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Receptor-Independent Activation of GABAergic Neurotransmission and Receptor-Dependent Nontranscriptional Activation of Phosphatidylinositol 3-kinase/Protein Kinase Akt Pathway in Acute Cardiovascular Actions of Dexamethasone at the Nucleus Tractus Solitarii of the Rat

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GABA and PI3K/Akt in cardiovascular actions of glucocorticoid

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ABBREVIATIONS: aCSF, artificial cerebrospinal fluid; Akt, serine/threonine protein kinase; Dex, dexamethasone-21-phosphate sodium; DMSO, dimethyl sulphoxide; GABA, γ-aminobutyric acid; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GR, glucocorticoid receptor; HO-2, heme oxygenase-2; L-NMMA, N^G-monomethyl-L-arginine acetate; LY294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; MSAP, mean systemic arterial pressure; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NTS, nucleus tractus solitarii; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-polymerase chain reaction; 2-OH saclofen, 2-hydroxy saclofen.

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ABSTRACT

Whereas glucocorticoids are important blood pressure regulators via an action on peripheral circulation, their roles in central cardiovascular regulation are less known. This study evaluated the acute cardiovascular effect of glucocorticoid in the nucleus tractus solitarii (NTS) and delineated the underlying molecular mechanisms. In Sprague-Dawley rats maintained under propofol anesthesia, microinjection bilaterally into the NTS of a synthetic glucocorticoid, dexamethasone (Dex, 12.5, 25, 50 or 100 pmol) elicited hypertensive and tachycardiac responses. The initial cardiovascular responses, which lasted 15-30 min, were blunted by co-administration of a selective $GABA_A$ or $GABA_B$ receptor antagonist, bicuculline (15 pmol) or 2-hydroxy saclofen (150 pmol). The delayed responses, which endured at least 90 min and entailed maintained hypertension and tachycardia, was reversed by selective glucocorticoid type II receptor (GR) antagonist, mifepristone (100 or 200 pmol), phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002 (20 nmol), or nitric oxide synthase inhibitor, L-NMMA (5 nmol), but not by the RNA synthesis inhibitor, actinomycin D (20 nmol). Moreover, Dex induced an association of GR with the regulatory subunit of PI3K, p85α, in a ligand-dependent manner, and promoted serine/threonine kinase Akt phosphorylation that was blocked by co-administration of mifepristone or LY294002. These cardiovascular and molecular responses occurred when translocation of activated GR into the nucleus was minimal. Our results indicate that Dex acts on the NTS to elicit hypertension and tachycardia via both a GR-independent interaction with $GABA_A$ and $GABA_B$ receptors and a GR-dependent but nontranscriptional mechanism that involves activation of PI3K/Akt pathway.

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Most known effects of glucocorticoids, a major subclass of steroid hormones, are mediated by binding of the hormone to glucocorticoid receptor (GR) (Beato et al., 1995; Beato and Klug, 2000). As an intracellular protein, GR belongs to a phylogenetically conserved superfamily of nuclear hormone receptors known as ligand-inducible transcriptional activators (Evans, 1988). On binding to glucocorticoids, GR translocates from the cytoplasm to the nucleus where it activates or suppresses gene transcription through direct interactions with DNA or other transcription factors (Beato et al., 1995). This transcriptional mode of action is responsible for the well-defined long lasting cellular responses to glucocorticoids.

Recent studies also suggest that activated GR may regulate physiological functions via nontranscriptional mechanisms. Thus, administration of a synthetic glucocorticoid, dexamethasone (Dex), promotes relaxation of the isolated aorta within minutes after GR activation (Limbourg et al., 2002). A non-nuclear effect of GR reportedly mediates the acute cardioprotective effects of corticosteroids (Hafezi-Moghadam et al., 2002). At the signal transduction level, activation of phosphatidylinositol (PI) 3-kinase (PI3K) (Solito et al., 2003) and serine/threonine kinase Akt (Akt) (Buren et al., 2002; Solito et al., 2003) has been demonstrated to mediate the nongenomic cellular responses elicited by GR activation. In addition, glucocorticoids may act directly on membrane rather than cytosolic receptors to elicit rapid changes in cellular responses (Sakai et al., 2000). In this regard, natural and synthetic glucocorticoids increase binding affinity of γ-aminobutyric acid (GABA) receptors to their agonists in cerebral synaptosomal membrane (Majewske, 1987; Marrow et al., 1990). They also increase the inhibitory postsynaptic potential recorded from pyramidal neurons, in a process that is not affected by the GR antagonist (Teschemacher et al., 1995).

It is well established that glucocorticoids are important regulators of blood pressure via an action on peripheral circulation (Korte et al., 1993; Lin et al., 1999). Of the much less available information on the role of glucocorticoids in central cardiovascular regulation, administration of glucocorticoid into the cerebral ventricle (van den Berg et al., 1989) or the dorsal hindbrain (Scheuer et al., 2004) increases

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arterial pressure. Both GR mRNA (Comer et al., 1999) and protein (Harfstrand et al., 1986) are distributed in neurons of the nucleus tractus solitarii (NTS), the principal terminal site of baroreceptor afferents in the medulla oblongata for blood pressure regulation (Ciriello, 1983). As such, NTS is an appropriate neural substrate to subserve the central cardiovascular regulatory actions of glucocorticoids. The present study was undertaken to evaluate this hypothesis, using the synthetic glucocorticoid, Dex, as our experimental tool. We also delineated the molecular mechanisms that underlie this regulatory process. Our results indicate that Dex acts on the NTS to elicit hypertension and tachycardia via both a GR-independent interaction with GABAergic neurotransmission and a GR-dependent but nontranscriptional mechanism that involves rapid activation of PI3K/Akt pathway.

Materials and Methods

A**nimals.** Experiments were carried out in adult male Sprague-Dawley rats (190 to 240 g, n = 219) purchased from the Experimental Animal Center of the National Science Council, Taiwan. They were housed in an animal room under temperature control (24±0.5°C) and 12-h light-dark (lights on during 08:00-20:00) cycle. Standard laboratory rat chow (Purina) and tap water were available *ad libitum*. All animals were allowed to acclimatize for at least 7 days prior to experimental manipulations. All experimental procedures were carried out in compliance with the guidelines of our institutional animal care committee.

General Preparation. Rats were anesthetized initially with pentobarbital sodium (50 mg/kg i.p.) to perform preparatory surgery (Li et al., 2001; Chan et al., 2003, 2004). This routinely included intubation of the trachea to facilitate ventilation and cannulation of the femoral artery and vein to measure SAP or administer test chemicals. All surgical procedures were performed under a surgical plane of

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anesthesia as indicated by the absence of withdrawal reflex to hindpaw pinch. Animals received thereafter continuous intravenous infusion of propofol (30 mg/kg/h), which provided satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation (Yang et al., 1995). Pulsatile and mean systemic arterial pressure (MSAP), as well as heart rate (HR), were recorded on a polygraph (Gould RS3400). Animals were mechanically ventilated to maintain an end-tidal CO_2 to be within 4 to 5%, as monitored by a capnograph (Datex Normocap). All data were collected from animals with a maintained rectal temperature of 37 ± 0.5°C. Since diurnal change in plasma corticosterone concentration affects the degree of GR activation and translocation (Spencer et al., 1993), all physiological experiments were conducted in the morning between 09:00 to 12:00. At the end of each experiment, rats were killed with intravenous injection of an over-dose of pentobarbital sodium (100 mg/kg).

