Deletion of the bis gene results in a marked increase in the production of corticosterone that is associated with thymic atrophy in mice

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Youn DY, Yoon JS, Kim YK, Yeum CE, Lee SB, Youn HJ, Tsujimoto Y, Lee JH. Deletion of the bis gene results in a marked increase in the production of corticosterone that is associated with thymic atrophy in mice. Am J Physiol Endocrinol Metab 301: E223–E231, 2011. First published May 3, 2011; doi:10.1152/ajpendo.00604.2010.—Bis (Bag3) is known to be involved in cell survival, migration, the regulating of chaperones, and protein quality control. We reported recently on the production of bis gene-deleted mice, which show early lethality within 3 wk after birth with a phenotype showing severe malnourishment and shrinkage of the thymus. In this report, we provide evidence to show that an intrinsic problem of adrenal gland is the primary cause for the severe atrophy of the thymus in bis−/− mice. The bis−/− mice show significantly higher levels of corticosterone, but CRH and ACTH levels were considerably lower than those of wild littermates. The transcription of steroidogenic enzymes was increased in the adrenal glands of bis−/− mice, accompanied by an increase in the thickness of the zona reticularis. An analysis of thymus tissue from bis−/− mice revealed that the severe atrophy of the thymus is due to the specific loss of immature double-positive (CD4+/H11002/CD8+) cortical thymocytes, by apoptosis, as evidenced by immunohistochemical examination and flow cytometric analysis, which were restored by injection of an inhibitor of glucocorticoid synthesis. In vitro cultures of thymocytes with increasing doses of dexamethasone exhibited a similar degree of apoptosis between wild and bis−/− thymocytes. The corticosterone levels from fasted wild littermates were one-half those of bis−/− mice, although serum glucose levels were similar. Thus, the deletion of the bis gene resulted in the intrinsic defect in the adrenal gland, leading to a marked increase in glucocorticoid levels, probably upon starvation stress, which accounts for the massive apoptosis of the thymus.

Bis, also known as Bag3 or CaIr-1, was identified originally on the basis of its ability to bind Bcl-2, which enhances the antiapoptotic activity of Bcl-2 (15, 30, 43). The prosurvival activity of Bis was supported by later studies showing that Bis is overexpressed in several types of cancer and that the downmodulation of Bis sensitizes the apoptosis of tumor cells induced by chemotherapeutic agents and proteasome inhibitors (9, 32, 38, 39). In addition to the prosurvival activity of Bis, its induction under a variety of stresses in vitro as well as in vivo, including hypoxia and viral infections (3, 4, 25, 31, 33, 40), suggests that Bis might function as an antistress protein to modulate cellular response, thereby protecting cells from unfavorable environmental stresses. Other biological functions in which Bis is involved include the promotion of differentiation as shown in promyelocytes or E19 embryonic stem cells (45), the regulation of motility migration and invasion (20, 24, 26), and protein quality control via the promotion of autophagy (5, 13, 16, 19). Thus, under pathophysiological conditions, Bis appears to have multiple roles in addition to its survival-related activity.

Recently, we and another group generated bis gene-deleted (bis−/−) mice using a Cre-loxP system and retroviral insertion, respectively (23, 46). The bis−/− mice from both systems exhibited early lethality within 25 days after birth as a common phenotype, which verifies, not only at the cellular level but also in organisms, that the absence of Bis results in critical consequences for survival. Homma et al. (23) proposed respiratory difficulties or decreased cardiac performance as the cause of death. These conclusions were based on the massive apoptosis observed and a marked degeneration of the diaphragm, intercostal muscles, heart, and other skeletal muscles without any observable abnormalities in other organs. However, our study, in which the bis gene was deleted by a Cre-loxP system, revealed Z-disc disruption in skeletal muscles, but massive degeneration or apoptotic features were not observed. Instead, the most obvious phenotypes observed in our bis gene knockout model were serious hypoglycemia and fatty livers. Quantitative analysis of the hepatic mRNA of several enzymes involved in glucose and lipid metabolism revealed an increase in gluconeogenesis and β-oxidation as well as a decrease in lipogenesis, indicating a typical adaptive response to malnutrition, which was supported by serum profiles of metabolites as well as the scarcity of peripheral fat (46). Thus, the serious nutritional problem could be associated with the early lethality, but the exact reason for the metabolic derangement by bis deletion is unclear at this time. The bases for the differences in the phenotypes between our and the other group are not clear, although the methods used in gene targeting and the subsequent possibility that trace amounts of truncated Bis protein remained in our or the previous study cannot be completely excluded at this time.

