A Novel Model of Dexamethasone-Induced Hypertension: Use in Investigating the Role of Tyrosine Hydroxylase

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ABSTRACT
Our objective was to study hypertension induced by chronic administration of synthetic glucocorticoid, dexamethasone (DEX), under nonstressful conditions and examine the role of catecholamine biosynthesis. To achieve this, we did the following: 1) used radiotelemetry to record mean arterial pressure (MAP) and heart rate (HR) in freely moving rats, and 2) administered different doses of DEX in drinking water. To evaluate the involvement of tyrosine hydroxylase (TH), the rate-limiting step in catecholamine biosynthesis, we treated rats with the TH inhibitor, α-methyl-para-tyrosine (α-MPT), for 3 days prior to administration of DEX and assessed TH mRNA and protein expression by quantitative realtime polymerase chain reaction and Western blot in the adrenal medulla. We observed a dose-dependent elevation in blood pressure with a DEX dose of 0.3 mg/kg administered for 10 days, significantly increasing MAP by 15.0 ± 1.1 mm Hg, while concomitantly reducing HR. Although this DEX treatment also significantly decreased body weight, pair-fed animals that showed similar decreases in body weight due to lowered food intake were not hypertensive, suggesting that body weight changes may not account for DEX-induced hypertension. Chronic DEX treatment significantly increased the TH mRNA and protein levels in the adrenal medulla, and α-MPT administration not only reduced DEX pressor effects, but also inhibited TH (serine3) phosphorylation. Our study thus validates a novel model to study hypertension induced by chronic intake of DEX in freely moving rats not subject to the confounding factors of previous models and establishes its dependence on concomitant activation of peripheral catecholamine biosynthesis.

Introduction
Synthetic glucocorticoids are used in the chronic treatment of inflammatory diseases (Kirwan, 1995; Saag, 2002; van Everdingen et al., 2002). Dexamethasone (DEX) is one of the most commonly used synthetic glucocorticoids because of its potency (Kari et al., 1994; Kaal and Vecht, 2004; Piette et al., 2006). However, cumulative exposure to glucocorticoids increases the risk of cardiovascular outcomes, including hypertension (Souverein et al., 2004; Davis et al., 2007). Approximately 70% of the patients undergoing chronic glucocorticoid therapy become hypertensive, the severity being dependent on the dose and duration of the treatment (Souverein et al., 2004; Panoulas et al., 2008). Nevertheless, the mechanisms by which chronic glucocorticoid therapy induces hypertension are not well understood.

Cortisol, the endogenous glucocorticoid, can produce hypertension associated with increased sodium and water reten-
tion and cause renal impairment via interaction with both mineralocorticoid and glucocorticoid receptors (Whitworth et al., 1989, 2000; Mangos et al., 2003). DEX, in contrast, affects renal function (Bia et al., 1982) specifically via the glucocorticoid receptor (GR) (Reul et al., 1987). However, studies evaluating the kidney-specific homozygous deletion of GR in mice concluded that the GR in the distal nephron is not necessary for the development or maintenance of DEX-induced hypertension (Goodwin et al., 2010). A potential alternative site would be a central effect. However, DEX does not readily cross the blood brain barrier (De Kloet, 1997; Meijer et al., 1998), precluding direct activation of the central nervous system. Thus, the mechanism by which DEX raises blood pressure is most likely peripheral, possibly through activation of the peripheral sympathoadrenal system. It is long known that sympathetic hyperactivation can result in elevated cardiac output and total peripheral resistance (Ulrich et al., 1968; Fletcher et al., 1976). Increased sympathetic nerve activity and elevated circulating catecholamine concentrations are common characteristics of essential hypertension (Eslser et al., 1980), as well as in deoxycorticosterone-salt hypertension and in spontaneously hypertensive rats (Sakaguchi et al., 1983; Lange et al., 1998). Despite this knowledge on the involvement of sympathetic nervous system

ABBREVIATIONS: α-MPT, α-methyl-para-tyrosine; BPM, beats per minute; DEX, dexamethasone; GR, glucocorticoid receptor; HR, heart rate; MAP, mean arterial pressure; SCG, superior cervical ganglion; TH, tyrosine hydroxylase; VEH, vehicle.
in blood pressure development (Esler et al., 2008; DiBona and Esler, 2010), how chronic glucocorticoid exposure affects it is largely unknown.