Microinjection of Test Agents into the NTS. Test agents were microinjected bilaterally and sequentially, at a volume of 50 nL, into the NTS (Li et al., 2001; Chan et al., 2003, 2004). The coordinates for the NTS were -0.5 to +0.5 mm from the obex, 0.3 to 0.8 mm lateral to the midline and 0.5 to 1.0 mm below the dorsal surface of the medulla oblongata. These coordinates were selected to cover subdivisions of NTS in which functionally identified barosensitive neurons reside (Chan and Sawchenko, 1998). Test agents used included a synthetic glucocorticoid, dexamethasone-21-phosphate sodium (Dex, Sigma); a selective glucocorticoid type II receptor antagonist (Clapham and Turner, 1997; Limbourg et al., 2002), mifepristone (RU486, Sigma), that does not bind to the type I mineralocorticoid receptor or to aldosterone receptors (Brogden et al., 1993); a selective $GABA_A$ receptor antagonist (Galvez-Ruano et al., 1995), bicuculline methiodine (Sigma); a $GABA_B$ receptor antagonist (Al-Dahan et al., 1990), 2-hydroxy saclofen (2-OH saclofen, Sigma); a non-selective nitric oxide synthase (NOS) inhibitor (Reif and McCreedy, 1995), N^G-monomethyl-L-arginine acetate (L-NMMA, Sigma); a PI3K inhibitor (Vlahos et al., 1994),

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2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002, Calbiochem); or a RNA synthesis inhibitor (Limbourg et al., 2002), actinomycin D (AMD, Calbiochem). The dose and treatment scheme were adopted from previous reports (Clapham and Turner, 1997; Ouyang and Wang, 2000; Hafezi-Moghadam et al., 2002; Limbourg et al., 2002; Moreno et al., 2002; Chan et al., 2003), which used the same test agents for the same purpose as in this study. With the exception of mifepristone, LY294002 or AMD, which used respectively 1% or 0.2% DMSO or 3% methanol as the solvent, all test agents were prepared with aCSF. To avoid the confounding effects of drug interaction, each animal received only one drug treatment of Dex, given alone or in combination with one test agent. Pretreatment of AMD by microinjection bilaterally into the NTS of rats anesthetized with pentobarbital sodium was carried out 24 h prior to Dex application. The wound was closed and animals were allowed to recover in individual cages.

Extraction of Protein and Western Blot Analysis. To extract protein, tissues on both sides of the dorsomedial part of the medulla oblongata, at the level of caudal NTS (1mm rostral to 1 mm caudal from the obex) were collected by micropunches made with a stainless-steel bore (1 mm i.d.), and were frozen immediately in liquid nitrogen (Li et al., 2001; Chan et al., 2002; Chan et al., 2003, 2004). Medullary tissues thus obtained from 5 to 6 rats that received the same treatment were homogenized, centrifuged and the cytosolic fraction was saved in liquid nitrogen until use. Pellets obtained after the first centrifugation were used to prepare purified nuclear protein. Nuclear protein was extracted by modifying previously reported procedures (Hutchison et al., 1994). In brief, the pellet was resuspended in a buffer that contains 10 mM HEPES, pH 7.2, 15 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 2 mM NaF, 15 μg/ml leupeptin, and 1 mM sodium orthovanadate, followed by centrifugation at 4000 rpm for 10 min. The pelleted nuclei were resuspended in an extraction buffer (100 mM HEPES, pH 7.2, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.8 M NaCl, 15% glycerol, 2 mM NaF, 1 mM PMSF, 15 μg/ml leupeptin, and 1 mM sodium orthovanadate), incubated

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on ice for 2-4 h, and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was stored at -85°C until further use. Protein concentrations were determined by the Bradford assay.

Western blot analysis of GR protein in the nuclear extract from the dorsomedial medulla was performed in control animals, or at 0, 1, 2, 4 or 8 h after Dex treatment. Phosphorylation of Akt (P-Akt) was determined in control animals, or at 5, 10, 20, 30 or 60 min after Dex treatment. The primary antisera used included a mouse monoclonal anti-GR (1:500; Oncogene), an anti-Akt (1;1000; Cell Signaling Technology), an anti-P-Akt (Ser^{473}) (1:1000; Cell Signaling Technology) or an anti-γ-tubulin (1:5000; Sigma) antiserum. The secondary antiserum used was a horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000; Jackson). Specific antibody-antigen complex was detected by an enhanced chemiluminescence Western blot detection system (NEN™, Life Science Products). The amount of protein was quantified by the Photo-Print Plus software (ETS Vilber-Lourmat, France), and was expressed as the ratio (%) to γ-tubulin protein, which served as the internal control to demonstrate equal loading of proteins.

Immunoprecipitation. For immunoprecipitation assay, protein A/G-agarose beads were added to the cytosolic protein extracts. Immunoprecipitation with either a mouse monoclonal anti-GR antiserum or a rabbit polyclonal anti-p85α antiseum against the regulatory subunit of PI3K (Gout et al., 1992) was performed at 4°C overnight and the precipitated beads were washed with an ice-cold lysis buffer followed by a kinase buffer (25 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, 2 mM dithiothreitol). Western blot analysis of p85α PI3K (1:1000; Santa Cruz) or GR (1:500) was carried out as described above.

Akt kinase assay. The dorsal medulla was homogenized in a kinase extraction buffer (KP31210, Calbiochem), and the Akt kinase activity was determined by detecting the ability of Akt to phosphorylate its downstream target, glycogen synthase

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kinase (GSK-3), using an Akt kinase assay kit (Calbiochem) according to the manufacturer's instructions.

Isolation of Total RNA and Reverse Transcription-Polymerase Chain Reaction Analysis. To verify the effectiveness of AMD as a transcription inhibitor, medullary tissues obtained from 6 to 8 rats was pooled for isolation of total RNA. Quantification of neuronal NOS (nNOS) or heme oxygenase (HO)-2 mRNA was carried out by reverse transcription-polymerase chain reaction (RT-PCR) (Chan et al., 2001; Scapagnini et al., 2002). These genes were selected because their transcription is regulated by Dex via activation of GR (Weber et al., 1994; Reagan et al., 1999). The gel was stained with ethidium bromide (1 μg/ml), visualized by an ultraviolet transilluminator and photographed. The density of each PCR band was measured and analyzed by the ImageMaster VDS analysis software (Pharmacia Biotech, Uppsala, Sweden).

Histology. For verification of microinjection sites, the brain stem, except those used for collection of tissues to extract total RNA or protein, was removed from animals after the physiological experiments and were fixed in 30% sucrose in 10% formaldehyde-saline solution for ≥ 72 h. Frozen 25-μm sections of the medulla oblongata were stained with 1% Neural red for histological verification of the location of microinjection sites. One percent Evans blue was added to the microinjection solution to facilitate this process.

Statistical Analysis. All values are expressed as mean ± SEM. One-way or 2-way ANOVA with repeated measures was used, as appropriate, to assess group means. This was followed by the Scheffé multiple range test for post hoc assessment of individual means. A value of $P < 0.05$ was taken to indicate statistical significance.

