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

KOICHI ASABA, YASUMASA IWASAKI, MASANORI YOSHIDA, MASATO ASAII, YUTAKA OISO, TOYOAKI MUROHARA, AND KOZO HASHIMOTO

Departments of Clinical Pathophysiology (K.A., Y.I.) and Internal Medicine (M.Y., M.A., Y.O., T.M.), Nagoya University Graduate School of Medicine and Hospital, Nagoya 466-8550, Japan; and Second Department of Internal Medicine (K.A., K.H.), Kochi Medical School, Nankoku, Kochi 783-8505, Japan

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$_2$O$_2$, glucocorticoid suppression on the proopiomelanocortin gene promoter activity was attenuated in a dose-dependent manner.

H$_2$O$_2$ also inhibited the ligand-stimulated nuclear translocation of glucocorticoid receptor. The released glucocorticoid suppression by H$_2$O$_2$ 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)

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-C1, 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 μg/ml penicillin and 50 μg/ml streptomycin; Invitrogen) under 5% CO$_2$/95%
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, H2O2 (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 H2O2-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 H2O2 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 H2O2, but no inhibition was observed at or above 1.5 mM.

**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 H2O2, however, the movement was significantly inhibited and most of GFP-GR was observed in the cytosol (Fig. 3C). Thus, our data show that H2O2 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 H2O2 observed above were mediated by ROS, we treated the cells with membrane-permeable radical scavenger TEMPOL (11). As shown in Fig. 4, H2O2 again abolished the dexamethasone suppression of POMC gene promoter activity. On the other...
hand, the effect was partly reversed by a simultaneous treat-
ment 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 ac-
tivity (data not shown). In addition, a similar effect was
observed when the cells were treated with LPS, a represen-
tative bacterial endotoxin. These results suggest that the
abolished glucocorticoid suppression by either H2O2 or in-
flammatory 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 regu-
lation of HPA axis.

It is well known that HPA axis is activated by acute in-
fecion 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 sup-
presses 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 main-
tained 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 cyto-
kines, may be involved in this process. Indeed, proinflam-
matory 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 H2O2 treatment inhibits GC-induced nuclear transloca-
tion of GR through changes in the intracellular redox state.
In this paper, we confirmed a similar phenomenon in corti-
troph cells as well, suggesting that impaired transloca-
tion 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 reg-
ulated by intracellular oxidative stress (16). For example,
ROS is known to activate nuclear factor-
κB (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 inflam-
mation, 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 ox-
idative stress. Further studies will clarify the intracellular
events responsible for the host-defense mechanism against a
variety of environmental stresses.

Acknowledgments

We are indebted to Ms. Machiko Kambayashi and Tatsuyo Miura
for excellent technical assistance.

Received March 26, 2003. Accepted October 3, 2003.

Address all correspondence and requests for reprints to: Yasumasa
Iwasaki, M.D., Ph.D., Department of Pathophysiology, Nagoya Univer-
sity Graduate School of Medicine and Hospital, 65 Tsurumai-cho,
References


Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.