Thyroid Hormone Activation in Vascular Smooth Muscle Cells Is Negatively Regulated by Glucocorticoid

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Background: Type 2 iodothyronine deiodinase (D2) catalyzes the production of triiodothyronine from thyroxine. D2 is present in rat aorta media, and there is a circadian variation in the D2 expression. In rat aorta media, the D2 activity exhibited the maximal value at 1200 hour and low value between 1800 and 2400 hour. To understand the mechanisms that induce the circadian variation in the D2 expression, we examined the effects of glucocorticoid on the D2 activity and mRNA in rat aorta media and cultured vascular smooth muscle cells (VSMCs).

Methods: The effects of intrinsic and extrinsic glucocorticoid on the D2 activity and mRNA in rat aorta media were studied using metyrapone, a corticosterone synthesis inhibitor, and dexamethasone (DEX). Further, the effects of DEX on D2 expression were studied using the cultured rat VSMCs.

Results: The trough values of D2 activity and mRNA at 2100 hour were increased by the treatment with metyrapone. On the other hand, the peak values of D2 activity and mRNA were decreased by the treatment with DEX. D2 activity and mRNA in cultured rat VSMCs were increased by the addition of 10^{-3} M dibutyryl cyclic adenosine monophosphate [(Bu)₂cAMP]. The increments were reduced by coinubation with 10^{-6} M DEX.

Conclusions: These results suggest that glucocorticoids might directly suppress the D2 expression in rat VSMCs induced by a cAMP-dependent mechanism.

Introduction

THYROID HORMONE HAS SIGNIFICANT EFFECTS ON the peripheral vascular system (1). Systemic vascular resistance is high in patients with hypothyroidism, and is low in patients with hyperthyroidism (1). The high systemic vascular resistance in patients with hypothyroidism is rapidly reversed with thyroid hormone treatment (2). Thyroid hormone is known as a vasodilator that acts directly on vascular smooth muscle cells (VSMCs) (3).

Thyroxine (T₄), a major secretory product of the thyroid gland, needs to be monodeiodinated to triiodothyronine (T₃) to exert its full biological activity. There are two isoenzymes that catalyze the monodeiodination, type 1 and type 2 iodothyronine deiodinase (D1 and D2, respectively) (4–6). The Michaelis–Menten constant (K_m) of D2 is approximately 1–10 nM for T₄, which is 100 times lower than that of D1 (5). D2 is relatively insensitive to inhibition by 6-propyl-2-thiouracil (PTU), which inhibits D1. D2 is expressed in a limited number of tissues, including anterior pituitary, brain, and brown adipose tissue, in rat. In human, D2 is expressed not only in

pituitary and brain, as it is in the rat, but also in thyroid, myocardium, and skeletal muscle, which is not the case in the rodent (7,8). D2 is important in the generation of intracellular T₃, thus locally controlling thyroid status (9), and also has been recently appreciated to provide a significant fraction of the circulating T₃ in humans (10). Targeted disruption of the *dio2* gene in mice impairs pituitary thyroid-stimulating hormone feedback, adaptive thermogenesis, and cochlear development (11–13).

We and others found that D2 is present in human VSMCs and rat aorta media (14–16). The presence of D2 in VSMCs suggests that VSMCs are physiological targets for the action of thyroid hormone. Interestingly, there is a circadian variation in the D2 expression in rat aorta media (16). The peak D2 mRNA level at 0900 hour was approximately fourfold higher than the nadir value at 2100 hour. Circadian variation of D2 activity has been observed in rat pineal, harderian gland, and central nervous system (17–19). It is reported that the rhythmic changes in rat pineal and harderian gland D2 activities are regulated mainly at the pretranslational level by a β -adrenergic mechanism transmitted through superior cervical

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ganglia (20,21). It is reported that rat pineal D2 mRNA reached a peak 3 hours after the onset of darkness (20). On the other hand, the aorta media D2 mRNA began to rise approximately 7 hours after the beginning of darkness (16). Thus, it appears unlikely that same controlling mechanism is involved in the circadian variation of the aorta media D2 mRNA as those of pineal and harderian gland. Although we have previously reported that both α 1- and β -adrenergic mechanisms may be involved, at least partly, in the circadian variation of the D2 activity in rat aorta media (16), the mechanisms that induce the circadian variation of D2 expression are not clearly understood.

