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Attenuation by Reactive Oxygen Species of Glucocorticoid Suppression on Proopiomelanocortin Gene Expression in Pituitary Corticotroph Cells

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Up-regulation of hypothalamo-pituitary-adrenal axis is maintained during acute inflammation and/or infection, in the face of sustained elevation of plasma glucocorticoid hormone. Inflammatory stress is usually associated with high plasma cytokine levels and increased generation of reactive oxygen species (ROS) as well. In this study, we examined the effect of ROS on the negative feedback regulation of glucocorticoid in hypothalamo-pituitary-adrenal axis using AtT20 corticotroph cells *in vitro*. When the cells were treated with H_2O_2, glucocorticoid suppression on the proopiomelanocortin gene promoter activity was attenuated in a dose-dependent manner. H_2O_2 also inhibited the ligand-stimulated nuclear translocation of glucocorticoid receptor. The released glucocorticoid suppression by H_2O_2 was not observed when the cells were cotreated with antioxidants. Together, these results suggest that increased ROS generation in the oxidative redox state attenuates the glucocorticoid negative feedback system, at least in part, by interfering with the nuclear translocation of glucocorticoid receptor and eliminating the repression on proopiomelanocortin gene expression. (*Endocrinology* 145: 39–42, 2004)

HYPOTHALAMO-PITUITARY-ADRENAL (HPA)-AXIS IS activated by a variety of stresses, and the resultant increase in adrenal glucocorticoid (GC) hormone mediates body defense responses by its antistress effect (1). In addition to the well-known physical or mental stresses, inflammation and/or infection are known to activate HPA axis as well, through the so-called immune-endocrine interaction (2). Indeed, plasma GC level is elevated after infectious/inflammatory stress, which in turn exerts a suppressive effect on the immune function to prevent the overactivation of the system.

If the high GC suppresses the activated immune system, it may also inhibit HPA axis through the well-known negative feedback effect. Nonetheless, the activation of HPA axis is maintained until the inflammation/infection subsides, and thus there should be some mechanism by which GC suppression of neuroendocrine regulation is reduced or abolished in the face of high plasma GC levels.

It is well known that systemic or local generation of reactive oxygen species (ROS) is increased during inflammation and/or infection with increased cytokine production (3–5), which influences the redox regulation of various intracellular signaling systems. In this study, we have hypothesized that GC suppression of pituitary ACTH expression is somehow reduced so that the set point of negative feedback is shifted to allow continuous stimulation of GC secretion under a high plasma GC level. We especially focused on the effect of ROS on GC receptor (GR) function, because recent studies suggest an impaired function of GR during oxidative stress through redox regulation (6–8). We found that the impaired nuclear translocation of GR is observed in corticotrophs under high hydroxyradical generation, which may account for the reduced or eliminated effect of glucocorticoid on the expression of the proopiomelanocortin (POMC) gene that encodes ACTH.

Materials and Methods

Plasmids and stable transfection

The expression vector for the green fluorescent protein (GFP)-GR fusion protein was constructed using enhanced GFP expression vector (EGFP-CI; CLONTECH, Palo Alto, CA). When the human GR cDNA (9) was fused with the C terminus of the EGFP gene, two tandem repeats of an amino acid sequence (Gly-Gly-Gly-Ser) were artificially added between the GFP and GR to maintain the flexibility of the fusion protein and the function of GR. For making a stable transformant of GFP-GR, AtT20 murine corticotroph cells were transfected with the expression vector using the lipofection method (TransIT, PanVera, WI), and a clonal cell line, designated as AtT20GG, which expresses the GFP-GR fusion protein, was obtained.

Cell culture

AtT20PL, a clone of the AtT20 cell line in which an approximately 0.7 kb of the rat POMC gene 5′-promoter (−708 to +64; +1 indicates the transcription start site)-luciferase fusion gene is stably incorporated, is described elsewhere (10). Both the AtT20PL and AtT20GG cells were maintained in a T_{75} culture flask with DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) and antibiotics (50 μU/ml penicillin and 50 μg/ml streptomycin; Invitrogen) under 5% CO_2/95%

Abbreviations: EGFP, Enhanced GFP; GC, glucocorticoid; GFP, green fluorescent protein; GR, GC receptor; HPA, hypothalamo-pituitary-adrenal; LPS, lipopolysaccharide; POMC, proopiomelanocortin; ROS, reactive oxygen species.