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Results

Cardiovascular Responses to Microinjection of Dex into the NTS. Compared to the aCSF controls, microinjection bilaterally into the NTS of Dex (12.5, 25, 50 or 100 pmol) resulted in a dose-related increases in MSAP and HR (Fig. 1A). At the lower doses (12.5 or 25 pmol), Dex induced a transient hypertension and tachycardia that lasted 15-30 min. At the higher doses (50 or 100 pmol), pressor and tachycardiac effects of Dex persisted for at least 90 min posttreatment. Histological assessment confirmed that Dex was delivered to sites within the anatomical confines of the NTS (Fig. 1B). Microinjection of Dex into areas adjacent to the NTS, e.g., nucleus gracilis, nucleus cuneatus, or nucleus hypoglossi, elicited minimal effects on MSAP or HR (data not shown).

Reversal of Cardiovascular Responses to Microinjection of Dex into the NTS By GABA Receptor Antagonists. Co-administration bilaterally into the NTS of a $GABA_A$ receptor antagonist, bicuculline methiodine (15 pmol) (Fig. 2A) or a $GABA_B$ receptor antagonist, 2-OH saclofen (150 pmol) (Fig. 2B) significantly blunted the first 30 min of pressor and tachycardiac responses elicited by Dex (25 or 100 pmol). Furthermore, combined treatment with both antagonists totally eliminated those initial cardiovascular responses to Dex (25 or 100 pmol) to levels that were not statistically different from vehicle control (Fig. 3). The persistent hypertension or tachycardia promoted by a high dose of Dex (100 pmol), on the other hand, was not affected by either GABA receptor antagonist (Figs. 2 and 3). Bicuculline or 2-OH saclofen, at the dose used, induced a significant but short-lasting (< 10 min) decrease in baseline MSAP (bicuculline: -15.4 ± 3.8 mmHg, n = 8; 2-OH saclofen: -13.7 ± 3.1 mmHg, n = 7) or HR (bicuculline: - 18 ± 6 bpm, n = 8; 2-OH saclofen: -13 ± 4 bpm, n = 7).

Reversal of Cardiovascular Responses to Microinjection of Dex into the NTS By GR Antagonist. Co-microinjection of a type II GR receptor antagonist, RU486

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(100 or 200 pmol) significantly and dose-dependently reversed the delayed cardiovascular excitatory effects of Dex (100 pmol) (Fig. 4A) that commenced 30 min postinjection and endured the remaining observation period. Comparable results were obtained in animals that received RU486 30 min prior to Dex microinjection (data not shown). On the other hand, RU486 given together or 30 min before Dex did not affect the initial hypertension or tachycardia induced by the glucocorticoid. Microinjection bilaterally into the NTS of RU486 (200 pmol) alone resulted in no significant changes in baseline MSAP (peak change: -8.9 ± 2.4 mmHg, n = 6) or HR (peak change: -6 ± 4 bpm, n = 6).

Lack of Effect on Cardiovascular Responses to Microinjection of Dex into the NTS By RNA Synthesis Inhibitor. Both the pressor and tachycardiac effects (Fig. 4B) induced by microinjection bilaterally into the NTS of Dex (25 or 100 pmol) persisted in animals that received pretreatment with the RNA synthesis inhibitor, AMD (20 nmol), administered bilaterally into the NTS 24 h prior to Dex administration. At the dose used, AMD induced minimal effect on baseline MSAP (peak change: -1.9 ± 1.3 mm Hg, n = 6) or HR (peak change: - 6 ± 5 bpm, n = 6). The effectiveness of AMD as a transcription inhibitor was verified in a separate series of experiments (Fig. 4C). Microinjection of Dex (100 pmol) bilaterally into the NTS induced upregulation of HO-2 or downregulation of nNOS mRNA expression, measured 24 h later in the dorsomedial medulla. Both actions of Dex were blocked by co-administration of RU486 (150 pmol). Co-microinjection of AMD (20 nmol) with Dex (100 pmol) bilaterally into the NTS also inhibited the GR-dependent upregulation of HO-2 or downregulation of nNOS mRNA expression in the dorsomedial medulla, detected 24 h posttreatment.

Temporal Expression of GR protein in the Dorsomedial Medulla After Microinjection of Dex into the NTS. Microinjection bilaterally into the NTS of Dex (100 pmol) resulted in a gradual increase in the expression of GR protein in

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nuclear extract from the dorsomedial medulla that included the NTS (Fig. 5A). From a low level detected immediately or at 1 or 2 h after glucocorticoid treatment, this GR expression exhibited a significant increase at 4 or 8 h after Dex application.

Activation of PI3K/Protein Kinase Akt Pathway in the Dorsomedial Medulla after Microinjection of Dex into the NTS. Microinjection bilaterally into the NTS of Dex (100 pmol) induced the formation of a GR/p85α PI3K complex (Fig. 5B) and increased phosphorylation of Akt (Fig. 5C) in the dorsomedial medulla, measured respectively at 20 and 30 min posttreatment. The Dex-promoted GR/p85α PI3K association or Akt phosphorylation was significantly inhibited by co-administration with RU486 (200 pmol). Dex treatment also led to an increase in Akt kinase activity (Fig. 5D) detected 30 min after administration of the glucocorticoid. Likewise, this Dex-promoted Akt activation was inhibited by co-administration of RU486 (200 pmol) or LY294002 (20 nmol), but not by the transcriptional inhibitor AMD (20 nmol), delivered 24 h prior to Dex administration.

Reversal of Cardiovascular Responses to Microinjection of Dex into the NTS by PI3 Kinase Inhibitor. Co-microinjection of a PI3K inhibitor, LY924002 (20 nmol) into the NTS significantly suppressed the delayed cardiovascular excitatory effects of Dex (100 pmol) (Fig. 6A). On the other hand, the initial hypertension and tachycardia promoted by Dex was not affected. Microinjection bilaterally into the NTS of LY294002 (20 nmol) alone resulted in no significant change in baseline MSAP (peak change: +3.5 ± 1.9 mmHg, n = 6) or HR (peak change: +7 ± 3 bpm, n = 6).

Inhibition of Cardiovascular Responses to Microinjection of Dex into the NTS By Nitric Oxide Synthase Inhibitor. **C**o-microinjection of a non-selective NOS inhibitor, L-NMMA (5 nmol), into the NTS significantly reversed the delayed cardiovascular excitatory effects of Dex (100 pmol) (Fig. 6B) that commenced 30 min

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postinjection and endured the remaining observation period. The initial pressor and tachycardiac responses by Dex, on the other hand, were not affected by the same treatment. Similar to the previous findings (Chan et al., 2004), microinjection bilaterally into the NTS of L-NMMA (5 nmol) alone resulted in no significant change in baseline MSAP (peak change: -5.8 ± 2.6 mmHg, n = 6) or HR (peak change: -9 ± 3 bpm, n = 6).

Discussion

The present study provides novel evidence to support a nongenomic role for glucocorticoids at the NTS in central cardiovascular regulation. Microinjection bilaterally of Dex into the NTS elicited cardiovascular excitatory responses within our 180-min observation period. The initial response, which lasted 15-30 min and included elevations in SAP and HR, was blunted by either $GABA_A$ or $GABA_B$ receptor antagonist. The delayed response, which endured the remaining 100-120 min and entailed a maintained pressor and tachycardiac response, was reversed by a GR receptor antagonist. Together, these results suggest that both GR-independent and GR-dependent mechanisms are involved in the elicitation of acute cardiovascular responses by Dex in the NTS.