Of note is the fact that there is a circadian variation in circulating corticosterone levels in rats ranging from trough values at 1100 hour to peak values at 2000 hour (22). This circadian variation of circulating corticosterone level is opposite to that of D2 expression in rat aorta media. It has been reported that dexamethasone (DEX), a synthetic glucocorticoid, reduces the D2 activity in mouse neuroblastoma cells (23), in human placental cells (24), and in the epithelial cells of the mouse mammary gland (25). These results suggest that glucocorticoid may repress the D2 expression in aortic media *in vivo*. In the present study, we have investigated the effect of glucocorticoids on D2 activity and mRNA in rat aortic media, and also examined the direct effects of glucocorticoids using cultured rat VSMCs.

Materials and Methods

Materials

[α -³²P]UTP, [α -³²P]dCTP, and [¹²⁵I]T4 were purchased from New England Nuclear Corporation (Boston, MA). Sephadex LH20 was from Pharmacia Biotech (Uppsala, Sweden), T7 RNA polymerase from Promega Corporation (Madison, WI), AG 50W-X2 from Bio-Rad Laboratories (Hercules, CA), and Methyrapone from Biomol (Plymouth Meeting, PA). All other chemicals of the highest quality were obtained from Sigma Chemical (St. Louis, MO) or Nakarai Tesque (Kyoto, Japan) unless otherwise indicated.

Animals

All procedures were performed in accordance with institutional guidelines for animal research at Kansai Medical University. Male Sprague-Dawley rats ranging in age from 8 to 10 weeks were used. Rats were acclimated to a 12-hour light, 12-hour dark schedule (lights between 0800 and 2000 hour), and controlled temperature ($25 \pm 1^\circ\text{C}$). Rat chow and tap water were provided *ad libitum*.

Experimental design

Metyrapone, an inhibitor of 11- β -hydroxylase and a corticosterone synthesis inhibitor, was injected intraperitoneally at a dosage of 50 mg/kg body weight in 500 μL of 0.9% sterile NaCl. The control group received an equal volume of 0.9% NaCl. All injections were administered at 6 and 3 hours before the dissection. The dose and time lags for metyrapone treatments correspond to those used in previous report (26). DEX was injected intraperitoneally at a dosage of 1–100 $\mu\text{g}/\text{kg}$ body weight in 500 μL of 0.9% sterile NaCl. The control group received an equal volume of 0.9% NaCl. All injections were administered at 8 hours before the dissection. The dose and

time lags for DEX treatments correspond to those used in previous reports (27–29). We analyzed the D2 mRNA and D2 activity levels in rat aorta media at both 0900 and 2100 hour and at 1200 and 2100 hour, respectively, because the peaks for D2 mRNA and activity were previously demonstrated to occur at 0900 and 1200 hour, respectively (16).

Preparation of aorta media

Rats were anesthetized with ether, and their thoracic aortae were dissected. Each was cleaned of fat and connective tissue. After the removal of tunica intima and adventitia, medium was isolated and immediately frozen and stored at -70°C .

Cell cultures

Rat VSMCs were isolated from thoracic aorta media of male Sprague-Dawley rats. Briefly, the aortae were removed to sterile dishes containing medium and stripped of adventitia with a sterile forceps. The aortic media were dispersed into single cells by incubation with 2 mg/mL collagenase (type 1) and 0.5 mg/mL elastase (type III) for 60 minutes. Cells were sedimented and plated on tissue culture dishes and were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin. All cell cultures were kept in a humidified 5% CO_2 /95% air incubator at 37°C . The growth medium was changed every 2 days. The subcultures of VSMCs from passage 2 to 3 were used in all experiments. All the experiments were performed on 70% confluent cells of identical passages.

Measurement of iodothyronine deiodinase activity

Tissue was homogenized in a 10-fold volume of ice-cold buffer containing 100 mM potassium phosphate (pH 7.0), 20 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid (homogenization buffer). After centrifugation at 3000 rpm for 10 minutes, the supernatants were used for the deiodination assay. Iodothyronine deiodinase activity was measured as previously described (15,16). Tissue homogenates were incubated with 2 nM [¹²⁵I]T4, which was purified using LH-20 column chromatography on the day of the experiment, in the presence of 1 mM PTU in homogenization buffer in a total volume of 300 μL at 37°C for 2 hours. The reaction was terminated by adding 200 μL horse serum and 100 μL of 50% trichloroacetic acid. The released ¹²⁵I[−] was separated by column chromatography using AG 50W-X2 resin and counted with a γ -counter. The protein concentration was measured according to the method of Bradford using bovine serum albumin as a standard (30). The deiodinating activity was calculated as femtomols of I[−] released/(mg protein $\cdot$ h).

