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Role of rat adrenal antioxidant defense systems in the aldosterone turn-off phenomenon

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Abstract

The mechanism(s) of the ''aldosterone turn-off phenomenon'', hypoaldosteronemia following chronic ACTH administration, remains unclear. Our previous observation that antioxidants prevented turn-off prompted us to evaluate the chronic effect of ACTH on the enzymatic antioxidant system as well as P450aldo activity and expression of CYP11B2 in adrenal zona glomerulosa. Male Wistar rats were administered ACTH-Z for 5 days with or without antioxidants, vitamin E or DMSO. Adrenal capsules were prepared for P450aldo activity measurement and mRNA content determination by competitive RT-PCR, and immunoreactivity of Mn-SOD in whole adrenals was evaluated. ACTH decreased the P450aldo activity and mRNA level of CYP11B2 in adrenal capsules, while co-administration of vitamin E or DMSO partially blocked this inhibition. ACTH increased Mn-SOD mRNA and immunoreactivity but decreased GPx mRNA. These results suggest that prolonged ACTH treatment increases oxidative stress in the zona glomerulosa and an imbalance in the ratio of Mn-SOD to GPx, possibly via corticosterone overproduction in the zona fasciculata, resulting in the downregulation of CYP11B2. Vitamin E and DMSO might thus protect CYP11B2 expression through their antioxidant actions.

Keywords: Superoxide dismutase; Glutathione peroxidase; ACTH; Zona glomerulosa; Vitamin E

1. Introduction

The enzyme essential for aldosterone biosynthesis is P450aldo, a mitochondrial cytochrome P450 encoded by CYP11B2, present only in the zona glomerulosa and mediating the last three steps of aldosterone synthesis, deoxycorticosterone (DOC) to aldosterone, in rodents and humans [1,2]. ACTH, as well as other stimuli such as angiotensin II or potassium, acutely stimulates aldosterone secretion from the zona glomerulosa, while long-term ACTH treatment dramatically decreases the plasma aldosterone concentration. ACTH increases CYP11B2 expression levels transiently with a decrease after 24 h of treatment [3]. This paradox is called the ''aldosterone escape phenomenon'' or ''aldosterone turn-off phenomenon'' [4–6]. Such a condition is also observed in critically ill patients, in whom hypoaldosteronemia is observed despite elevated plasma levels of renin, ACTH and cortisol [7].

Several possible mechanisms in this complicated phenomenon have been proposed. Prolonged ACTH treatment causes morphological and functional changes in the glomerulosa of experimental animals [8,9], loss of glomerulosa-specific characteristics with acquisition of fasciculata-reticularis-like ones. ACTH induces 17α-hydroxylase mRNA in cultured bovine adrenal glomerulosa cells, thereby shifting the steroidogenic pathway to cortisol production [10]. It has also been shown that ACTH interferes with the renin-

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Nomenclature

DOC	deoxycorticosterone	NF-κB	nuclear factor kappa
Mn-SOD	manganese superoxide dismutase	AP-1	activator protein-1
GPx	glutathione peroxidase	GR	glucocorticoid receptor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase		

angiotensin system by reducing the number of angiotensin II receptors in glomerulosa cells [6] and/or by blocking angiotensin II receptor second messenger pathway(s) [11].

We have demonstrated that the co-administration of vitamin E or DMSO as an antioxidant significantly attenuates the decline in plasma aldosterone concentration and aldosterone output from rat adrenal capsules following 5 days of ACTH treatment [12]. Our results thus strongly suggest the involvement of oxidative stress in this phenomenon. Even under physiological conditions, mitochondria in adrenocortical cells are a major source of free radicals [13] which can cause critical damages in the tissues. In order to accomodate such oxidative stresses, the adrenal cortex has abundant non-enzymatic antioxidants such as ascorbic acid and vitamin E [14]. Depletion of vitamin E or ascorbic acid impairs aldosterone secretion in response to sodium depletion, without changes in plasma renin activity, ACTH and serum potassium [15,16]. The adrenal cortex also expresses enzymes such as manganese superoxide dismutases (Mn-SOD) and glutathione peroxidase (GPx) [14] to detoxify superoxide eventually to H_2O. However, the effects of chronic ACTH treatment on enzymatic antioxidants in adrenal cortex have not been studied.