Activation of $GABA_A$ or $GABA_B$ receptors in the NTS results in short-lasting hypertension and tachycardia (Sved and Sved, 1990; Barron et al., 1997). It is therefore intriguing that the initial GR-independent cardiovascular actions of the glucocorticoid engaged an interaction with both $GABA_A$ and $GABA_B$ receptors in the NTS. Our results revealed that the Dex-induced pressor or tachycardiac response exhibited a parallel time-course of early onset (0-5 min), rapid peaking time (10-15 min) and short duration (< 30 min). More importantly, they were similarly blunted by bicuculline or 2-OH saclofen, and were completely eliminated by combined treatment with the $GABA_A$ and $GABA_B$ receptor antagonists. A GR antagonist RU486, on the

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other hand, was ineffective. Steroids may exert receptor-independent actions on the cell membrane (Teschemacher et al., 1995; Ouyang and Wang, 2000; Sakai et al., 2000), or elicit direct allosteric modulation of GABA receptors (Turner et al., 1989). Contrast to the natural glucocorticoids that increase binding affinity of muscimol to GABA receptor, synthetic glucocorticoid does not directly affect muscimol binding (Majewska et al., 1985). Thus, it is unlikely that the Dex-induced initial cardiovascular responses are due to direct increase in binding affinity of ligands to GABA receptors in the NTS. Alternatively, the antagonistic effects of bicuculline or 2-OH saclofen on Dex-induced cardiovascular response indicates the involvement of GABAergic neurotransmission, probably in an upstream event that results in more GABA having access to the receptors. In support of this suggestion, Dex decreases the excitability of barosensitive NTS neurons via activation of GABA receptors and augmentation of the delayed rectifying potassium current (Ouyang and Wang, 2000). These actions of Dex are short-lasting and are not mediated by GR in the NTS (Ouyang and Wang, 2000). It is thus conceivable that the initial cardiovascular responses to Dex may be attributable to a GR-independent mechanism that involves interactions with GABAergic neurotransmission in the NTS. Of note is that a GR-independent interaction between Dex and the GABAergic system in the NTS is responsible for the abolishment of cardiovascular depression promoted by neuropeptide Y (Ouyang and Wang, 2000) or norepinephrine (Ouyang et al., 1999).

Another important contribution of this study is the establishment of a nontranscriptional mechanism of GR-dependent cardiovascular actions by glucocorticoids in the NTS. Translocation of activated GR to the nucleus contributes mainly to the well-established cellular responses of steroid hormones (Beato et al., 1995). This genomic mechanism of steroid hormones usually takes hours or days to effect. Our results indicated that the delayed hypertension and tachycardia, which was blunted by RU486, occurred within 30-45 min after microinjection of high doses of Dex into the NTS. More importantly, these manifested phenotypic effects of Dex were not affected by a blockade of *de novo* RNA synthesis with AMD (Fig. 4B), and took

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place during a time period when translocation of activated GR protein into the nucleus was minimal (Fig. 5A). Together, these observations indicate that the delayed cardiovascular effects after activation of the GR in the NTS by Dex may not involve a transcriptional mechanism. Our observation that expression of GR in the nucleus occurred 4-8 h after Dex treatment further suggests that this nontranscriptional activation of GR by the glucocorticoid in the NTS may not be a consequence of untoward stress, since acute stress evokes a rapid GR nuclear translocation within one hour after stress (Spencer et al., 1993).

Instead of a transcriptional mechanism, this study provided novel evidence to suggest that the delayed cardiovascular excitation after nontranscriptional activation of GR in the NTS may involve activation of the intracellular PI3K/Akt signaling pathway. We found that Dex induced an association of GR with the regulatory subunit of PI3K, p85α, in a ligand-dependent manner (Fig. 5B), followed by in vivo phosphorylation of Akt (Fig. 5C) and activation of Akt kinase activity (Fig. 5D) in the dorsomedial medulla. It is noteworthy that this Dex-induced activation of PI3K/Akt pathway in the NTS does not involve transcriptional induction of target genes after GR activation. Our results showed that the transcriptional inhibitor AMD, at a dose that significantly inhibits Dex-induced transcriptional upregulation of HO-2 mRNA or downregulation of neuronal NOS mRNA expression in the dorsomedial medulla (Fig. 4C) (Weber et al., 1994; Reagan et al., 1999), did not affect activation of Akt by the glucocorticoid. Nontranscriptional activation of endothelial NOS is reportedly mediated by the PI3K/Akt pathway (Hafezi-Moghadam et al., 2002; Limbourg et al., 2002; Limbourg and Liao, 2003). Our demonstration that PI3K or NOS inhibitor similarly reversed the Dex-induced delayed cardiovascular excitation (Fig. 6) further suggests that these cardiovascular responses may be mediated by nontranscriptional activation of NOS through the PI3K/Akt pathway.

The role of glucocorticoids in central regulation of blood pressure remains poorly understood. Earlier studies demonstrated that intracerebroventricular infusion of low doses of glucocorticoid is ineffective (Gomez-Sanchez et al., 1990), whereas an

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intermediate dose causes a decrease in arterial pressure (Tonolo et al., 1993). Other studies reported that bolus injection of higher doses of glucocorticoid into the cerebral ventricle (van den Berg et al., 1989) or the dorsal hindbrain (Scheuer et al., 2004) increase arterial pressure. The types and/or doses of steroids used to activate GR and brain regions affected may account for those discrepancies. Our results provide the first demonstration that glucocorticoids participate in central cardiovascular regulation by acting directly on the NTS, the major neural substrate in caudal medulla that integrates inputs from baroreceptor afferents and forebrain nuclei (Mifflin et al., 1988).

The selectivity of bicuculline methiodine and 2-OH saclofen as $GABA_A$ and $GABA_B$ receptor antagonists (Al-Dahan et al., 1990; Galvez-Ruano et al., 1995), or AMD as a RNA synthesis inhibitor (Limbourg et al., 2002), has been documented. Activation of type II, but not type I, glucocorticoid receptors contributes to hypertension induced by the agonists (Calpham and Turner, 1997). RU486 is a type II glucocorticoid receptor antagonist that does not bind to type I mineralocorticoid receptors or aldosterone receptors (Brogden et al., 1993). We also recognize that our results showed that $GABA_A$ or $GABA_B$ receptor antagonist by itself evoked hypotension and bradycardia. Thus, reversal of the initial phase of Dex-induced pressor and tachycardiac responses by either GABA receptor antagonist may simply result from cancellation of two physiological responses. This possibility, however, is deemed unlikely, since the cardiovascular inhibitory actions of bicuculline or 2-OH saclofen lasted less than 10 min, whereas their effects to reverse the Dex-induced cardiovascular excitation endured 30 min after microinjected into the NTS. The lack of effect of RU486 to antagonize the initial cardiovascular response to Dex might result from a delay but higher affinity binding of RU486 to GR as compared to Dex. That microinjected bilaterally into the NTS of RU486 30 min prior to Dex administration had no effect on the Dex-induced initial hypertension and tachycardia reduces greatly this possibility. In addition, the comparable temporal profiles of GR antagonist and PI3K inhibitor in reversing Dex-induced cardiovascular responses

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suggest that activation of GR is engaged in the delayed Dex-induced cardiovascular excitation in the NTS. Activation and translocation of GR can be affected by diurnal change in plasma concentration of corticosterone (Spencer et al., 1993; Scheuer et al., 2004) and by acute stress (Spencer et al., 1993). As such, all experiments were conducted between 9:00 and 12:00. In addition, our preliminary results indicate no significant difference in plasma level of corticosterone between control and Dex-treated animals (1.5-4.3 μg/dL vs 1.2-4.7 μg/dL, n = 3), detected at 120 min posttreatment. These observations, which are comparable to those determined in the morning in conscious rats (Spencer et al., 1993; Scheuer et al., 2004), reduce the possible confounding effect of acute stress on Dex-induced cardiovascular responses.