RNA preparation and Northern analysis

Total RNA was extracted from tissues by acid guanidinium thiocyanate phenol-chloroform method according to the method of Chomczynski and Sacchi (31). Plasmid rDII 5-1/pBluescript SK, which contains rat D2 cDNA, was kindly provided by Dr. St. Germain (7). Briefly, rat D2 complementary RNA (cRNA) probe was synthesized by *in vitro* transcription of linearized rDII 5-1/pBluescript SK using T7 RNA polymerase and [α -³²P]UTP. Total RNA (30 $\mu\text{g}/\text{lane}$) was electrophoresed on a 0.8% agarose gel and transferred to

FIG. 1. Effects of metyrapone on the plasma corticosterone level. Metyrapone (50 mg/kg body weight) or normal saline (vehicle) was injected intraperitoneally (i.p.) twice at 6 and 3 hours before the harvest. Plasma was obtained at both 0900 and 2100 hour. The plasma corticosterone level shown is the mean $\pm$ SD of six animals. * $p < 0.05$.

nylon membranes (Biodyne, Pall, NY). The membrane was prehybridized with the hybridization buffer (50% formamide, 5 $\times$ SSC [1 $\times$ SSC; 150 mM NaCl and 15 mM sodium citrate], 200 μ g/mL denatured salmon sperm DNA, 0.2% sodium dodecyl sulfate (SDS), 5% dextran sulfate, and 1 $\times$ Denhardt's solution [1 $\times$; 0.2% bovine serum albumin, 0.2% Ficoll, and 0.2% polyvinyl pyrrolidone]) at 68°C for 3 hours. Subsequently, the membrane was hybridized at 68°C overnight with the hybridization buffer containing a rat D2 cRNA probe. The membrane was washed twice in 2 $\times$ SSC-0.1% SDS at 25°C for 15 minutes and twice in 0.1 $\times$ SSC-0.1% SDS at 68°C for 1 hour. D2 mRNA level was determined by Fujix Bioimage Analyzer (BAS 2000; Fuji Photo Film, Tokyo, Japan). After detection of D2 mRNA, the probe was stripped off, and blots

were rehybridized with glyceraldehyde-3-phosphate dehydrogenase cDNA probe, which was synthesized using [α -³²P]dCTP, as a control as previously described (16). D2 mRNA level was corrected by the mRNA for glyceraldehyde-3-phosphate dehydrogenase.

Determination of plasma corticosterone concentrations

Rats were anesthetized, and blood was drawn via cardiac puncture. The level of plasma corticosterone was measured using a radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA).

Determination of plasma T4 and T3 concentrations

Plasma T4 and T3 concentrations were assayed by commercial kits (Elesys Electrochemiluminescence; Roche Diagnostics Corporation, Indianapolis, IN).

Statistical analysis

All results are presented as means $\pm$ SD. Differences between groups were analyzed by analysis of variance with multiple comparison using Dunnett's method. Statistical significance was accepted at $p < 0.05$.

Results

Effect of metyrapone on D2 activity and mRNA

To address the question of whether endogenous glucocorticoids regulate D2 expression, we initially analyzed the D2 activity and mRNA in aorta media of the rats treated with metyrapone. In control rats treated with vehicle, the plasma level of corticosterone at 2100 hour (460 $\pm$ 135 ng/mL) was significantly higher than that at 0900 hour (178 $\pm$ 112 ng/mL) as described previously (Fig. 1). In rats treated with metyrapone, plasma corticosterone level at 2100 hour (131 $\pm$ 40 ng/mL) was significantly lower than in control, but the level at 0900 hour (139 $\pm$ 54 ng/mL) was not significantly different from controls (Fig. 1). On the other hand, plasma concentrations of T4 and T3 in rats treated with metyrapone were not significantly different compared with those values in control rats at both 0900 and 2100 hour (Table 1).