The present study was undertaken to elucidate the role of oxidative stress and antioxidants in the "aldosterone turn-off phenomenon" in more detail, by evaluating P450aldo and the intracellular enzymatic reduction systems in adrenal capsules during ACTH and/or antioxidant treatment in vivo.

2. Materials and methods

2.1. Animals and tissue preparation

Adult male Wistar rats weighing about 200g (SLC, Nagoya, Japan) were given standard chow and free access to water, and housed under controlled temperature (22–28°C) and lighting conditions, 14 h light (06.00–20.00 h) and 10 h darkness (20.00–06.00 h). They were divided into six groups ($n = 6$ for P450aldo activity, 4 for mRNA quantitation). Each group was treated daily for 5 days with a subcutaneous injection of saline or ACTH-Z (10 μg/100 g body weight) with or without vitamin E (2 mg/100 g body weight) or DMSO (50 μl (0.65 mmol)/100 g body weight). Care and treatment of the rats were approved by the institutional animal experiment committee of the Gifu University School of Medicine.

The rats were sacrificed by decapitation and trunk blood collected in a prechilled tube containing EDTA. Plasma was stored at −70°C for hormone measurements. The adrenal glands were quickly removed, cleaned, weighed and placed in Krebs–Ringer phosphate buffer solution (127.5 mM NaCl, 5.1 mM KCl, 12.5 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 1.4 mM $CaCl_2$, 11.1 mM glucose, 0.2% BSA, pH 7.4) on ice for brief periods before separation into capsular and inner zones by the method of Giroud et al. [17]. Each pair of adrenal capsules originating from one rat was collected into one tube and immediately homogenized in pre-chilled ISOGEN (Nippongene, Toyama, Japan) by POLYTRON (Kinematica, Luzern, Switzerland) and stored at −80°C until RNA extraction. Whole adrenal samples for immunohistochemistry were quickly frozen in liquid nitrogen and stored at −80°C until further processed.

2.2. Measurement of plasma aldosterone concentration and vitamin E

Plasma aldosterone concentrations were measured with an aldosterone RIA kit (Shionogi Pharmaceuticals, Tokyo, Japan) following the appropriate dilution with aldosterone-free serum. Cross-reaction of antiserum against other corticosteroids were as follows: 0.00016% for corticosterone, 0.000026% for progesterone, 0.015% for 18-hydroxycorticosterone and 0.00012% for DOC. Plasma vitamin E was measured by fluorometric determination [18].

2.3. Measurement of P450aldo enzyme activity

After preincubation for 30 min in Krebs–Ringer phosphate buffer, adrenal capsules were incubated in glass tubes with 0.5 ml Krebs–Ringer phosphate buffer containing 0.72 nM ^{14}C-DOC and 0.6 mM NADPH at 37°C for 24 h. Reactions were stopped by adding 5 ml chilled dichloromethane, and 2 μl each of steroids

(DOC, 11-dehydrocorticosterone, corticosterone, 18-hydroxy DOC, aldosterone and 18-hydroxycorticosterone) dissolved in ethanol (1 mg/ml) was added to the medium after removing the adrenal capsules. Each tube was shaken vigorously for 10 min and centrifuged for another 5 min. The aqueous supernatant was removed, and the inner walls of the tubes were also washed with 200 μl acetone. The dried compounds were dissolved in 50 μl acetone, applied to thin layer chromatography plates (Whatman, NJ, USA), and developed with dichloromethane–methanol–water (300:20:1). Six bands consistent with each steroid were identified under ultraviolet light, scraped and counted in a scintillation counter. P450aldo activity catalyzing DOC to aldosterone in adrenal capsules was expressed as %conversion = (aldosterone in sample (counts)/all steroids in sample (counts) − aldosterone in blank (counts)/all steroids in blank (counts)) × 100(%).