Both naturally occurring and synthetic glucocorticoids are important regulators of blood pressure, and excessive glucocorticoid is associated with hypertension in animals and in human. Much of the earlier work on the blood pressure-raising effects of the steroids focuses on GR-dependent transcriptional mechanisms. Recent findings, however, suggest the presence of rapid, nontranscriptional effects of the steroids in both cardiac (Hafezi-Moghadam et al., 2002) and vascular systems (Limbourg et al., 2003). Our demonstration that both GR-independent and GR-dependent but nontranscriptional mechanisms are involved in the acute hypertensive and tachycardiac responses to glucocorticoid in the NTS further expands the current mechanistic views on glucocorticoid-induced hypertension. Moreover, the identification of a nontranscriptional link between GR and PI3K/Akt pathway expands the properties of glucocorticoids beyond a ligand-inducible transcriptional activator in the regulation of blood pressure.

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Footnotes

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Fig. 1. (A). Temporal changes in mean system arterial pressure (MSAP) or heart rate (HR) in rats that received microinjection bilaterally into the NTS (at time 0; n = 7–8 animals per group) of aCSF or dexamethasone-21-phosphate sodium (Dex). Values are mean ± SEM. $^{*}P < 0.05$ vs aCSF group in the Scheffé multiple-range analysis. (B). Diagrammatic representations of three levels of the NTS with reference to the obex illustrating the location of sites where microinjection of Dex elicited significant (●) or insignificant (○) effect on MSAP or HR. Numbers on the left side indicate distance from the obex. AP, area postrema; NTS, nucleus tractus solitarii; cc, central canal; ts, tractus solitarii; X, nucleus dorsalis nervi vagi; XII, nucleus hypoglossi.

Fig. 2. Temporal changes in MSAP or HR in rats that received co-microinjection bilaterally into the NTS (at time 0; n = 6–7 animals per group) of Dex and aCSF, bicuculline (A) or 2-hydroxy saclofen (2-OH saclofen) (B). Data on aCSF from Figure 1 are duplicated for comparison. Values are mean ± SEM. $^{*}P < 0.05$ vs aCSF group, $^{\#}P < 005$ vs respective Dex group in the Scheffé multiple-range analysis.

Fig. 3. Temporal changes in MSAP or HR in rats that received microinjection bilaterally into the NTS (at time 0; n = 5–7 animals per group) of a low dose (25 pmol) (A) or a high dose (100 pmol) (B) of Dex, in combination with aCSF, or bicuculline plus 2-hydroxy saclofen. Data on aCSF or Dex+aCSF from Figure 2 are duplicated for comparison. Values are mean ± SEM. $^{*}P < 0.05$ vs aCSF group, $^{\#}P < 005$ vs respective Dex group in the Scheffé multiple-range analysis.

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Fig. 4. Temporal changes in MSAP or HR in rats that received co-microinjection bilaterally into the NTS (at time 0; n = 6–7 animals per group) of Dex and 1% DMSO or mifepristone (RU486) (A), or Dex and 3% methanol (MeOH) or actinomycin D (AMD) (B). Data on aCSF from Figure 1 are duplicated for comparison. Values are mean ± SEM. $^{*}P < 0.05$ vs aCSF group, $^{\#}P < 005$ vs respective Dex group in the Scheffé multiple-range analysis. Also shown are representative gels for RT-PCR products of heme oxygenase (HO)-2, neuronal nitric oxide synthase (nNOS) or GAPDH mRNA, detected from the dorsomedial medulla 24 h after microinjection bilaterally into the NTS of aCSF (Conotrol) or Dex (100 pmol), given alone or with RU486 (150 pmol) or AMD (20 nmol) (C). These results are representative of four independent experiments.

Fig. 5. Representative Western blots (inset) of glucocorticoid receptor (GR) protein or GR level detected from the nuclear extracts of dorsomedial medulla in rats at 0, 1, 2, 4 or 8 h after microinjection bilaterally into the NTS of Dex (100 pmol) (A). Western blot of γ-tubulin was carried out to verify that all lanes contain equal amounts of nuclear protein. Values are mean ± SEM, n = 5–6 animals per group. $^{*}P < 0.05$ vs pretreatment control (C) group in the one-way ANOVA. (B) shows representative results from immunoprecipitation (IP) followed by immunobloting (IB) assays on association of GR with p85α PI3K in the dorsomedial medulla 20 min (Dex-20) after microinjection bilaterally into the NTS of aCSF (Control), Dex (100 pmol), alone or with RU486 (150 pmol). Also shown are representative Western blot gels showing the expression of phosphorylated Akt (P-Akt) (C) or Akt activity (D) from the cytosolic extracts of dorsomedial medulla in rats at 5, 10, 20, 30 or 60 min after microinjection bilaterally into the NTS of Dex (100 pmol) alone or with AMD (20 nmol), RU486 (150 pmol) or LY294002 (20 nmol). Data in (B), (C) and (D) are representative of four independent experiments.

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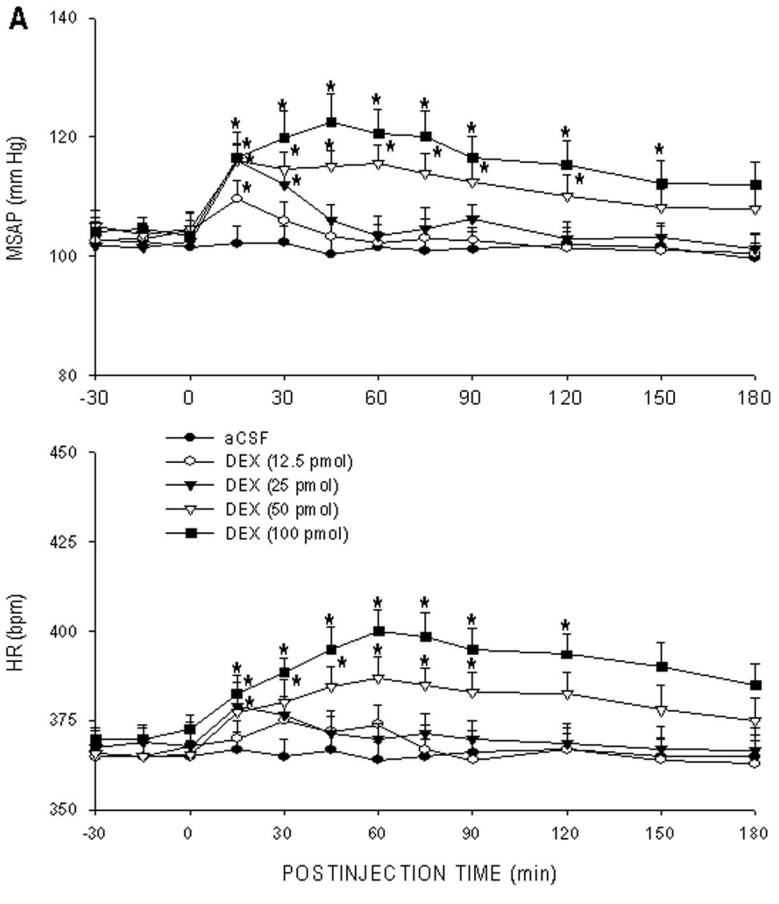
Fig. 6. Temporal changes in MSAP or HR in rats that received co-microinjection bilaterally into the NTS (at time 0; n = 6–7 animals per group) of Dex and 0.2% DMSO or LY294002 (A), or Dex and aCSF or L-NMMA (B). Data on aCSF from Figure 1 are duplicated for comparison. Values are mean ± SEM. $^{*}P < 0.05$ vs aCSF group, $^{\#}P < 005$ vs Dex group in the Scheffé multiple-range analysis.