In control rats, D2 activities at 1200 hour were significantly higher than those values at 2100 hour (Fig. 2A). In rats treated with metyrapone, D2 activities at 1200 hour were not significantly different compared with those values in control, but D2 activities at 2100 hour were significantly higher than those values in control (Fig. 2A). Northern analysis of total RNA prepared from four pooled aorta media using rat D2 cRNA probe clearly demonstrated a single hybridization signal of

TABLE 1. EFFECT OF METYRAPONE ON PLASMA THYROXINE AND TRIIODOTHYRONINE CONCENTRATIONS

	0900 hour		2100 hour	
	Control	Metyrapone	Control	Metyrapone
T4 (μ g/dL)	4.7 $\pm$ 0.3	4.8 $\pm$ 0.4	4.8 $\pm$ 0.3	4.6 $\pm$ 0.4
T3 (ng/dL)	97 $\pm$ 5	98 $\pm$ 6	99 $\pm$ 3	97 $\pm$ 5

Metyrapone (50 mg/kg body weight) or normal saline (vehicle) was injected intraperitoneally twice: at 6 and 3 hours before the harvest. Plasma was obtained at both 0900 and 2100 hour. The plasma T4 and T3 concentrations shown are the mean $\pm$ SD of six animals.

T3, triiodothyronine; T4, thyroxine.

FIG. 2. Effects of metyrapone on type 2 iodothyronine deiodinase (D2) activity and mRNA in rat aorta media. Metyrapone (50 mg/kg body weight) or normal saline (vehicle) was injected i.p. twice at 6 and 3 hours before the dissection. Aorta media were dissected at both 1200 and 2100 hour for D2 activity measurements, and at both 0900 and 2100 hour for mRNA measurements. **(A)** D2 activity in aorta media obtained as described above. The results of D2 activity shown is the mean $\pm$ SD of four animals. **(B)** Northern analysis of D2 mRNA in aorta media. Each lane represents four pooled aorta media. **(C)** D2 mRNA (D2 mRNA/glyceraldehyde-3-phosphate dehydrogenase [GAPDH] mRNA ratio) in aorta media obtained as described above. The optical density of the D2 mRNA was corrected for GAPDH mRNA, and the results were expressed as a percentage of the control value obtained at 2100 hour. The results of D2 mRNA shown are the mean $\pm$ SD of three different experiments. * $p < 0.05$.

approximately 7.5 kb in size as described previously (Fig. 2B). In control rats, D2 mRNA levels at 0900 hour were significantly higher than those values at 2100 hour (Fig. 2B, C). In rats treated with metyrapone, D2 mRNA levels at 0900 hour were not significantly different, but D2 mRNA at 2100 hour were significantly higher than those values in control (Fig. 2B, C).

Effects of DEX on D2 activity and mRNA in aorta media

Next, we examined the effect of exogenous glucocorticoid on the D2 activity and mRNA level in aorta media. Rats were treated with a single dose of DEX (100 μ g/kg body weight, intraperitoneally) or normal saline (vehicle) 8 hours before the dissection. D2 activity levels in aorta media in rats treated with DEX harvested at both 1200 and 2100 hour were significantly decreased to 10–20% of those levels in the control rats (Fig. 3A). On the other hand, D2 mRNA levels in rats treated with DEX harvested at both 0900 and 2100 hour were also significantly decreased to 10–20% of the levels in the control rats (Fig. 3B, C).

Next, we investigated the dose dependency of the effects of DEX on D2 activity at 1200 hour, and mRNA level at 0900 hour in aorta media. As shown in Figure 4A and B, D2 activity and mRNA significantly decreased to $\sim$ 30% by the treatment with 10 μ g/kg body weight DEX and reached the lowest level, $\sim$ 10% of the control, by the treatment with 100 μ g/kg body weight DEX.

Analysis of iodothyronine deiodinase activity in rat VSMCs

To clarify the effect of DEX on D2 activity in rat aorta media, we used the cultured rat VSMCs. Significant deiodinating

activity was detectable in the sonicate of rat VSMCs. The T4 deiodination was dependent on the incubation period up to 2 hours and the protein concentration of rat VSMCs (data not shown). Incubation at 4°C or preheating the cell sonicate at 56°C for 30 minutes completely abolished the deiodination. The deiodinating activity was not influenced by 1 mM PTU but was completely inhibited by 1 mM iopanic acid. From the double reciprocal plot, kinetic constants were calculated to be $K_m = 3.1$ nM and $V_{max} = 32.8$ fmol I⁻ released/(mg protein $\cdot$ h) (Fig. 5). These results indicate that all the characteristics of the deiodinating activity in rat VSMCs are compatible with D2.