2.4. Measurement of abundance of CYP11B2, Mn-SOD, and GPx mRNA in adrenal capsules by competitive RT-PCR

Because only a small amount of RNA could be obtained from a pair of adrenal capsules, we have employed the quantitative competitive RT-PCR method to measure mRNA levels of CYP11B2, Mn-SOD and GPx. Further, the mRNA levels were normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, to minimize variations in DNase digestion and reverse transcription between samples. Briefly, 1.75 μg of total RNA was treated with DNase (Gibco-BRL, USA) and reverse transcribed with 50 U Superscript II reverse transcriptase (Gibco-BRL) in a 10-μl reaction volume containing 2.5 mM random 9 mers, 1 mM each dNTP, 4 U placental RNase inhibitor, and the manufacturer's buffer. Each reaction was allowed to proceed at room temperature for 10 min followed by incubation at 37°C for 1.5 h. Competitors for each cDNA were prepared following the PCR MIMIC™ KIT protocol (Clontech, Palo Alto, USA). Gene specific primers for each cDNA are as follows; CYP11B2 [19]: 5′ GGA TGT CCA GCA AAG TCT CTT C 3′ (sense, nt 558–579) and 5′ CAC CGT CGT GAT TAT TGA GTC C 3′ (antisense, nt 868–889), Mn-SOD [20]: 5′ TGA CCT GCC TTA CGA CTA TG 3′ (sense, nt 98–117) and 5′ CGA CCT TGC TCC TTA TTG AA 3′ (antisense, nt 446–465), GPx [21]: 5′ CCA CCG TGT ATG CCT TCT CG 3′ (sense, nt 107–126) and 5′ ACC GGG GAC CAA ATG ATG TA 3′ (antisense, nt 492–511), GAPDH [22]: 5′ GCC AAG GTC ATC CAT GAC AAC 3′ (sense, nt 482–497) and 5′ AGT GTA GCC CAG GAT GCC CTT 3′ (antisense, nt 812–832). Quantitative competitive PCR was performed by addition of 0.5 mM of a sense and a antisense primer to 0.5 μl of reverse-transcribed samples in 5 μl of 67 mM Tris–HCl (pH 8.5), 16 mmol/l $(NH_4)_2SO_4$, 2 mmol/l $MgCl_2$, 17 μg/ml bovine serum albumin and 5% glycerol. Samples were subjected to initial denaturation at 96°C for 1 min, followed by 38–48 cycles of 96°C, denaturation for 20 s, 48–60°C, annealing for 30 s, and 72°C extension for 30 s. PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide and evaluated by NIH Image 1.60 (W. Rasband, NIH). Measured values for CYP11B2, Mn-SOD and GPx were normalized against those of GAPDH.

2.5. Immunohistochemistry

The protocol for the immunohistochemistry followed the procedure of Wood [23]. A frozen tissue block was sectioned at 4 μm, mounted on siliconized slides (DAKO Japan, Kyoto, Japan), air dried for 30 min, fixed for 10 min in 4% paraformaldehyde at 4°C, blocked with 0.3% H_2O_2, and incubated with rabbit

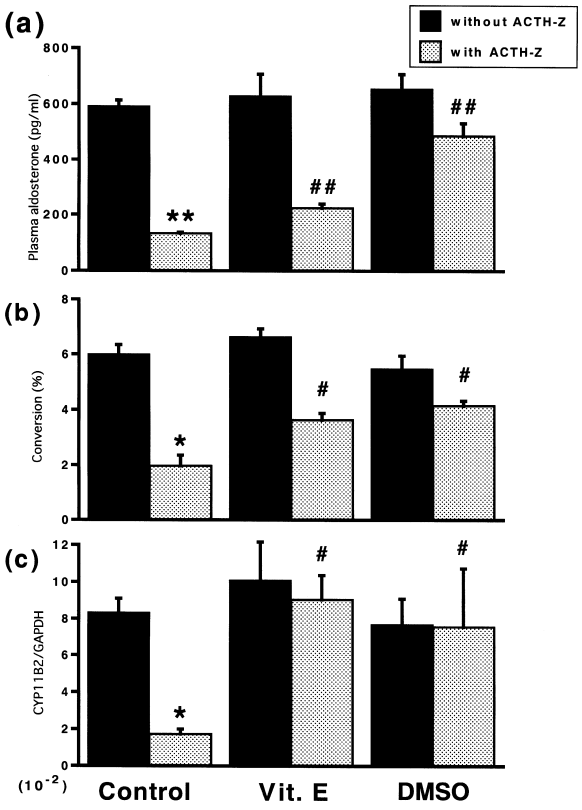

Fig. 1. Plasma aldosterone concentration ($N = 6$) (a), conversion rate of DOC to aldosterone in adrenal capsules ($N = 4$) (b), and CYP11B2 mRNA content normalized for GAPDH in adrenal capsules ($N = 4$) (c) of rats treated daily for 5 days with 2 mg vitamin E/100 g body weight, or 100 μl (1.3 mmol) DMSO/100 g body weight, with or without 5 μg ACTH-Z/100 g body weight. All data are means ± SE. $**P < 0.01$ vs. normal control (saline), $*P < 0.05$ vs. normal control, $\#\#P < 0.01$ vs. ACTH-Z alone, $\#P < 0.05$ vs. ACTH-Z alone.