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atmosphere at 37 C. Culture medium was changed twice a week, and the cells were subcultured once a week.

Experimental protocols

All experiments were performed more than twice to confirm their reproducibility. The culture condition of the cells is mentioned elsewhere (10). For each experiment, forskolin (Sigma, St. Louis, MO) and/or dexamethasone (Sigma) were added to the culture medium of each dish, and the cells were harvested after the defined time interval for luciferase assay. When used, H_2O_2 (Wako Chemical, Tokyo, Japan) and TEMPOL (Sigma) were added into the culture medium 1 h before the addition of forskolin/dexamethasone. Alternatively, lipopolysaccharide (LPS; from *Escherichia coli* 055:B5, Sigma) and TEMPOL were added 6 h before the addition of forskolin/dexamethasone.

Measurements and statistics

Luciferase activity was measured as described (10). Results are expressed as mean ± SEM of triplicate or quadruplicate dishes in each group. The statistical significance of differences among the groups was analyzed by ANOVA, followed by a Fisher's protected least significant difference test at a significance level of 0.05.

Results

GC suppression of POMC gene expression is abolished under ROS excess

We first examined the negative feedback effect of GC on the POMC gene 5′-promoter activity under the environment of ROS overproduction. As shown in Fig. 1, under the control condition, dexamethasone (100 nM) potently suppressed the forskolin-induced POMC gene transcription, which is in accordance with the previous reports by Aoki *et al.* (10). In contrast, the inhibition was completely eliminated in the H_2O_2-treated group, suggesting the impaired negative feedback regulation under the circumstance with high oxidative stress.

The ROS interference of GC negative feedback is dose dependent

We then studied the effect of three different doses of H_2O_2 pretreatment on the suppressive effect of GC inhibition of the POMC gene. As shown in Fig. 2, attenuation of dexamethasone suppression was dose dependent. Dexamethasone still suppressed forskolin-induced POMC gene transcription at 1.0 mM of H_2O_2, but no inhibition was observed at or above 1.5 mM.

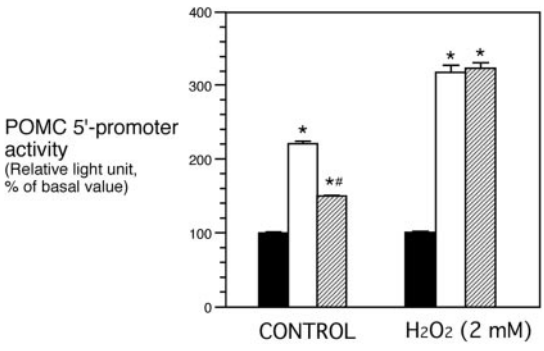

FIG. 1. The effect of H_2O_2 treatment on glucocorticoid suppression of the POMC gene 5′-promoter activity in AtT20PL cells. Cells were cultured with or without H_2O_2 (2 mM) for 1 h and then treated with vehicle (*closed bar*), forskolin (10 μM; *open bar*), or forskolin plus dexamethasone (100 nM; *shaded bar*) for 5 h. Each value is shown as a percentage of the basal value. *, $P < 0.05$ *vs.* basal value. #, $P < 0.05$ *vs.* forskolin alone.

ROS excess prevents nuclear translocation of GR by dexamethasone

Using AtT20GG cells, we also studied the influence of ROS excess on GC-induced nuclear localization of GR. GFP-GR was almost exclusively located in the cytosol in the control condition, and then translocated to the nucleus after the addition of dexamethasone (Fig. 3, A and B). Under treatment with H_2O_2, however, the movement was significantly inhibited and most of GFP-GR was observed in the cytosol (Fig. 3C). Thus, our data show that H_2O_2 treatment interferes with nuclear translocation of GR in corticotroph cells as well as in nonendocrine cells (7) and may partly explain the attenuation of dexamethasone suppression under ROS excess.