Figure 1


A
MSAP (mm Hg)
HR (bpm)
aCSF
DEX (12.5 pmol)
DEX (25 pmol)
DEX (50 pmol)
DEX (100 pmol)
POSTINJECTION TIME (min)

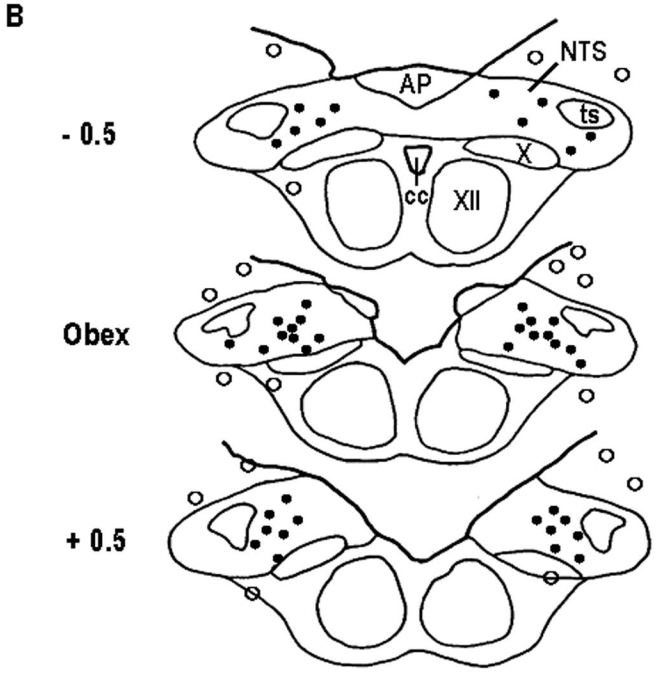

B
- 0.5
Obex
+ 0.5
AP
NTS
ts
X
cc
XII

Figure 2

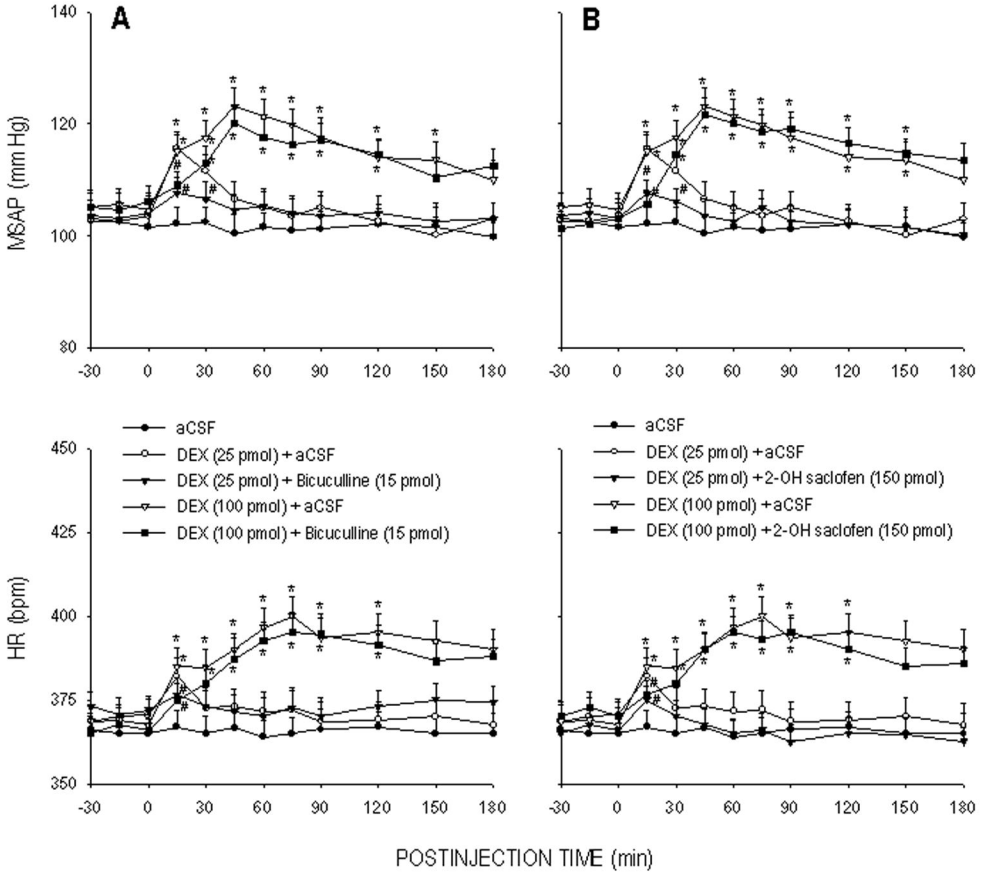

A
B
MSAP (mm Hg)
HR (bpm)
POSTINJECTION TIME (min)
aCSF
DEX (25 pmol) + aCSF
DEX (25 pmol) + Bicuculline (15 pmol)
DEX (100 pmol) + aCSF
DEX (100 pmol) + Bicuculline (15 pmol)
DEX (25 pmol) + 2-OH saclofen (150 pmol)
DEX (100 pmol) + 2-OH saclofen (150 pmol)