antiserum (1:100) against for rat Mn-SOD for 1 h at room temperature. Sections were washed, treated for 1 h with goat anti-rabbit IgG-biotin conjugate (Sigma, St. Louis, USA), and another 40 min with ABC (DAKO, Denmark), and developed by freshly prepared diaminobenzidene $+0.01\%$ H_2O_2 for 2–5 min.

2.6. Analysis of data

Results were expressed as the mean $\pm$ SE. The non-parametric Mann–Whitney U test was used to evaluate the differences in means between groups. A P value of <0.05 was considered significant.

3. Results

3.1. Plasma aldosterone and vitamin E concentration

ACTH treatment for 5 days decreased the plasma aldosterone concentration to 22% of that in saline-treated control rats (ACTH; 133 ± 4 pg/ml versus control; 593 ± 21, $P < 0.005$), whereas no significant changes in plasma aldosterone concentration were observed between control animals and rats treated with vitamin E (577 ± 83) or DMSO (697 ± 60). In ACTH treated rats, co-administration of vitamin E or DMSO, significantly increased the plasma aldosterone concentration (224 ± 15 or 487 ± 46, respectively, $P < 0.005$) (Fig. 1(a)).

Plasma vitamin E concentrations in vitamin E treated group were 1.7-fold higher than those in control (1.11 ± 0.05 versus 0.66 ± 0.05, mg/dl, $P < 0.0001$).

3.2. P450aldo activity in adrenal capsules

As shown in Fig. 1(b), P450aldo activity in ACTH treated animals fell to 33% of saline treated controls (2.0 ± 0.4 versus 6.0 ± 0.3 %conversion, $P < 0.05$). There was no significant difference in P450aldo activity between control and groups treated with vitamin E or DMSO (6.6 ± 0.3 or 5.5 ± 0.5). Co-administration of vitamin E or DMSO restored the reduced P450aldo activity by chronic ACTH treatment (3.6 ± 0.1, $P < 0.05$ or 4.2 ± 0.2, $P < 0.05$).

3.3. CYP11B2 mRNA content in adrenal capsules

A representative result of competitive RT-PCR is shown in Fig. 2. As shown in Fig. 1(c), CYP11B2 mRNA content in adrenal capsules of ACTH treated group was significantly reduced to 21% of control group ($1.7 \pm 0.3 \times 10^{-2}$ versus $8.3 \pm 0.8 \times 10^{-2}$ CYP11B2/GAPDH, $P < 0.05$). Vitamin E or DMSO alone did not affect the CYP11B2 mRNA levels ($10.1 \pm 2.1 \times 10^{-2}$ or $7.7 \pm 1.4 \times 10^{-2}$). Co-administration of vitamin E or DMSO significantly reversed the ACTH-induced reduction ($9.0 \pm 1.3 \times 10^{-2}$, $P < 0.05$, $7.6 \pm 3.2 \times 10^{-2}$, $P < 0.05$).