Another prominent observation in the bis−/−deleted mice in our study is the shrinkage of the thymus and spleen. Although these organs have been shown to be distressed by nutritional status (6, 8), the relationships between metabolic deterioration and involution of the thymus and spleen in bis−/− mice are not obvious at this point. Our recent report, which described the role of Bis in the integrity of the vascular niche for hematopoietic stem cells, demonstrated that mice with a bis gene deletion exhibited a selective loss of β-cell lineage in bone marrow and spleen (27). Thus, the atrophy of the spleen could be due, at least in part, to a defect in β-cell development in addition to malnutrition. However, the finding that the T cell lineage in bone marrow was comparable for bis−/− and bis+/+ littermates raises the possibility that the thymic involution is
related to the serious hypoglycemia in bis⁻/⁻ mice rather than to a functional defect in bone marrow. Thymic atrophy, which is considered to be a barometer of malnutrition, can be attributed to the depletion of thymocytes, particularly affecting immature CD4⁺CD8⁺ cells, through an increase in the production of glucocorticoid (2, 11, 36). Therefore, in the present study, to determine whether the thymic atrophy observed in bis⁻/⁻ mice is caused by higher corticosterone levels, we examined corticosterone levels and adrenal phenotypes of both wild and bis⁻/⁻ deficient mice. We also examined the issue of whether thymic alterations are restored by inhibiting corticosterone synthesis. We also provide evidence to show that thymic phenotypes are not the result of an unusually high susceptibility of bis⁻/⁻ thymocytes to glucocorticoid by showing that the proportion of apoptotic thymocytes from wild and bis⁻/⁻ mice are comparable, after in vitro incubation, with increasing doses of dexamethasone (DEX). Finally, to address the issue of whether the deletion of the bis gene influences the synthesis of glucocorticoid under hypoglycemic stress, we determined the serum corticosterone levels as well as the phenotypes of thymus and adrenal glands in wild littermates that had been fasted for 72 h.

METHODS

Mice and injection of the corticosteroid synthesis inhibitor. The mice in which exon 4 of the bis gene was deleted in both chromosomes (bis⁻/⁻) were generated by mating heterozygous mice, as described previously (46). The heterozygous mice that were maintained in C57BL/6 mixed background by crossing C57BL/6 wild mice >13 generations were used in this study. The bis genotypes were identified by PCR analysis using DNA isolated from tail samples (46). To suppress glucocorticoid synthesis, bis⁻/⁻ mice and wild littermates were injected subcutaneously behind the neck with DL-aminogluthemide (AMG; Sigma, St. Louis, MO) at 50 µg/g daily from day 11 after birth until day 16. AMG was dissolved in dimethyl sulfoxide (DMSO; Sigma) and diluted 1:20 with phosphate-buffered saline (PBS) before injection. Control mice were injected with an equal volume of 5% DMSO in PBS. For starvation experiments, the wild-type littermates from heterozygous parents at postnatal 12 days were deprived from mothers and then fasted for 72 h. All research procedures involving animals were performed in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Experiments provided by the Institutional Animal Care and Use Committee at the College of Medicine, The Catholic University of Korea, which approved the issue of whether the deletion of the bis gene influences the synthesis of glucocorticoid under hypoglycemic stress, we determined the serum corticosterone levels as well as the phenotypes of thymus and adrenal glands in wild littermates that had been fasted for 72 h.

Measurement of glucose, corticosterone, and ACTH levels. Whole blood was obtained by retroorbital sampling under ether anesthesia at 9 AM, 2 h after the lights were turned on in the morning. The concentration of glucose in the blood was determined by Hemocue Glucose 201+ (Hemocue, Angelholm, Sweden). Plasma corticosteroid levels and ACTH levels were measured with the Corticosterone EIA (Cayman, Ann Arbor, MI) and ACTH ELISA kits (MD Bioscience, St. Paul, MN), respectively, according to the manufacturers’ instructions.