The ROS/LPS interference of GC suppression is reversed by treatment with radical scavenger

Finally, to confirm whether the effects of H_2O_2 observed above were mediated by ROS, we treated the cells with membrane-permeable radical scavenger TEMPOL (11). As shown in Fig. 4, H_2O_2 again abolished the dexamethasone suppression of POMC gene promoter activity. On the other

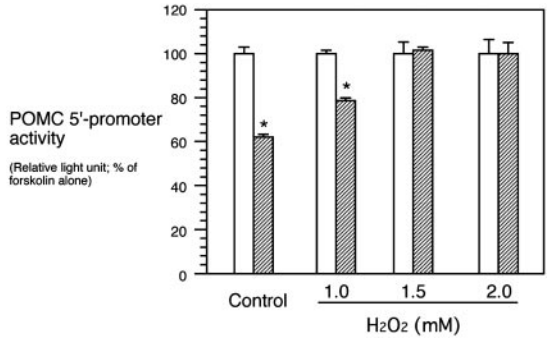

FIG. 2. The dose-response effect of H_2O_2 treatment on glucocorticoid suppression of the POMC gene 5′-promoter activity in AtT20PL cells. Cells were cultured with or without various concentrations of H_2O_2 for 1 h and then treated with forskolin (10 μM; *open bar*) or forskolin plus dexamethasone (100 nM; *shaded bar*) for 5 h. Each value is shown as a percentage of the value with forskolin alone. *, $P < 0.05$ *vs.* forskolin alone.

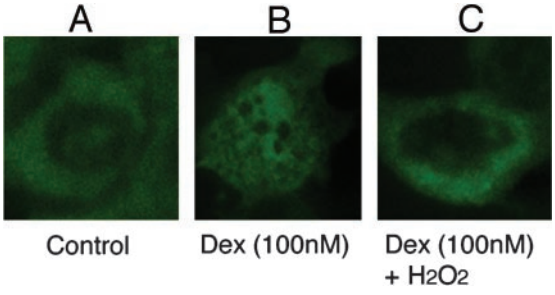

FIG. 3. The effect of H_2O_2 treatment on glucocorticoid-induced nuclear translocation of GR in AtT20GG cells. The cells, which express GFP-GR fusion protein, were treated with vehicle (A), dexamethasone (Dex; 100 nM) (B), or Dex plus H_2O_2 (2 mM) (C), and subcellular localization of GFP-GR chimeric protein was examined by confocal fluorescence microscopy.

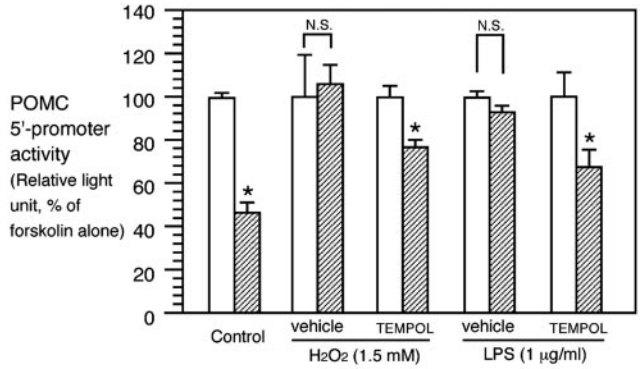

FIG. 4. The effects of TEMPOL on H_2O_2 or LPS-induced elimination of dexamethasone suppression on the POMC gene 5′-promoter activity in AtT20PL cells. Cells were incubated with vehicle, H_2O_2, or H_2O_2 and TEMPOL (2 mM) for 1 h, and then treated with forskolin (10 μM; *open bar*) or forskolin plus dexamethasone (100 nM; *shaded bar*) for 5 h. Alternatively, cells were incubated with LPS (1 μg/ml) or LPS and TEMPOL (2 mM) for 6 h, and then treated with forskolin/dexamethasone for 5 h. Each value is shown as a percentage of the value with forskolin alone. *, $P < 0.01$ *vs.* forskolin alone.

