators, whereas HPA axis activation is more important in regulating Ucn in the thymus. Because changes in Ucn peptide were not measured in this study, it is not clear how LPS or HPA axis activation affects the peripheral production of CRH-like peptides (54). Nevertheless, this study indicates that expression of Ucn in peripheral tissues may be responsive to stress, particularly those involving inflammatory signals.

In addition to expression changes in the periphery, Ucn

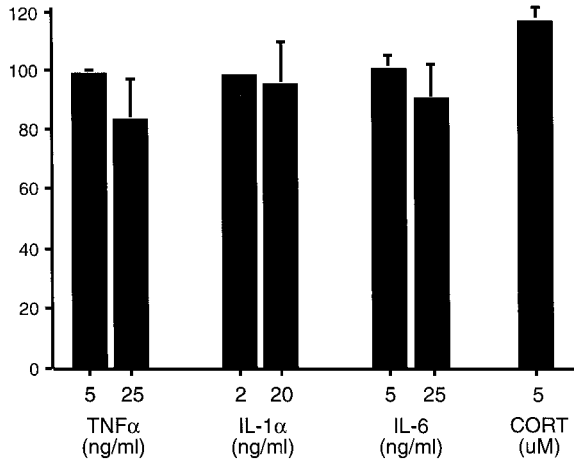

FIG. 5. Cytokines and corticosterone do not regulate CRH-R2 in adult mouse ventricular cardiomyocytes. Myocytes were treated with the indicated concentrations of recombinant mouse TNFα, IL-1α, IL-6, corticosterone or medium alone for 9 h. CRH-R2 mRNA was quantitated by RNase protection and normalized to L3 mRNA, a ribosomal gene used to control for RNA loading. *Bars* represent the mean of duplicate samples + SD and are expressed as the percent of CRH-R2 mRNA in medium (control) treated cells. The experiment was performed twice yielding similar results. One-way ANOVA with Newman-Keuls posthoc analysis and *t* test revealed no significant differences in the means of control *vs.* treatment samples.

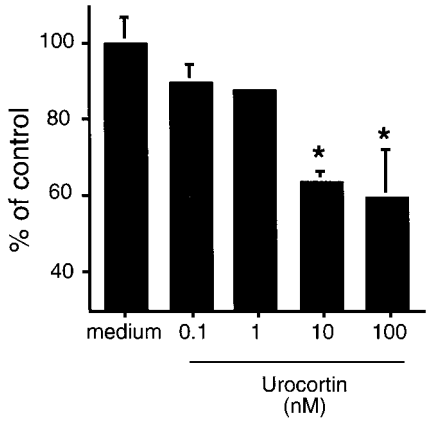

FIG. 6. Ucn regulation of CRH-R2 mRNA in adult mouse ventricular cardiomyocytes. Myocytes were treated with the indicated concentrations of Ucn or medium alone for 9 h. CRH-R2 mRNA was quantitated by RNase protection and normalized to L3 mRNA, a ribosomal gene used to control for RNA loading. *Bars* represent the mean of duplicate samples + SD (except for the 1.0 nM dose of Ucn) and are expressed as the percent of CRH-R2 mRNA in medium (control) treated cells. The experiment was performed three times yielding similar results. *, $P < 0.05$ by one-way ANOVA with Newman-Keuls posthoc analysis in medium *vs.* Ucn-treated cardiomyocytes.

expression in several regions of the central nervous system are altered by stimuli that influence cardiovascular function. For example, Ucn expression in the Edinger-Westphal nucleus of the mouse is increased following restraint stress. This effect was blocked by prior, chronic glucocorticoid infusion (55) indicating that glucocorticoids may desensitize or down-regulate pathways that mediate stress-responsive regulation of Ucn mRNA in the Edinger-Westphal nucleus. Furthermore, the number of Ucn-containing neurons in the supraoptic nucleus (SON) and fibers in the median eminence of the rat brain is increased by dehydration, again demonstrating that Ucn may be regulated by physiologic stress (56). Interestingly, neurons in the SON are known to be activated by several vascular stressors (*e.g.* changes in blood pressure, volume, and osmolality) (57–59); thus dehydration, which increases plasma osmolality, may lead to Ucn induction in the SON and subsequent modulation of the vascular system.

Based on our findings, we propose a model in which systemic inflammation induces the peripheral production of Ucn or CRH, which activate CRH-R2 on cardiac myocytes in the mouse heart. Following prolonged stimulation, CRH-R2 mRNA is down-regulated. Based on the stimulatory activity of Ucn and CRH on heart function, we posit that CRH-R2 may represent an inotropic pathway that contributes to an early hyperdynamic response of the heart in response to hypotension caused by cytokines during sepsis. The studies presented here provide the basis for future studies using the power of genetic mouse models that harbor disruptions in the immune or CRH systems as a means to further dissect the interaction between immune mediators and CRH pathways in the heart. We are currently studying the early cardiac response to inflammation in Crhr2$^{-/-}$ mice. If Crhr2$^{-/-}$ mice show attenuated cardiac contractility in response to inflammation, this would lend support to our model, revealing a critical role for CRH-R2 in the regulation of heart function during stress. To date, our findings (17) and those of others (5, 7) indicate that Ucn and/or CRH are members of a family of neuropeptides (60–62) that affect heart function. Thus, CRH and Ucn may represent a mode of regulation of the cardiovascular system distinct from catecholamines, that complements or fine tunes cardiac responses during adverse conditions that threaten homeostasis.

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