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in catecholamine biosynthesis and is highly expressed in the adrenal medulla and sympathetic neuronal terminals. At the molecular level, our laboratory identified an atypical glucocorticoid response element in the promoter region of rat and human TH gene that is stimulated by DEX (Rani et al., 2009; Sheela Rani et al., 2013). In the present study, we hypothesized that an underlying mechanism for DEX-induced hypertension involves increases in TH gene expression in the adrenal medulla, and perhaps in other sympathetic terminals, upon chronic DEX treatment; this in turn leads to a sustained increase in catecholamine synthesis, higher enzyme activity, and finally to hypertension. Indeed, DEX administration is known to increase plasma catecholamine levels in hypertensive patients (Watanabe et al., 1995). We chose to study adrenal medulla as a representative of peripheral sympathetic ganglia because medullary cells are, indeed, modified postganglionic cells of the autonomic nervous system that receive innervation from corresponding preganglionic fibers (Perlman and Chalfie, 1977).

Furthermore, we used α-methyl-para-tyrosine (α-MPT), an irreversible TH inhibitor (Udenfriend et al., 1965; Ankenman and Salvatore, 2007), to explore the possible role of adrenal TH in DEX-induced hypertension, because earlier studies indicated that this drug is effective in treating certain types of hypertension involving increased serum catecholamine levels (Perry et al., 1990; Chiuo-Tan et al., 1994; Shimizu et al., 2001). In addition to adrenal medulla, α-MPT would also allow us to access the role of sympathetic nerve terminals because it has been shown to cause catecholamine depletion in terminals as well (Nagatsu et al., 1964; Spector et al., 1965).

The primary aim of this study was to generate a novel in vivo model to study hypertension following chronic DEX administration without the involvement of stressors due to the methodologies used for drug administration and blood pressure recording. To accomplish this, we delivered DEX in drinking water and used a radiotelemetry approach to continuously record blood pressure. This method has the advantage of measuring blood pressure directly in freely moving/resting animals, thus avoiding handling-related changes in their arousal state (Kurtz et al., 2005). We evaluated the dose-response relationship between chronic administration of DEX in drinking water and changes in blood pressure, and also examined TH expression and phosphorylation at serine40 (pSer40) as possible mechanisms in the generation of DEX-induced hypertension by α-MPT systemic administration.

Materials and Methods

Animals. Male Fisher 344 rats, 8–10 weeks old and weighing 250–300 g, were purchased from Harlan (Houston, TX). They were maintained on a 14/10-hour light/dark cycle and fed ad libitum for the whole study. Rats were allowed to acclimate to housing conditions for 3 days and then underwent telemetric implant surgery. After the surgery, the rats were individually housed in plastic cages with continuous access to rat chow (Harlan Teklad 7912) and water. The experiments were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Radiotelemetry Implantation and Measurement of Blood Pressure and Heart Rate. Implantation surgery was done under isoflurane anesthesia. A catheter attached to a CA11PA-C40 radiotelemetry transmitter (Data Science International, St. Paul, MN) was implanted in the peritoneal aorta and secured to the abdominal muscle. The animals were allowed to recover for 8 days. Each animal was housed in a plastic cage, placed on top of a RPC-1 receiver that was attached to a Data Exchange Matrix (Data Science International). After recovery, systolic blood pressure, diastolic blood pressure, and mean arterial pressure (MAP), as well as heart rate (HR), were monitored for 3–5 days to establish the baseline. Recordings were performed every 10 minutes; data were collected using Dataquest A.R.T. 4.1 software (Data Science International). Because systolic blood pressure and diastolic blood pressure responded similarly to MAP, we present our data only as MAP. Results of change in Δ MAP, ΔHR, and Δbody weight were calculated by subtracting the 24-hour mean values from the average of the mean of 3 days of baseline recording for DEX dose-response curves and pair-feeding experiments, and 5 days of baseline recording for the α-MPT experiment. Day 1 indicates the first day of recording to establish the baseline, and the numeration continues until the last day of recording/treatment on day 13 for DEX dose-response and pair-feeding experiments, and on day 17 for the α-MPT study.