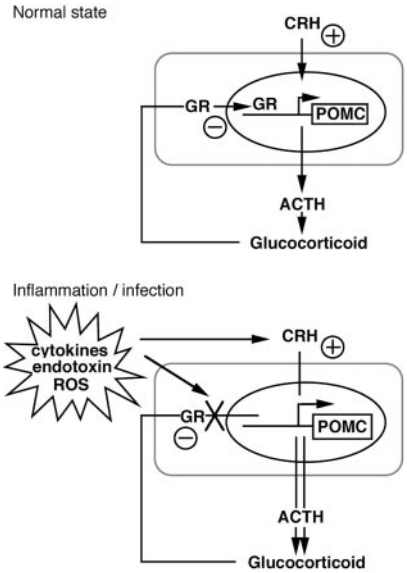

FIG. 5. Schematic representation of the effect of oxidative stress on glucocorticoid suppression of POMC gene expression in the corticotroph cells.

hand, the effect was partly reversed by a simultaneous treatment with TEMPOL (2 mM), and a significant inhibitory effect of dexamethasone was recovered. TEMPOL alone had no effect on basal or forskolin-induced POMC promoter activity (data not shown). In addition, a similar effect was observed when the cells were treated with LPS, a representative bacterial endotoxin. These results suggest that the abolished glucocorticoid suppression by either H_2O_2 or inflammatory stimulus is indeed caused by oxidative stress.

Discussion

Our results show that ROS diminishes or abolishes GC negative feedback regulation, with impaired GR nuclear translocation probably caused by altered intracellular redox regulation. This is the first evidence suggesting that oxidative stress may also be one of the stresses influencing the regulation of HPA axis.

It is well known that HPA axis is activated by acute infection and/or inflammation. In this situation a variety of cytokines or inflammatory substances produced has been shown to stimulate the expression of CRH and ACTH through immune-neuroendocrine interaction (2, 12), with the resultant increase in adrenal GC production which suppresses overactivation of the immune system. At the same time, increased plasma glucocorticoid hormone is expected to suppress HPA axis as well through negative feedback regulation at the pituitary level, whereas in fact it is maintained to be activated until the end of the event. Thus, some unknown mechanism(s) that release the GC suppression and allow the continuous expression of POMC/ACTH should exist. Our results in this study suggest that ROS, possibly derived from increased leukocyte or activated plasma cytokines, may be involved in this process. Indeed, proinflammatory cytokines such as TNFα, or endotoxins like LPS, have previously been shown to induce ROS formation in a variety of cells or tissues (13–15).

It is not completely clear how ROS diminishes or abolishes GC suppression. Okamoto *et al.* (7) showed that ROS induced by H_2O_2 treatment inhibits GC-induced nuclear translocation of GR through changes in the intracellular redox state. In this paper, we confirmed a similar phenomenon in corticotroph cells as well, suggesting that impaired translocation of GR may partly explain the release of GC suppression under oxidative stress. Furthermore, recent studies suggest that many transcription factors are redox-sensitive and regulated by intracellular oxidative stress (16). For example, ROS is known to activate nuclear factor κ-B (17), which is known to interfere with GR function (18). ROS may also influence other transcription factors regulating POMC gene expression (19, 20). We speculate that changes in the redox state following inflammation are somehow involved in the diminished or abolished GC repression.

Collectively, our results strongly suggest that the set point of GC negative feedback is shifted to maintain activated HPA axis during inflammation/infection. As shown in Fig. 5, in the normal state, CRH stimulation and GC suppression are balanced at the pituitary level, whereas GC suppression is released and CRH stimulation is enhanced during inflammation, with maintenance of up-regulated HPA axis function even in the face of high plasma GC. Our data, although obtained *in vitro*, may at least partly explain the molecular mechanism of chronic up-regulation of HPA axis under oxidative stress. Further studies will clarify the intracellular events responsible for the host-defense mechanism against a variety of environmental stresses.

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