Morphological analysis. Paraffin sections (4 µm) from thymus or adrenal gland were processed for hematoxylin and eosin (H & E) staining. To examine the state of apoptosis in situ in thymus, the frozen sections of thymus were stained with the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN), based on a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, as well as with the antibody against cleaved caspase-3 (1:200; Cell Signaling Technology, Danvers, MA). After incubation with Cy3- or Alexa 488-conjugated secondary antibodies (1:1,000; Molecular Probes, Eugene, OR; and 1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA), the specimens were examined under fluorescence microscopy (Olympus, Tokyo, Japan). The effect of AMG injection on the apoptosis in thymus in bis⁻/⁻ mice as well as detection of apoptotic cells in the adrenal gland were investigated with the ApopTag Peroxidase In Situ Apoptosis Detection Kit S7100 (Chemicon, Temecula, CA) under a light microscope (Axioskop40; Carl Zeiss, Gottingen, Germany).

Flow cytometric analysis. Single-cell suspensions were prepared from thymus, and ~10⁶ cells were stained for 10 min at 4°C with specific monoclonal antibodies against CD4 and CD8 labeled with PE and FITC, respectively (BD Bioscience, San Diego, CA), in PBS containing 2% fetal bovine serum. After washing with PBS containing 5% fetal bovine serum and 0.05% sodium azide, expression of each cell surface marker was analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA), and data were processed using Cell Quest software (BD Bioscience). To determine the susceptibility to apoptosis, the isolated thymocytes from wild and bis⁻/⁻ mice were incubated in vitro with indicated doses of DEX (Sigma) and VP-16 (Sigma) for 4 or 8 h. The apoptotic fractions were obtained by staining with annexin V and propidium iodide (PI) using the ApoScan Apoptosis Detection kit (BioBud, Seoul, South Korea), followed by flow cytometric system using Cell Quest software (BD Bioscience).

Quantitative real-time PCR. Total RNA was extracted from the adrenal gland or hypothalamus of bis⁻/⁻ mice and wild-type littermates using RNA-Be (Tel-Test). Two micrograms of total RNA from each preparation was reverse transcribed to CDNA by units of Moloney murine leukemia virus reverse transcriptase (Fermentas) and 0.2 µg of random hexamer oligonucleotide (Roche Diagnostics) and processed for quantitative real-time PCR on an Mx3000P cycler (Stratagene, La Jolla, CA), using SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan) and appropriate primers for steroidogenic acute regulatory protein (StAR), steroidogenic factor-1 (SF-1), cytochrome P450 21α-hydroxylase (CYP21A1), cytochrome P450 11β-hydroxylase (CYP11B1), or corticotropin-releasing hormone (CRH) (Table 1). The relative levels of PCR products were determined after normalizing to an endogenous β-actin control.

Western assay. Proteins were extracted from tissues or cultured cells with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) with protease inhibitor (Roche Diagnostics), and equal amounts of protein were

Table 1. Primers used for real-time PCR analysis

<table>
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<tr>
<th>Gene</th>
<th>Primer Sequences (5’-3’)</th>
<th>Accession No.</th>
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<td>NM_205769</td>
</tr>
<tr>
<td></td>
<td>Reverse TCC GAG CTC GGA TAT GAT ACA GAA</td>
<td></td>
</tr>
<tr>
<td>StAR</td>
<td>Forward TCA CTT GGC TGC TCA G7A TTG AC</td>
<td>NM_011485</td>
</tr>
<tr>
<td></td>
<td>Reverse GGC ATA GGA CCT GGT TGA TGA</td>
<td></td>
</tr>
<tr>
<td>SF-1</td>
<td>Forward TCC TGA ACA ACC ACA GCC TGG TAA</td>
<td>NM_139051</td>
</tr>
<tr>
<td></td>
<td>Reverse TGG TTC AAA TGC TGG TAC ACG</td>
<td></td>
</tr>
<tr>
<td>CYP21A1</td>
<td>Forward GGG AAC TGC CCA GCA AGT T</td>
<td>NM_009995</td>
</tr>
<tr>
<td></td>
<td>Reverse GGG TGG TTT GGG ATT CCT C</td>
<td></td>
</tr>
<tr>
<td>CYP11B1</td>
<td>Forward TCA GAT GGC ATG TGG TCC AGC TTA</td>
<td>NM_001033229</td>
</tr>
<tr>
<td></td>
<td>Reverse AAG TCT ACA GCT TGT CCT GCC ACA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b-actin Forward TTC GTC GCC GGT CCA A</td>
<td>NM_007393</td>
</tr>
<tr>
<td></td>
<td>Reverse ACC AGC GCA GGC ATA TCG</td>
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CRH, corticotropin-releasing hormone; StAR, steroidogenic acute regulatory protein; SF-1, steroidogenic factor-1; CYP21A1, cytochrome P450 21α-hydroxylase; CYP11B1, cytochrome P450 11β-hydroxylase.
separated through 10% SDS-PAGE and then blotted onto PVDF membranes (Millipore, Bedford, MA). The membranes were incubated antibodies recognizing StAR (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), CYP11B1 (1:500; Santa Cruz Biotechnology), active caspase-3 (1:1,000; Cell signaling Technology), or α-tubulin (1:2,000; Sigma), followed by incubation with horseradish peroxidase-linked secondary antibodies (1:2,000; Millipore). The bound antibodies were visualized with an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL).