RNA samples prepared from adrenal capsules con-

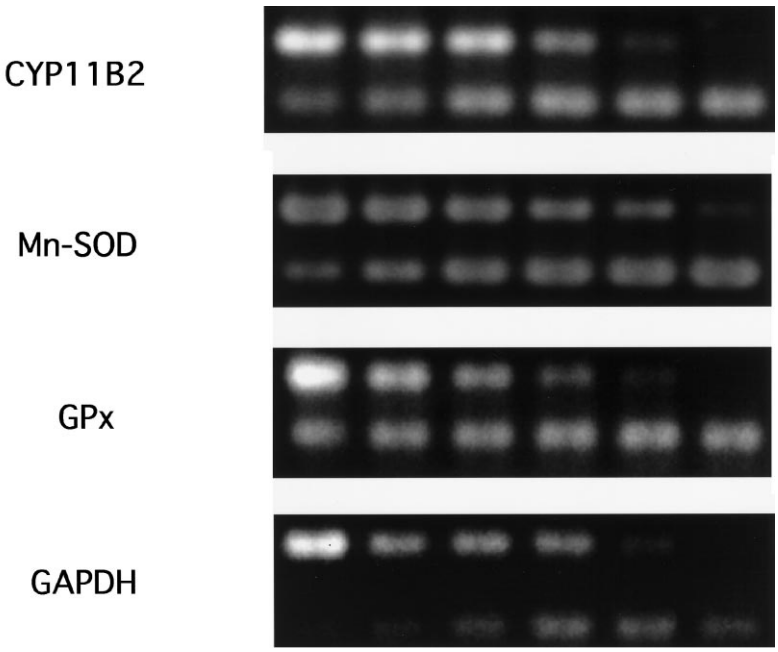

Fig. 2. Representatives of competitive RT-PCR for CYP11B2, Mn-SOD, GPx, and GAPDH mRNA. The upper bands are competitors and the lowers are endogenous products, respectively. The highest concentration of each competitor (the lefthand band) is as follows; CYP11B2 0.1 pmol/l, Mn-SOD 0.01 pmol/l, GPx 1 pmol/l, and GAPDH 10 pmol/l. Each competitor was diluted serially up to $1/(2.5)^5$ of the highest concentration (the righthand band).

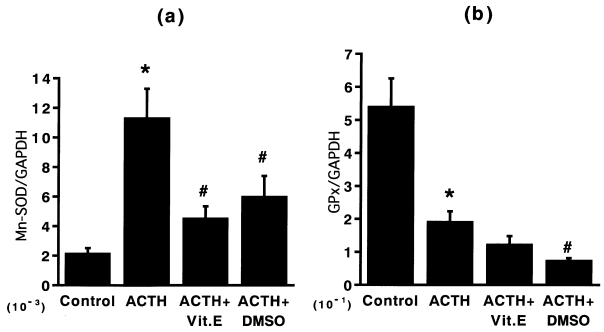

Fig. 3. Mn-SOD (a) and GPx (b) mRNA content normalized for GAPDH in adrenal capsules in rats treated daily for 5 days with 2 mg vitamin E/100 g body weight or 100 μl (1.3 mmol) DMSO/100 g body weight with 5 μg ACTH-Z/100 g body weight. All data are means ± SE ($N = 4$). $*P < 0.05$ vs. normal control (saline), $\#P < 0.05$ vs. ACTH-Z alone.

tain CYP11B1 mRNA which encodes 11β-hydroxylase, at much lower levels than those from adrenal cores, indicating subtle contamination of fasciculata cells. However, no significant difference was observed in capsular CYP11B1 mRNA levels between control and ACTH treated group (data not shown), suggesting that ACTH does not cause differentiation from glomerulosa cells to fasciculata cells in adrenal capsules.

3.4. Mn-SOD mRNA content and immunoreactivity in adrenal capsules

Mn-SOD mRNA content in ACTH treated rat adrenal capsule was five-fold that of control ($11.3 \pm 2.0 \times 10^{-3}$ versus $2.4 \pm 0.1 \times 10^{-3}$ Mn-SOD/GAPDH, $P < 0.05$) (Fig. 3(a)). Vitamin E and DMSO partially abolished Mn-SOD mRNA upregulation by ACTH ($4.6 \pm 0.4 \times 10^{-3}$ and $6.0 \pm 1.4 \times 10^{-3}$, $P < 0.05$).

Fig. 4 depicts the typical adrenal localization of Mn-SOD protein in control and ACTH treated group. Chronic ACTH treatment markedly increased Mn-SOD immunoreactivity in the zona glomerulosa as well as in the zona fasciculata-reticularis. There was no obvious change in cell number and morphological feature in zona glomerulosa by ACTH treatment in our current study.

3.5. GPx mRNA content in adrenal capsules

Changes in GPx mRNA content in adrenal capsules (Fig. 3(b)) were different from those of Mn-SOD. GPx mRNA levels in the ACTH treated group fell to a third of those in control ($1.9 \pm 0.3 \times 10^{-1}$ versus $5.4 \pm 0.8 \times 10^{-1}$ GPx/GAPDH, $P < 0.05$). Co-administration of vitamin E or DMSO further lowered GPx mRNA levels ($1.2 \pm 0.2 \times 10^{-1}$, n.s.; $0.8 \pm 0.1 \times 10^{-1}$, $P < 0.05$).