Figure 3

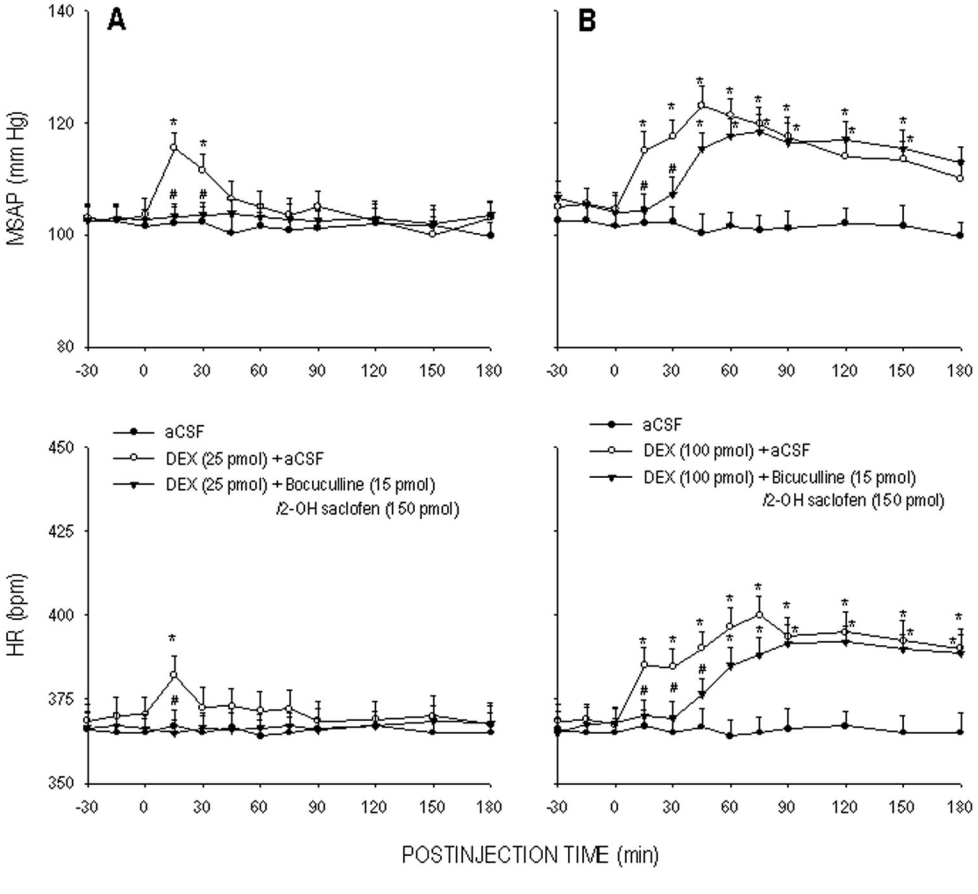

A
B
MSAP (mm Hg)
HR (bpm)
aCSF
DEX (25 pmol) + aCSF
DEX (25 pmol) + Bocuculline (15 pmol)
/2-OH saclofen (150 pmol)
aCSF
DEX (100 pmol) + aCSF
DEX (100 pmol) + Bicuculline (15 pmol)
/2-OH saclofen (150 pmol)
POSTINJECTION TIME (min)

Figure 4

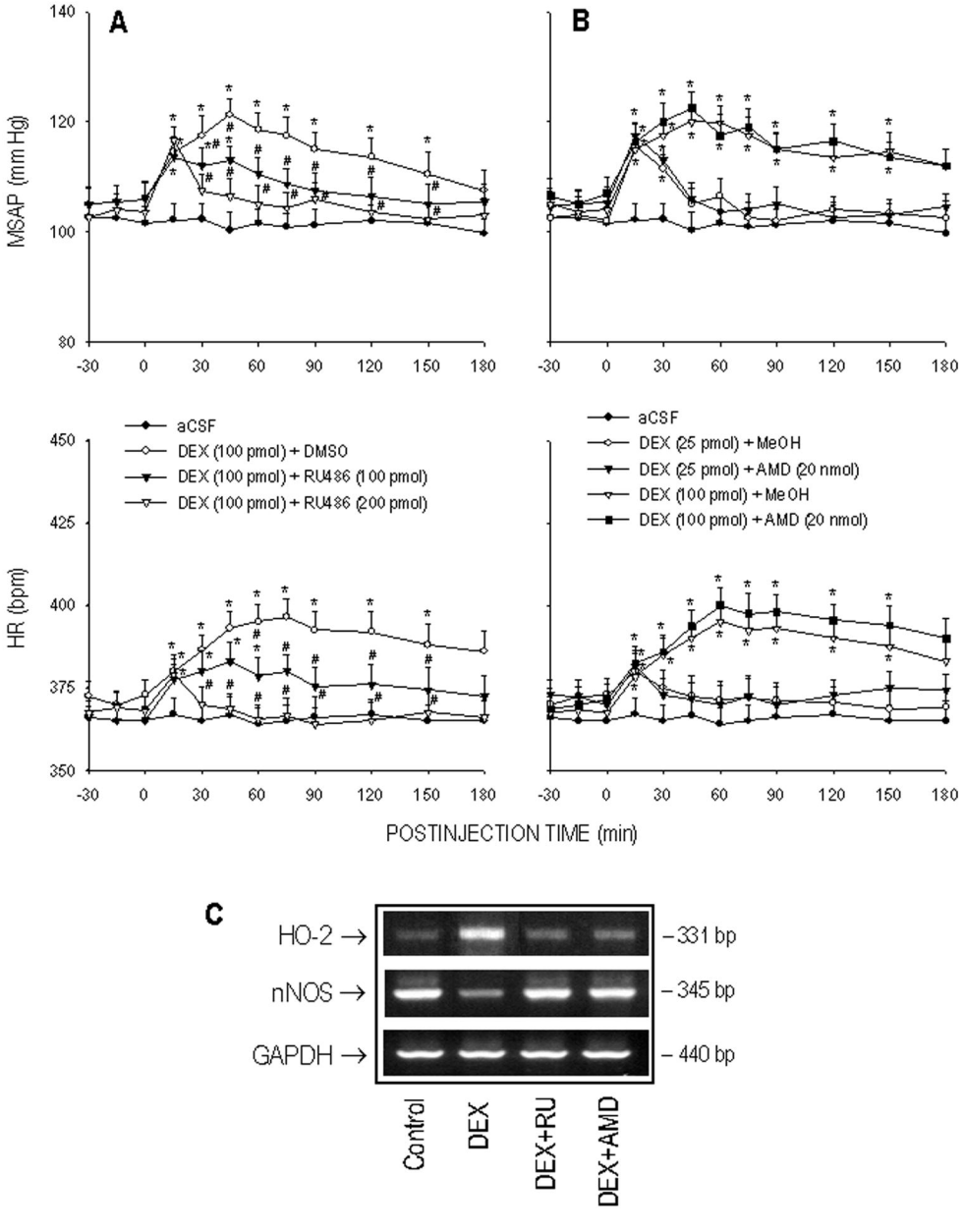

A
B
MSAP (mm Hg)
HR (bpm)
POSTINJECTION TIME (min)
aCSF
DEX (100 pmol) + DMSO
DEX (100 pmol) + RU486 (100 pmol)
DEX (100 pmol) + RU486 (200 pmol)
aCSF
DEX (25 pmol) + MeOH
DEX (25 pmol) + AMD (20 nmol)
DEX (100 pmol) + MeOH
DEX (100 pmol) + AMD (20 nmol)
C
HO-2 →
nNOS →
GAPDH →
– 331 bp
– 345 bp
– 440 bp
Control
DEX
DEX+RU
DEX+AMD