Effect of DEX on D2 activity and mRNA in rat VSMCs

D2 activity was low in VSMCs, and the values of the cells cultured with 10^{-6} M DEX for 12 hours were not significantly different compared with in the control cells (Fig. 6A). Northern analysis of total RNA from rat VSMCs clearly demonstrated a single hybridization signal of approximately 7.5 kb in size as shown in Figure 6B, indicating the presence of D2 mRNA, as well as D2 activity, in rat VSMCs. D2 mRNA was also low in VSMCs, and the value in the cells cultured with 10^{-6} M DEX for 12 hours was not significantly different from that in the control cells (Fig. 6B, C). The administration of 10^{-3} M dibutyryl cyclic adenosine monophosphate [(Bu)₂cAMP] significantly increased D2 activity and mRNA (Fig. 6A–C). The D2 activity and mRNA increased approximately 20-fold, respectively, in the cells cultured with 10^{-3} M (Bu)₂cAMP for 12 hours. Next, we investigated the effect of DEX on D2 activity and mRNA in cultured rat VSMCs incubated with (Bu)₂cAMP. The D2 activity and mRNA in the cells cultured with 10^{-3} M (Bu)₂cAMP plus 10^{-6} M DEX decreased to less than 40% of the levels in the cells incubated with (Bu)₂cAMP

FIG. 3. Effects of dexamethasone (DEX) on D2 activity and mRNA in rat aorta media. DEX (100 μ g/kg body weight) or normal saline (vehicle) was injected i.p. 6 hours before the dissection. Aorta media were dissected at both 1200 and 2100 hour for D2 activity measurements, and at both 0900 and 2100 hour for mRNA measurements. **(A)** D2 activity in aorta media obtained as described above. The results of D2 activity shown are the mean $\pm$ SD of four animals. **(B)** Northern analysis of D2 mRNA in aorta media. Each lane represents four pooled aorta media. **(C)** D2 mRNA (D2 mRNA/GAPDH mRNA ratio) in aorta media obtained as described above. The optical density of the D2 mRNA was corrected for GAPDH mRNA, and the results were expressed as a percentage of the control value obtained at 2100 hour. The D2 mRNA shown is the mean $\pm$ SD of three different experiments. * $p < 0.05$.

alone. The significant inhibitory effect of DEX on the (Bu)₂cAMP-induced increase of D2 activity and mRNA was observed at 10^{-7} M DEX and at higher concentrations (Fig. 6A–C). The significant inhibitory effect of 10^{-6} M DEX on the 10^{-3} M (Bu)₂cAMP-induced increase of D2 activity and mRNA was observed after 3 hours of incubation (Fig. 7A, B). The D2 activity and mRNA level in the cells cultured with 10^{-3} M (Bu)₂cAMP plus 10^{-6} M corticosterone for 12 hours (D2 activity, 48 ± 4 fmol/[mg protein $\cdot$ h]; D2 mRNA, 4.2 ± 0.5 arbitrary unit) were similar to those values in the cells cultured with 10^{-3} M (Bu)₂cAMP plus 10^{-6} M DEX. In contrast, the D2 activity and mRNA in the cells cultured with 10^{-3} M (Bu)₂cAMP plus were not affected by 10^{-6} M aldosterone, 10^{-6} M estradiol, or 10^{-6} M testosterone (data not shown).