Dexamethasone Treatment. To evaluate the dose of DEX that produces a significant increase in blood pressure, animals received either regular drinking water (controls) or a water-soluble form of synthetic glucocorticoid (GC), dexamethasone 21-phosphate disodium salt (Sigma Aldrich, St. Louis, MO), in drinking water. Three different groups of rats received DEX in drinking water for 10 days: low (0.03 mg/kg/d), medium (0.3 mg/kg/d), and high (3 mg/kg/d). Water intake was measured every day and to calculate daily DEX doses was adjusted to it as well. Body weight was monitored daily to determine possible catabolic DEX effects.

Pair-Feeding. To determine the role of body weight loss on the blood pressure effects of DEX, we treated a group of rats with DEX (0.16 mg/kg/d) for 10 days and performed pair feeding of a group of untreated rats with the same amount of food previously determined to be consumed by the DEX-treated group. We used this single mid-range dose (0.16 mg/kg/d) in this control study because both 0.03 and 0.3 mg/kg/d had earlier produced elevations in blood pressure. Food intake was calculated as the difference of weight of the food container between the day when the container was filled and 24 hours after that.

Measurement of TH mRNA Content in the Adrenal Medulla and Brainstem. After 10 days of DEX treatment, the animals were euthanized, and the adrenal medullae were removed, flash frozen, and stored at −80°C. Brains were removed, and the brainstem was separated from the encephalon. A 4-mm section of the medulla oblongata was cut. The left adrenal medullae and the brainstem regions were homogenized in 1 ml TRizol reagent (Life Technologies/Invitrogen, Carlsbad, CA). Total RNA was isolated and treated with DNase and used for synthesizing cDNA using the High Capacity cDNA synthesis kit (Life Technologies/ Applied Biosystems, Foster City, CA), according to manufacturer’s instructions. cDNA (10 ng) was used for quantitative real-time polymerase chain reaction, as described previously (Green et al., 2011), using a TaqMan gene expression master mix and 6-carboxyfluorescein–labeled probe for rat TH (Gene Expression Assay ID Rn00662500_m1; Life Technologies/ Applied Biosystems) and 18S rRNA, which was used as the endogenous normalizer and amplified simultaneously using TaqMan Eukaryotic 18S rRNA endogenous control VIC MGB probe (primer limited, 4319413E). The assays were performed in duplicate using the ABI PRISM 7900 Sequence Detection System. The relative expression was calculated from the average difference in cycle threshold between the vehicle (veh) control and DEX-treated samples using the ΔΔ cycle threshold method (Livak and Schmittgen, 2001).

Pharmacological Inhibition of TH. To determine the role of TH in DEX hypertension, separate groups of rats were treated with the TH inhibitor, α-methyl-DL-tyrosine methyl ester hydrochloride...
showed an elevation of MAP by day 11 (+9.4 ± 3.5 mmHg) with respect to the VEH control group (\(P < 0.01\)) and continued increasing to 13.5 ± 3.8 mmHg by day 13 (\(***P < 0.001\) versus day 3 and \(\%P < 0.001\) versus VEH). The group that received the medium dose of 0.3 mg/kg/d (\(n = 5\)) showed an average MAP at baseline of 102.2 ± 2.7, and, at day 9, MAP increased 11.0 ± 1.4 mmHg, reaching earlier a high significant level (\(***P < 0.001\) versus day 3 and \(\%P < 0.001\) versus VEH) than the group with lowest dose. This level was maintained until day 13 (+15.0 ± 1.1 mmHg, \(***P < 0.001\) versus day 3 and \(\%P < 0.001\) versus VEH). Animals treated with the highest dose of DEX, 3 mg/kg/d (\(n = 4\)), presented an average baseline of 101.1 ± 0.9 mmHg. Nonetheless, they did not survive beyond 7 days of DEX treatment. By day 10, there were three animals alive. They also showed significant increases in MAP from days 8 to 10 that ranged from 10.2 ± 1.8 mmHg (\(***P < 0.001\) versus day 3) to 17.9 ± 3.3 mmHg (\(***P < 0.01\) versus day 3 and \(\%P < 0.01\) versus VEH), respectively.