Statistical analysis. The difference between the two groups was analyzed by unpaired two-tailed Student’s t-test. Values of \( P < 0.05 \) were considered significant. All values are presented as means ± SE.

RESULTS AND DISCUSSION

Significantly high levels of corticosterone and the adrenal alterations in bis-deficient mice. It has been shown that glucocorticoid regulates apoptosis of thymocytes during normal development (1, 12). Concerning the malnutrition or acute infection-induced thymus atrophy, the elevated glucocorticoid levels can be also regarded as one of hormonal impact on the induction of apoptosis of thymocytes (2, 11, 42). Furthermore, atrophy of the spleen and thymus can be induced by treatment with DEX (21, 22, 35). Therefore, as a possible biological explanation for the shrinkage of the thymus in mice carrying a homozygous bis deletion, we measured levels of serum corticosterone, which is the predominant glucocorticoid in rodents (14, 29, 41), of the wild-type and bis/−/− mice. As shown in Fig. 1A, the range of serum corticosterone levels in wild-type littermates at 16–18 days was 72–136 ng/ml (mean 119.4 ng/ml; \( n = 10 \)), whereas the range was 154–698 ng/ml in bis/−/− mice (mean 389.9 ng/ml; \( n = 10 \)), suggesting that the involution of the thymus is likely to be caused by elevated serum corticosterone levels rather than by the absence of antiapoptotic Bis protein directly.

Fig. 1. Serum corticosterone levels and adrenal alterations in bis-deleted mice. A: serum corticosterone levels were determined in wild and bis/−/− mice at 16–18 days. Means ± SE are presented (\( n = 10 \)). B: the quantization of corticotropin-releasing hormone (CRH) mRNA from the hypothalamus of wild and bis/−/− mice was performed by real-time quantitative PCR, as described in Methods (\( n = 7 \)). Plasma ACTH levels from bis/−/− mice were measured and are represented as percentage of those of wild mice (\( n = 8 \)). C: mRNA levels for the steroidogenic enzymes steroidogenic factor-1 (SF-1) as well as steroidogenic acute regulatory protein (StAR) were determined from adrenal tissues of wild and bis/−/− mice on day 16 after birth (\( n = 9 \)). Data are normalized relative to β-actin mRNA in the same samples, and wild-type values were arbitrarily set as 1.0. D: hematoxylin and eosin (H & E) staining of adrenal gland of wild and bis/−/− mice on day 16. The thickness of the zona reticularis is indicated by a white arrow. E: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay shows that apoptotic cells were very rare in both wild and bis/−/− adrenal glands. The bar represents 100 μm in D and E. ***P < 0.01, ****P < 0.001 compared with wild-type littermates in A–C.
Then, to investigate whether the significantly high levels of corticosterone in bis-deficient mice are attributable to an increase in signals from a higher cascade, the hypothalamus, or pituitary glands, we compared CRH mRNA levels in the hypothalamus between wild and bis-deficient littermates. A quantitative analysis of CRH mRNA indicated that the bis-deficient mice express almost one-half the amounts of CRH mRNA levels compared with wild littermates (Fig. 1B). In addition, the ACTH levels of bis-deficient mice were significantly reduced to 37% of wild littermates (Fig. 1B), indicating that the negative feedback regulation appears to be intact in bis-deficient mice. Thus, it is not the constitutive release of signals from the hypothalamus or pituitary gland but the intrinsic problem of the adrenal gland that is the primary source of the elevated corticosterone levels in bis-deficient mice. We then determined the relative amounts of mRNA of several enzymes that are involved in corticosterone synthesis in the adrenal gland of bis/− mice as well as in that of the wild littermates. As shown in Fig. 1C, the mRNA expressions CYP11B1 and CYP21A1 were all increased in bis/− mice 3.6- and 2.9-fold, respectively, compared with those of wild littermates. Furthermore, SF-1, a common transcription factor for steroidogenic enzymes (28) as well as StAR, which transports cholesterol into mitochondria (10, 18), was also higher in bis/− mice compared with wild littermates ~1.5- and 1.7-fold, respectively, in mRNA levels.