Discussion

The present results clearly show that glucocorticoid downregulates the D2 activity and mRNA in rat aorta media and VSMCs. We have previously shown that there is a circadian variation in the D2 activity and mRNA in rat aorta media (16). The rat aorta media D2 activity at 1200 hour was approximately sixfold higher than at 2100 hour, and D2 mRNA at 0900 hour was approximately fourfold higher than at 2100 hour. In rats treated with metyrapone, the peak levels of plasma corticosterone at 2100 hour decreased, and the nadir values of D2 activity and mRNA in rat aorta media at 2100 hour increased. These results suggest that corticosterone suppresses the D2 activity by suppression of D2 mRNA in rat aorta media. In rats treated with metyrapone, the plasma levels of T4 and T3 at 2100 hour were not significantly different compared with those levels in control rats at 2100 hour. These results suggest that the increase of D2 activity of aorta media in rats treated with metyrapone at 2100 hour is not due

to the change of thyroid hormone status. Further, the D2 activity and mRNA in rat aorta media were suppressed by DEX administration. These results indicate that both endogenous and exogenous glucocorticoid suppress the D2 expression in rat aorta media, and that the circadian variation of plasma corticosterone level, at least partly, may induce the circadian variation of the D2 expression in rat aorta media.

To investigate the effect of glucocorticoid on D2 activity and mRNA in vascular smooth muscle, we employed the rat VSMCs. We have found that D2 activity and mRNA were present not only in human but also in rat VSMCs. These results suggest that rat VSMCs can serve as a model for investigation of the regulatory mechanisms of D2 expression in VSMCs. The Vmax of the rat VSMCs was approximately 10% of the value of the human VSMCs (14,15). The D2 activity and mRNA were markedly stimulated by (Bu)₂cAMP, consistent with those results in human VSMCs (14,15), human skeletal muscle cells (32), human thyroid follicular cells (33), and rat astrocytes (34). Recently, a cAMP response element (CRE) was identified in the human and rat *dio2* promoter region, suggesting that the transcriptional regulation of D2 expression by cAMP regulatory mechanisms might be present in rat VSMCs (35–37).

The increment of both D2 activity and mRNA by (Bu)₂cAMP in rat VSMCs was reduced by DEX and corticosterone to a similar extent, suggesting pretranslational regulation. It has been reported that DEX reduces the D2 activity in cultured NB41A3 mouse neuroblastoma cells (23), in human cultured placental cells (24), and in HC11 cells, which are derived from the epithelial cells of the mouse mammary gland (25). In HC11 cells, both D2 activity and mRNA were reduced by DEX (25). The reporter assay using the proximal promoter region of mouse *dio2* gene indicated that DEX down-regulated mouse *dio2* gene expression, at least in part, at

FIG. 4. Effects of various doses of DEX on D2 activity and mRNA in rat aorta media. DEX (1, 10, and 100 $\mu\text{g/kg}$ body weight) or normal saline (vehicle) was injected i.p. 6 hours before the dissection. Aorta media were dissected at 1200 hour for D2 activity measurements, and at 0900 hour for mRNA measurements. **(A)** D2 activity in aorta media obtained as described above. The D2 activity shown is the mean $\pm$ SD of four animals expressed as a percentage of the control value obtained at 1200 hour. D2 mRNA (D2 mRNA/GAPDH mRNA ratio) in aorta media obtained as described above. The optical density of the D2 mRNA was corrected for GAPDH mRNA, and the results were expressed as a percentage of the control value obtained at 0900 hour. The results of D2 mRNA shown are the mean $\pm$ SD of three different experiments. **(B)** Northern analysis of D2 mRNA in aorta media. Each lane represents four pooled aorta media. $*p < 0.05$.

a transcription level (25). The 5'-upstream region of the mouse *dio2* gene contains potential CRE but not the glucocorticoid response element (GRE) (38). On the other hand, both DEX and (Bu) $_2$ cAMP upregulated the D2 mRNA in GC cells, which is derived from rat pituitary tumor (39), and in adrenocorticotrophic hormone (ACTH)-secreting AtT-20 mouse pituitary tumor cells (40). Interestingly, it has been reported that D2 mRNA is synergistically upregulated by DEX and (Bu) $_2$ cAMP in AtT-20 cells (40). These results suggest that the effect of glucocorticoid on D2 expression could be cell type specific.