With regard to HR, there were significant dose-dependent decreases (Fig. 2B). The group with the lowest dose, 0.03 mg/kg/d, showed an average baseline of 368.9 ± 9.7 beats per minute (BPM); upon DEX treatment, there were significant decreases (\(***P < 0.01\) versus day 3) on days 6 (−9.2 ± 4.2 BPM), 8 (−10.2 ± 2.7 BPM), 9 (−11.5 ± 4.0 BPM), and 10 (−12.0 ± 4.4 BPM). Only day 10 was different from the VEH group (\(\%P < 0.05\)). The group that received the medium dose had a HR average at baseline of 368.8 ± 5.2 BPM. Specifically, at this dose, HR decreased significantly on days 8 (−22.7 ± 4.1 BPM) to 13 (−125.3 ± 3.1 BPM) with respect to day 3 of baseline recording (\(***P < 0.001\)) and from day 10 (−26.0 ± 3.2 BPM, \(\%P < 0.05\)) to day 12 (−24.5 ± 1.2 BPM, \(\%P < 0.05\)) with respect to the VEH group. The third group with the highest dose (3 mg/kg/d) had a baseline HR average of 373.9 ± 5.0 BPM, and it started decreasing significantly (\(***P < 0.01\) versus day 3 and \(\%P < 0.01\) versus VEH) from day 6 (−43.8 ± 4.6 BPM) until day 10 (−54.2 ± 9.3 BPM), being that latter the last day of survival in this group.

Moreover, body weight decreased upon DEX administration in the three groups (Fig. 2C). The VEH control group showed significant increases (\(***P < 0.001\) versus day 3) from day 4 (−18.4 ± 6.4 g) to 13 (−45.2 ± 5.2 g). Animals treated with the lowest dose had significant reductions (\(***P < 0.001\) versus day 3) on days 12 (−13.9 ± 1.3 g) and 13 (−18.1 ± 0.8 g). It was also significantly reduced from the VEH group from day 5 to 13 (\(\%P < 0.05\)). Furthermore, the group with the medium dose showed significant reductions on body weight by day 8 (−25.7 ± 1.1 g, \(***P < 0.001\) versus day 3) and continued until day 13 (−46.3 ± 7.3 g, \(***P < 0.001\) versus day 3). It was also significantly different from the VEH control group from day 4 to 13 (\(\%P < 0.01\)). Finally, the third group receiving the highest dose of DEX showed significant decreases from day 7 (−25.9 ± 0.9 g, \(***P < 0.001\) versus day 3) to 10 (−70.0 ± 3.2 g, \(***P < 0.001\) versus day 3) and from day 5 to 10 with respect to the VEH group (\(\%P < 0.01\)).

**Reduction in Body Weight Is Not Responsible for DEX-Induced Hypertension.** To isolate the effect of reduced body weight from blood pressure, we used pair feeding with reduced food intake and compared it with the effects of DEX administration (\(n = 3\) group). In Fig. 3A, DEX-treated rats showed a significantly reduced food intake on days 11–17.
6 (13.3 ± 0.5 g, ***P < 0.001) to 12 (13.2 ± 0.8 g, ***P < 0.001) and 13 (14.2 ± 0.5 g, **P < 0.01), with respect to day 2 of baseline. It was also significantly reduced on days 7, 8, 11, and 12 with respect to the VEH control group (**P < 0.01 versus VEH). Moreover, the pair-fed group also had a decreased food intake on days 9 (10.8 ± 1.6 g, %P < 0.01 versus day 3) to 13 (12.8 ± 0.8 g, %P < 0.01 versus day 3) as well as on days 8, 9, 11, 12, and 13 with respect to the VEH group (%P < 0.05). Regarding body weight (Fig. 3B), it was reduced (**P < 0.01 and ***P < 0.001 versus day 3) in both groups: the pair-fed (days 8–13) and DEX-treated animals (days 6–13). These were also significantly different from the VEH group, **P < 0.01 and %P < 0.001, respectively. Finally, MAP increased only in DEX group, on days 6 to 8 (**P < 0.01) and 9 to 13 (**P < 0.001) with respect to day 3 of baseline and on days 11 to 13 (**P < 0.001) with respect to the VEH group (Fig. 3C), whereas MAP in the pair-fed group was not significantly different from the VEH control group or baseline.