A morphological examination of adrenal glands from bis/− mice revealed a noticeable increase in the cellularity of the inner layer of the cortex in H & E staining. Examination with a higher magnification confirmed that the thickness of the zona reticularis was increased, which can be attributed to hyperplasia (Fig. 1D). Thus, the increase in the width of zona reticularis might be related to the enhanced synthesis or secretion of glucocorticoid in the adrenal gland of bis-deficient mice, thereby increasing peripheral glucocorticoid levels. Again, these results strongly suggest that the deletion of the bis gene caused a critical problem in adrenal glands and subsequently increased corticosterone levels, which could account for the catabolic phenotypes of bis/− mice. The molecular bases for the alterations in the adrenal gland from bis/− mice are not clear at this point. However, it is certain that it is not due to the absence of apoptotic response upon the suppression of ACTH secretion from hypophysis because a TUNEL assay revealed that the distribution of apoptotic cells was very limited in the adrenal glands from both wild and bis/− mice (Fig. 1E).

Thymic alterations in bis-deficient mice are related to increased serum corticosterone levels. Consistent with the atrophic phenotypes of thymus tissue in gross examination, a histological examination of the thymus at 16 days postnatal revealed a noticeable decrease in cellularity, especially in the cortex, which was thinner than in wild-type littermates (Fig. 2A). H & E staining also revealed the presence of a large number of pyknotic cells in the cortexes of bis/− mice thymus, which were determined to be apoptotic cells, as evidenced by labeling with TUNEL and with an antibody against active caspase-3. The apoptotic cells were found to be limited exclusively to the cortex, and none were found among medullary thymocytes (Fig. 2B). It is generally accepted that the major targets of glucocorticoid-induced apoptosis are CD4+CD8+ double-positive thymocytes, which are located mainly in the cortex, (2, 11, 42). Figure 2C showed a representative fluorescence-activated cell sorting analysis indicating a significant decrease in the population of CD4+CD8+ double-positive cells and a relative increase in the population of single-positive cells. This change in the relative proportion of each subtype is not due to an absolute increase in single-positive cells but rather to a profound loss of double-positive cells, as evidenced by the marked decrease in the total number of thymocytes. As shown in Table 2, the total number of thymocytes from bis/− mice was less than one-fourth that from wild littermates, and the numbers of each population of thymocytes were all decreased, among which the decrease in double-positive thymocytes was the most significant. The decrease in the number of single-positive cells might be the consequence of a massive reduction of double-positive cells. Thus, the intense loss of immature double-positive thymocytes, probably via apoptosis, may contribute to the hypocellularity of the thymic cortex of bis-deficient mice.

To confirm that the increased corticosterone is the primary cause of the apoptosis of double-positive thymocytes in bis-deficient mice, we injected AMG, an inhibitor for CYP11A1 that converts cholesterol to pregnenolone in the corticosterone synthesis pathway (7, 44). The injection of AMG from day 11 for 5 days significantly reduced the corticosterone levels in bis/− mice to 23% of those in 5% DMSO/PBS-injected bis/− mice from 358.2 to 81.3 ng/ml (Fig. 3A). In accordance with the corticosterone levels, AMG injections dramatically restored the population of double-positive thymocytes in bis-deficient mice from 23.3 to 81.8% (Fig. 3B). Apoptotic cells in the cortex were also significantly reduced by AMG injection in bis/− mice, as evidenced by TUNEL assay (Fig. 3C). In addition, the AMG injection also restored the adrenal phenotypes in bis/− mice, as evidenced by the finding that the thickness of the zona reticularis reverted to that of wild littermates (Supplemental Fig. S1; Supplemental Material for this article is available online at the AJP-Endocrinology and Metabolism web site). Thus, the hypertrophy in the zona reticularis in the adrenal gland might be associated with an increased level of corticosterone synthesis in bis/− mice. Collectively, the thymic phenotypes in bis/− mice clearly appear to be dependent on increased corticosterone levels.