Figure 5

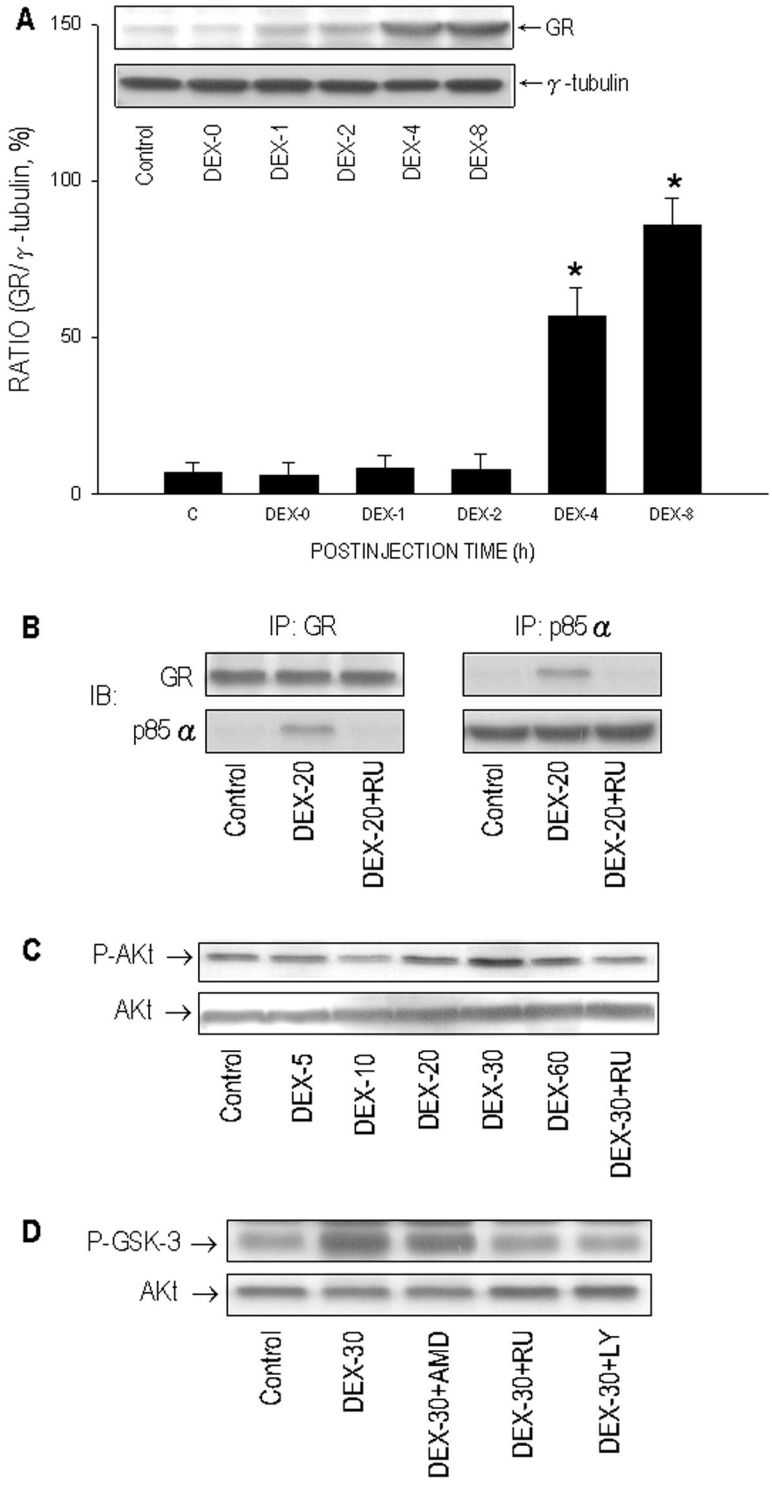
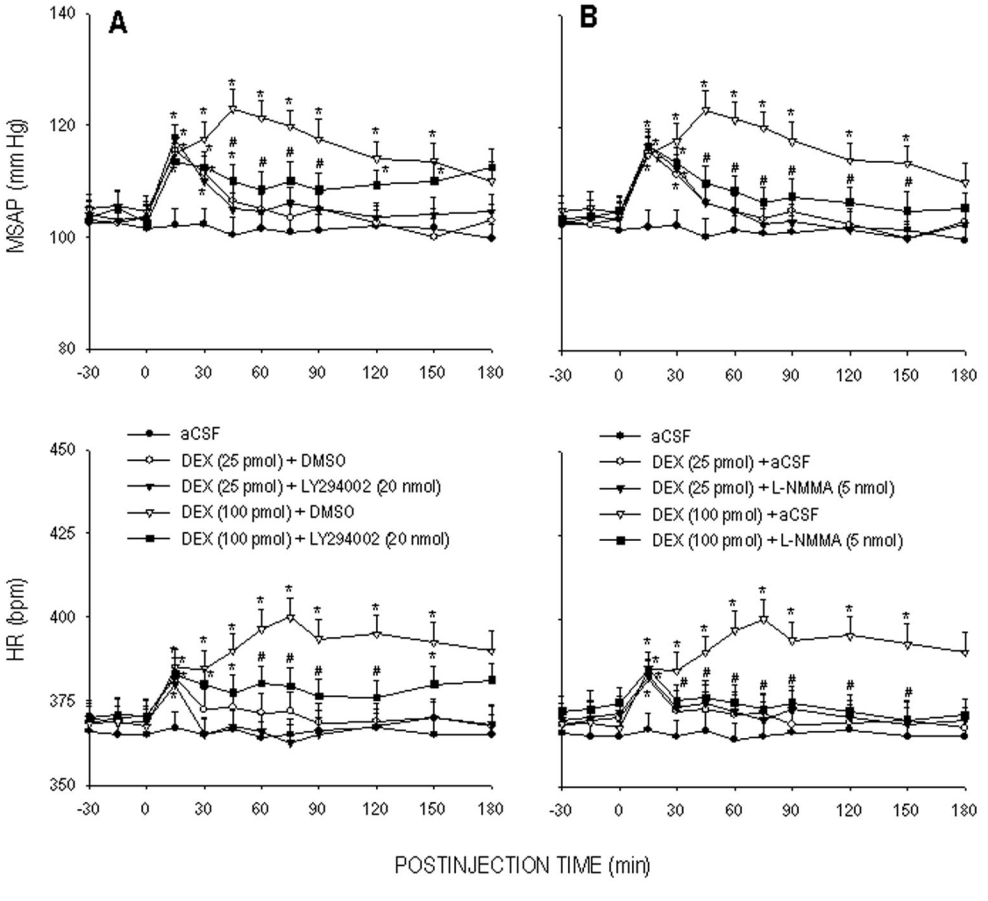

A
150
100
50
0
GR
γ-tubulin
Control
DEX-0
DEX-1
DEX-2
DEX-4
DEX-8
RATIO (GR/γ-tubulin, %)
C
DEX-0
DEX-1
DEX-2
DEX-4
DEX-8
POSTINJECTION TIME (h)
B
IP: GR
IP: p85α
IB:
GR
p85α
Control
DEX-20
DEX-20+RU
Control
DEX-20
DEX-20+RU
C
P-AKt
AKt
Control
DEX-5
DEX-10
DEX-20
DEX-30
DEX-60
DEX-30+RU
D
P-GSK-3
AKt
Control
DEX-30
DEX-30+AMD
DEX-30+RU
DEX-30+LY

Figure 6


A
B
MSAP (mm Hg)
HR (bpm)
POSTINJECTION TIME (min)
aCSF
DEX (25 pmol) + DMSO
DEX (25 pmol) + LY294002 (20 nmol)
DEX (100 pmol) + DMSO
DEX (100 pmol) + LY294002 (20 nmol)
aCSF
DEX (25 pmol) + aCSF
DEX (25 pmol) + L-NMMA (5 nmol)
DEX (100 pmol) + aCSF
DEX (100 pmol) + L-NMMA (5 nmol)