**DEX Increased TH Expression in the Adrenal Medulla, but Not in Brainstem.** We tested the effect of DEX at the medium dose of 0.3 mg/kg/d on TH expression because this dose was the one that produced a significant elevation in blood pressure required by our statistical power analysis. Furthermore, to identify alterations of TH expression between the peripheral and central nervous system, TH mRNA was measured in two of its main locations: the adrenal medulla and brainstem. There was a 2.5-fold increase in TH mRNA levels in the adrenal medulla (**P < 0.01), whereas TH mRNA in the brainstem was not modified (Fig. 4).

**Effect of α-MPT on DEX-Induced Hypertension.** In Fig. 5A, MAP increased in VEH/DEX on days 12–17 (**P < 0.01 versus day 5) and 14–17 with respect to the VEH/VEH group (**P < 0.01). After the administration of α-MPT, there were no changes in MAP baseline in any of the groups. Nonetheless, the α-MPT/DEX group exhibited significantly diminished pressor effect on days 14–17 (+5.9 ± 1.6, +9.1 ± 1.4, +11.1 ± 1.2, and +11.9 ± 1.8 mmHg, respectively; **P < 0.025) compared with the responses in the group treated with DEX alone on the same days (+12.0 ± 1.2, +14.8 ± 1.4, +17.5 ± 1.3, and +18.8 ± 1.7 mmHg, respectively). Despite that, there were increases in the α-MPT/DEX group against the VEH/VEH group on days 13–17 (**P < 0.05). Furthermore, a comparison of ΔMAP of the α-MPT/DEX and VEH/DEX groups, normalized to their respective control group (Fig. 5B), was performed to discard possible interferences with baseline, and significant differences were observed (βP < 0.05) between these groups on days 12–17. Finally, the administration of α-MPT did not change HR during the baseline recording, nor did it influence DEX-induced decreases in HR (Fig. 5C). It decreased in VEH/DEX group on days 12–17 compared with day 5 of baseline (***P < 0.001) and days 13–17 compared with the VEH/VEH group (**P < 0.01). The α-MPT/DEX group had decreases on days 13–17 against day 5 of baseline (**P < 0.01) and VEH/VEH group (βP < 0.01). No significant differences were observed...
when normalizing HR to the respective VEH control groups (Fig. 5D).

At the end of telemetric recordings, animals were euthanized and the adrenal medullae were isolated to measure TH protein. There was a significant increase in TH, 1.3-fold (**P < 0.01), after 7 days of DEX treatment compared with the VEH-treated group. Moreover, α-MPT pretreatment inhibited DEX-induced increase in TH protein (Fig. 6A). TH phosphorylation also increased in the VEH/DEX group, as shown by an elevation in the level of pSer40-TH (*P < 0.05 versus VEH/VEH), and this increase was completely inhibited by pretreatment with α-MPT (Fig. 6B).

Discussion

Our study describes the development of an animal model to study hypertension induced by the chronic administration of DEX. Mainly, by choosing radiotelemetry for recording blood pressure and an oral route for chronic administration of DEX in rats, our model circumvents the confounding factors seen in earlier studies (Iijima and Malik, 1988; Wallwork et al., 2003; Mondo et al., 2006; Ong et al., 2007). These include stress from restraint that was used for blood pressure recording and administration of DEX, which may have had an effect on the arousal state of the animal, possibly altering blood pressure.
results. We found that MAP increases in a cumulative and dose-dependent manner over the period of continuous oral DEX administration (Fig. 2A), with significant increases (+11 mmHg) seen after 6 days of treatment with a medium dose of DEX (0.3 mg/kg/d). In an attempt to relate our experimental protocol to a clinically relevant dose/time frame, we can say our treatment paradigm would be approximately equivalent to 6 months of medium-dose GC therapy in humans (Quinn, 2005; Andreollo et al., 2012). Interestingly, patients affected by rheumatoid arthritis receiving long-term GC treatment (>6 months) are more prone to develop hypertension when using a medium dose of prednisone (0.1-4 mg/kg/d) than those with limited GC exposure (<0.1 mg/kg/d for <6 months) (Panoulas et al., 2008).