Thymocytes from wild and bis/− mice show identical sensitivity to DEX. Although the phenotypes of the thymus shown in bis/− mice were recovered by the inhibition of glucocorticoid synthesis, the possibility that the thymocytes of bis/− mice exhibit increased sensitivity to corticosterone-mediated apoptosis cannot be excluded. To examine this possibility, we isolated thymocytes from the thymus of wild and bis/− mice 9–11 days after birth, when the body weights of the bis/− mice were still increasing and corticosterone levels in bis/− mice were not higher than wild mice, to avoid the sensitization by enhanced glucocorticoid levels shown in the later stage. And then the thymocytes were exposed to DEX as well as to VP-16 to for determination of the susceptibility to apoptotic stimuli. To quantify the apoptotic proportion, we performed annexin V and PI staining using flow cytometry after incubation with various doses of DEX for 8 h (Fig. 4A). Figure 4B shows that the proportion of thymocytes that were positive for annexin V but negative for PI, representing early apoptosis, was increased in a dose-dependent manner, which was almost identical in wild and bis/− mice for all of the tested doses of DEX. The proportion of the late stage of apoptosis, positive for both annexin V and PI, was not very significant after 8 h of
incubation. The total proportion for annexin V positive cells, at a DEX dose of 0.1 μM, was 53.4 and 51.2% in wild and bis⁻/⁻ thymocytes, respectively. The apoptotic fractions from bis⁻/⁻ thymocytes after treatment with VP-16 were also parallel with those of wild thymocytes. We also examined caspase-3 activation by Western blotting. Figure 4C shows that the density of the cleaved form of caspase-3 is increased depending upon the concentration of DEX, showing similar patterns in both wild and bis⁻/⁻ thymocytes. Thus, the sensitivity of the thymocytes to glucocorticoid is not apparently increased in bis⁻/⁻ mice, indicating that the significant loss of thymocytes observed in bis-deficient mice is not the result of an intrinsic problem associated with thymocytes caused by bis deletion but rather the result of increased levels of corticosterone in bis⁻/⁻ mice. However, considering that T cell development is affected by the microenvironment provided by thymic epithelial cells, which secrete survival factors as well as glucocorticoid (1, 34, 37), the susceptibility of thymocytes to glucocorticoid should be determined in vivo in future studies using inducible or conditional knockdown of the bis gene is totally deleted.

Table 2. Changes in the thymocyte subpopulation in bis⁻/⁻ mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cell Phenotype Cell No. × 10⁶ (%)</th>
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<tr>
<td></td>
<td>Total CD4⁺ CD8⁺ CD4⁺ CD8⁻ CD4⁻ CD8⁺ CD4⁻ CD8⁻</td>
</tr>
<tr>
<td>bis⁺/+</td>
<td>88.25 (100) 8.02 (9.09) 4.77 (5.40) 71.77 (81.32) 3.54 (4.02)</td>
</tr>
<tr>
<td>bis⁻/-</td>
<td>2.05 (100) 0.96 (46.9) 0.38 (18.3) 0.41 (20.0) 0.29 (14.3)</td>
</tr>
</tbody>
</table>

No. and percentage of various thymocyte phenotypes were determined by flow cytometric analysis after double-staining with anti-CD4 and anti-CD8 staining and counting total thymocyte no. per thymus. The mean values from 6 independent experiments are presented.
Fig. 3. Inhibition of glucocorticoid synthesis restored thymic phenotypes in bis⁻/⁻ mice. A: wild and bis⁻/⁻ mice were injected with DL-aminogluthethimide (AMG) at the dose of 50 μg/g daily from days 11 to 16 after birth (AMG−). As a control, the same concentration of 0.5% DMSO in PBS was injected (AMG⁺). Three parameters, body weight (top), serum glucose (middle), and corticosterone levels (bottom), were measured in each mouse, and the data are shown as means ± SE (n = 6). **P < 0.01. B: thymocytes were isolated from control or AMG-injected wild and bis⁻/⁻ mice, and the subpopulation of thymocytes was determined. The proportion of double-positive thymocytes from each group is presented as means ± SE (n = 5). The inhibition of glucocorticoid synthesis reversed the proportion of double-positive thymocytes in bis⁻/⁻ to that in wild mice. **P < 0.01. C: the effect of AMG on the induction of apoptosis in the thymic cortex was demonstrated by TUNEL assay. The TUNEL-positive apoptotic cells localized in the cortex were decreased significantly by AMG injection. The scale bar represents 20 μm.