4. Discussion

In this study, we have confirmed that the reduced aldosterone production after ACTH treatment is caused by decreased aldosterone synthase activity [5] and accompanied by the downregulation of CYP11B2 mRNA [3,24]. ACTH-induced decrease in CYP11B2 mRNA level was recovered with co-administration of vitamin E or DMSO, indicating that oxidative stress

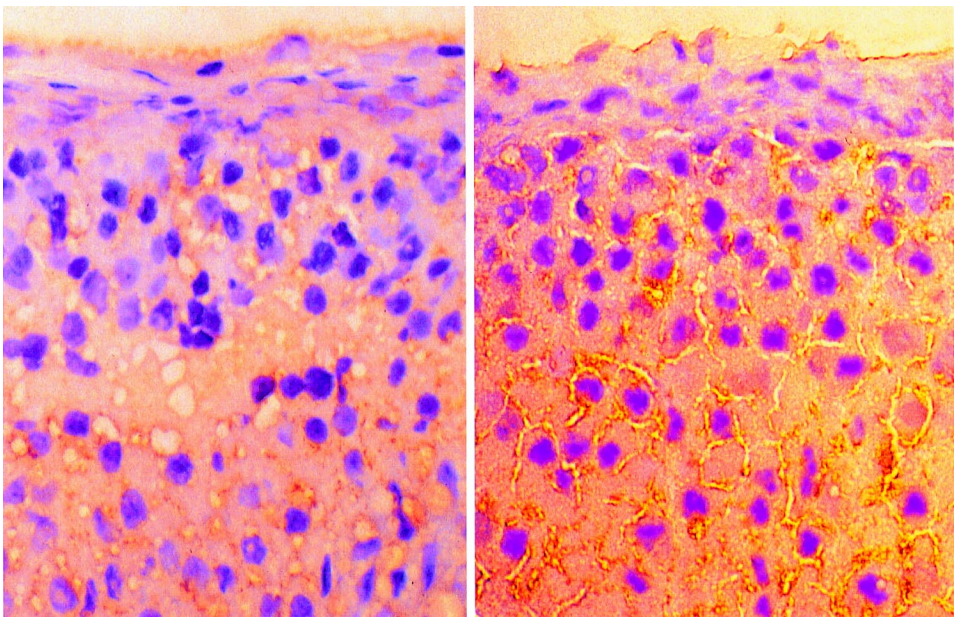

Fig. 4. Immunohistochemistry of Mn-SOD in rat adrenal glands treated with saline (left) or ACTH (right). Chronic ACTH treatment markedly increased Mn-SOD immunoreactivity in the zona glomerulosa as well as in the zona fasciculata-reticularis.

might decrease CYP11B2 gene transcription. Although certain genes of the cytochrome P450 superfamily are known to be antioxidant-inducible [25], antioxidants alone did not change the CYP11B2 mRNA level in this study. Further, there is a disjunction in effects of antioxidants on conversion and CYP11B2 expression. Co-administration of antioxidants recovered the decreased CYP11B2 mRNA levels, but plasma aldosterone level and P450aldo activity were not fully recovered. This may reflect mRNA lability, but perhaps effects of glucocorticoid or redox state on posttranslational processes should be considered, though the details are not available in our study. Anyway, our study strongly suggest that redox state modulates ACTH-induced changes in CYP11B2 gene transcription and/or stability.

Electrons escaping from the respiratory chain react with oxygen molecules, resulting in generation of superoxide and peroxynitrite. Superoxide itself and its metabolites (radical oxidative species) attack proteins, lipids and nucleic acids, thereby affecting their structure and function [26,27]. Superoxide dismutases catalyze the conversion of generated superoxide to H_2O_2 [28], which is then detoxified to H_2O by GPx [29]. If this detoxification is inappropriate, excess H_2O_2 is converted to toxic hydroxyl radicals through the Fenton reaction [30]. Thus, Mn-SOD and GPx are a critical two-step process occurring in mitochondria to scavenge free radicals. The decrease in GPx mRNA level concomitant with the increase in Mn-SOD mRNA induced by ACTH, in other words an imbalance in the ratio of Mn-SOD to GPx, results in the accumulation of H_2O_2 for the Fenton reaction, causing the formation of toxic noxious hydroxyl radicals. We propose that this imbalance is involved in the "turn-off phenomenon". Such a mechanism has been proposed in other conditions [31].