Concomitant with an increase in MAP, a 10-day continuous oral administration of DEX reduces food intake, but not water intake (data not shown), and is accompanied by a decrease in body weight in rats. However, the pair-fed group, which has similar reduction in body weight as in the DEX-treated group, does not show any change in MAP. This suggests food intake and body weight reductions due to DEX may not be directly related to its pressor effect through the sympathoadrenal system. Studies in the literature point to a complex interplay between GCs and other hormonal factors that regulate feeding behavior and subsequent body weight changes (Jahng et al., 2008; Cummings et al., 2013). For instance, DEX was shown to increase insulin secretion via improvement of β cell function (Cummings et al., 2013), in addition to affecting leptin release and its hypothalamic actions (Jahng et al., 2008), enhancing energy expenditure and anorexia. In contrast, GC therapy in humans is often associated with weight gain, in addition to increase in blood pressure (Whitworth et al., 2005).

Many factors, including diet, exercise, stress, and aging itself, affect adrenal expression of TH (Fluharty et al., 1983; Tumer et al., 1992; Kvetnansky et al., 2003; Patel et al., 2005). Several studies have explored to understand the relationship
between peripheral TH expression and hypertension (Guo et al., 2005; Burgi et al., 2011; Congo Carbajosa et al., 2015). Recent studies have uncovered natural polymorphic variations in the human TH gene that influence transcription, autonomic function, and hypertension (Rao et al., 2007, 2010). A causal relationship was revealed when acute DEX treatment in rats (1 mg/kg for 2 days) was shown to upregulate TH mRNA in the adrenal medulla and cause hypertension in rats (Kumai et al., 2000), and antisense TH gene therapy in rats was effective in causing hypotension in the SHR model (Kumai et al., 2001). Because our model allows monitoring long-term effects of DEX treatment on blood pressure and assessing the underlying mechanism, we find that, similar to acute DEX treatment, chronic DEX exposure also causes an increase in TH mRNA and protein levels, most likely via GR in chromaffin and ganglionic cells of the adrenal medulla (Phillips et al., 2001). This increase in TH transcription, perhaps, leads to synthesis of new enzyme molecules that in turn enhance TH activity and catecholamine synthesis. The exact medullary cell type involved in TH transcription in this model system is not known and will need to be determined in future studies. Of note, we did not find an increase in TH mRNA in the brainstem. Had DEX crossed into the brain, its effects on blood pressure may have been opposite, based on the report that intracerebroventricular injection of DEX reduces blood pressure (Tonolo et al., 1993). We never observed a decrease in blood pressure during the entire period of DEX administration, consistent with peripheral, not central effects.

We did not explore the role of TH in superior cervical ganglion (SCG) and carotid body, although they could also be potential peripheral sites for DEX effects; indeed, TH mRNA at these sites increases in response to stressful conditions (Chen et al., 1995). However, the duration and robustness of these increases are different, and, more importantly, they entail different signaling pathways (Nankova et al., 1999). For example, activator protein-1 has a lower binding to the TH promoter motif upon immobilization in the SCG than in the adrenal medulla (Sabban et al., 2004). The genomic effect of DEX on TH via the atypical glucocorticoid response element might not occur at the SCG, and was less likely to be of stimulatory nature that we saw in vitro (Rani et al., 2009; Sheela Rani et al., 2013). Consistent with this, systemic α-MPT administration generates a greater decrease in blood pressure than guanethidine, an inhibitor of the norepinephrine uptake transporter (Chiou-Tan et al., 1994). This suggests that noradrenergic chromaffin cells that contain TH, but lack this transporter (Phillips et al., 2001), contribute to blood pressure regulation to a greater extent than adrenergic
Our results could resemble studies in which the reduction of central catecholamine concentrations alters sympathetic outflow and elevates blood pressure (Doba and Reis, 1974; Fujino, 1984). In our study, such a consequence of α-MPT access into the brain may contribute to the maintenance of a normal baseline MAP and HR during the administration of α-MPT (Fig. 5A) on days 6–10 and also result in a side effect that prevents a complete reversal of DEX pressor response.

We did not observe significant alteration in HR upon α-MPT administration, although we had expected it to be reduced due to a decrease in cardiac sympathetic activity. As mentioned above, this could also be associated with α-MPT central effects. More importantly, HR maintenance could be due to opposing responses of the parasympathetic nervous system to effects. More importantly, HR maintenance could be due to small sample, may be necessary to tease of glucocorticoid therapy. According to measure due to small sample, may be necessary to tease translationally, perhaps involving the N terminus of the catalytic domain (Nakashima et al., 2009).

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