In genes that are negatively regulated by glucocorticoids, an imperfect copy of a GRE is found, and repression is probably due to competition between hormone receptor and other transcription factors or enhancer-binding proteins for binding to overlapping DNA sequences (41). The gene encoding the α -subunit of the glycoprotein hormones is regulated by cAMP and steroid hormones. In transfection experiments, the CRE has been identified in proximity to the promoter. Reporter genes containing this region of the α -subunit promoter linked to the CAT gene are negatively regulated by glucocorticoids (42). Elimination of the two 18 bp repeats that mediate cAMP regulation also leads to lack of glucocorticoid responsiveness (42). DNA-binding experiments have allowed the identification of three receptor-binding sites overlapping the CREs and the tissue specific enhancer (42). Although none of the three sites represents a perfect GRE consensus, all have essential homology to the receptor-binding sequences. Interestingly, when the reporter gene is introduced into cells in which its expression is not

affected by cAMP, glucocorticoids act as weak inducers rather than as repressors (42). Thus, it seems that inhibition by glucocorticoids is due to the fact that binding of the hormone receptor to the GRE competes for the binding of the cAMP mediator proteins to their elements. Therefore, it seems likely

FIG. 5. Kinetic analysis of deiodinating activity in cultured rat vascular smooth muscle cells (VSMCs). Double reciprocal plot of thyroxine (T4) deiodination. Incubation was performed with various concentration of T4. The results are representative of those obtained in a total of three experiments.

FIG. 6. Effects of DEX on D2 activity and mRNA in cultured rat VSMCs. (A) D2 activity in cultured rat VSMCs obtained as described above. The D2 activity shown is the mean $\pm$ SD of six dishes. (B) Northern analysis of D2 mRNA in cultured VSMCs incubated with various concentrations of DEX in the absence or presence of 10⁻³ M dibutylryl cyclic adenosine monophosphate [(Bu)₂cAMP] for 12 hour. Each lane represents 30 μ g total RNA obtained from cells in an individual dish. (C) D2 mRNA (D2 mRNA/GAPDH mRNA ratio) in cultured VSMCs as described above. The optical density of the D2 mRNA was corrected for GAPDH mRNA, and the results were expressed as a percentage of the value obtained for control cells. The results of D2 mRNA shown are the mean $\pm$ SD of six dishes. * p < 0.05.

FIG. 7. Time course of the effects of DEX on D2 activity and mRNA in cultured rat VSMCs. (A) D2 activity in cultured rat VSMCs obtained as described above. The D2 activity shown is the mean $\pm$ SD of six dishes. (B) D2 mRNA (D2 mRNA/GAPDH mRNA ratio) in cultured VSMCs as described above. The optical density of the D2 mRNA was corrected for GAPDH mRNA, and the results were expressed as a percentage of the value obtained for control cells. The results of D2 mRNA shown are the mean $\pm$ SD of six dishes. * p < 0.05.

that it is not the specific nucleotide sequence of the receptor-binding site that determines its functioning as positive or negative modulator of transfection, but rather the context surrounding the GRE which is important. Interestingly, it seems likely that an imperfect copy of a GRE overlapping a CRE is present in the rat *dio2* promoter (37). Further examination is necessary to clarify the mechanisms by which glucocorticoid induces the downregulation of rat *dio2* gene expression.

Glucocorticoids potentiate vasoconstrictive responses of catecholamines, angiotensin II, vasopressin, endothelin, and bradykinin, and increased glucocorticoid responsiveness has been associated with an increase in arterial contraction and vascular resistance (43). Although glucocorticoid-induced hypertension is characterized by sodium retention and volume expansion, increased peripheral vascular resistance is also thought to play a key role in glucocorticoid-induced hypertension (44). The cellular and molecular mechanisms of vascular smooth muscle in response to glucocorticoids are not

clearly understood. From our present results, we may hypothesize that the presumable decrease of T3 content in VSMCs due to suppression of D2 activity in VSMCs by glucocorticoid may at least partly contribute to the glucocorticoid induced peripheral vascular resistance, because T3 is a vasodilator that acts directly on VSMCs to cause a relaxation of arteries (3).

In conclusion, we have found that D2 activity and mRNA in rat aorta media were negatively regulated by glucocorticoid, and that the increments of D2 activity and mRNA induced by (Bu)₂cAMP were reduced by glucocorticoid in rat VSMCs. Our present results suggest that the circadian variation of plasma corticosterone contributes to the changes in D2 expression in rat aorta media and this might be true for other D2-expressing tissues as well. Further examination is necessary to clarify the physiological significance of the inhibitory effects of glucocorticoid on D2 expression in VSMCs.

Acknowledgments

This study was supported in part by the Smoking Research Foundation and by NIH Grant DK044128 to P.R.L.

Disclosure Statement

The authors declare that no competing financial interests exist.

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