Although Mn-SOD mRNA upregulation by ACTH seems to be suitable for scavenging intracellular oxidative stress, it does not always contribute to the avoidance of impaired steroidogenesis in the zona glomerulosa. Some reports suggest that upregulation of Mn-SOD does not always protect cells from oxidative stresses [32]. Antioxidants partially decrease the upregulation of Mn-SOD mRNA levels by ACTH, but at the same time they also attenuate GPx levels, so that Mn-SOD/GPx ratios do not alter to an optimal level. Therefore, antioxidants might elicit their antioxidative role by directly scavenging radical oxidative species.

The promoter region of Mn-SOD gene contains a putative cyclic AMP responsive element [33], so that ACTH may directly upregulate Mn-SOD transcription through protein kinase A activation. On the one hand, nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) are redox-sensitive transcription factors for Mn-SOD, and oxidation is required for their activation because H_2O_2 increases Mn-SOD gene transcription [33,34]. Mn-SOD upregulation might reflect oxidative stress induced by prolonged ACTH treatment. Mn-SOD gene transcription is also modulated by glucocorticoid, probably via glucocorticoid receptor (GR)-mediated suppression of transcription factors. Glucocorticoids inhibit DNA binding of NF-κB by upregulating inhibitor kappa B or protein–protein interaction through activated GR [35,36]. Further, the DNA binding ability of AP-1 is also regulated by GR [37]. In fact, glucocorticoids inhibit Mn-SOD transcription or activity in hepatocytes, intestinal epithelial cells and macrophages [38–40], but upregulates them in glomeruli [41] or under certain circumstances [38]. These observations indicate that effects of glucocorticoids on Mn-SOD transcription might differ between cell types. Vitamin E and DMSO decreased the Mn-SOD mRNA level upregulated by ACTH, possibly via reduction of transcription factors, since NF-κB is reported to be inactivated by antioxidants [42]. The changes in the GPx mRNA level are in contrast to those in Mn-SOD. Prolonged ACTH treatment decreased its mRNA level to one third of control. Although expression of mitochondrial GPx in adrenocortical cells of neonatal rats has been reported to depend on ACTH stimulation [43], ACTH decreased GPx mRNA level in glomerulosa cells of adult rats in this study.

In addition to free radical production accompanied by physiological steroidogenesis, glucocorticoids enhance the electron leakage from the electron transport chain in adrenal mitochondria [44]. P45011β is shown to be more leaky than P450scc [44], and thus P450aldo, the structure of which resembles P45011β, is also likely to be leaky. Further, corticosterone appears to have mild proxidant property [45]. In in vitro experiments using cultured glomerulosa cells, addition of cortisol or stimulation of steroidogenesis by ACTH depresses aldosterone synthesis [46], and lowering of O_2 concentration and addition of antioxidant maintain aldosterone synthesis [47]. On the contrary, the GR knockout mouse is shown to have greatly increased expression of CYP11B2 in the zona glomerulosa [48], supporting a negative regulation by glucocorticoids. Corticosterone concentrations in the zona glomerulosa, which is adjacent to the zona fasciculata, would be fairly high during prolonged ACTH treatment. Under such circumstances, oxidative stress can be supposed to be much greater, especially in mitochondria.

Finally, despite a higher concentration of corticosterone following chronic ACTH treatment, the steroidogenic functions in the zona fasciculata are preserved. Such a discrepancy may be explained by the difference in antioxidant systems between the zona glomerulosa and zona fasciculata. Munim et al. showed a different

distribution of immunoreactive Mn-SOD and copper zinc-SOD between the zona fasciculata and zona glomerulosa of rat adrenal gland [49]. The zona fasciculata may be equipped with more effective antioxidant systems than the zona glomerulosa.

In conclusion, prolonged ACTH treatment decreased the P450aldo activity and the mRNA level of CYP11B2, and vitamin E and DMSO inhibit the ''turn-off phenomenon'' by preventing their downregulation. Prolonged ACTH treatment is associated with an imbalance in the ratio of Mn-SOD to GPx expression, which apparently increases oxidative stresses in adrenal capsule. Our results probably offer the best current explanation for the oxidative stress hypothesis in aldosterone turn-off phenomenon in the adrenal gland.

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