The levels comparable with wild littermates by forced feeding, which was initiated on day 11 and continued to day 16, were unsuccessful. It is not certain whether the cause for the nutritional status of bis-deficient mice is associated with dietary intake or a problem associated with energy expenditure. Thus, it cannot be assured to increase glucose levels in bis⁻/⁻ mice with an appropriate and available procedure.

As an alternative route to examine the relationship between significant corticosterone levels and the undernourishment of bis⁻/⁻ mice, we performed starvation experiments using wild littersmates from bis⁺/+ hetero parents and compared the glucose levels and corticosterone levels with those from bis⁻/⁻ mice. The wild-type mice at 12 days of age and a body weight of ∼5.5 g, a weight similar to the peak body weight of bis⁻/⁻ mice, were deprived of their mothers’ milk for 72-h periods until 15 days of age. Following fasting, the weights of the wild-type mice were reduced (4.7 g; n = 6) similarly to that in bis⁻/⁻ mice at 16 days of age (4.3 g; n = 10) and showed hypoglycemia levels of ∼73 mg/dl, which is slightly higher than those of bis⁻/⁻ mice (64 mg/dl; n = 10) (Figs. IA and 5A). However, their range of corticosterone levels was 145–265 ng/ml (mean 203.9 ng/ml; n = 6), which is significantly lower than that for bis⁻/⁻ mice (mean value >350 ng/ml) (Figs. IA and 5A). On the other hand, a histological examination indicated that the thymus in fasted mice showed a decrease in cellularity in the cortex, but the proportion of apoptotic cells in the cortex appeared to be smaller than that in thymus in bis⁻/⁻ mice, as evidenced by TUNEL staining (Figs. 2, A and B, and 5B). Thus, the difference in the degree of thymic atrophy and apoptosis between bis-deficient mice and fasted wild littermates can be attributed to a difference in corticosterone levels.

An implication that can be presumed from these experiments is that hypoglycemia is not the only explanation for the increased corticosterone levels in bis⁻/⁻ mice. Another stress, such as respiration distress, in addition to nutritional deprivation, could enhance corticosterone synthesis in bis⁻/⁻ mice. If not, the catabolic phenotypes of bis⁻/⁻ mice might not be the essential cause of adrenal alterations and subsequent high glucocorticoid levels but the consequences of adrenal abnormalities conferred by deletion of the Bis gene. It should be noted that the suppression of glucocorticoid synthesis by AMG had no effect on body weight and serum glucose levels in bis-deficient mice (Fig. 3A), suggesting that growth retardation or hypoglycemia is not directly caused by a marked increase in corticosterone levels. However, it is possible that the inhibition of glucocorticoid production might later fail to alleviate the catabolic phenotypes caused by previous sustained exposure to glucocorticoid, probably because the endocrine organs or muscles were already sensitized to direct metabolic balance to catabolic status. One possible explanation is that the bis⁻/⁻ mice were subjected to starvation stress for an unidentified reason, and the absence of Bis protein in adrenal glands could contribute to the augmented production of corticosterone upon hypoglycemic stress.

A quantitative analysis for mRNA involved in corticosterone synthesis in fasted mice revealed a pattern similar to that of bis⁻/⁻ mice, showing a significant increase of CYP11B1 and CYP21A1 mRNA transcripts. The degree of their induction was comparable with that for in bis⁻/⁻ mice, a 3.3- and 2.9-fold induction of CYP21A1 in fasted wild and bis⁻/⁻ mice, respectively (Figs. 1C and 5C). Accordingly, the degrees of induction of STAR and CYP11B1 at protein levels in bis⁻/⁻
mice and fasted wild littermates were similar, as determined by a Western assay (Supplemental Fig. S2). Therefore, although hypoglycemic stress could be either inducer or consequence of corticosteroid production, it can be postulated that the significantly high levels of corticosterone levels in bis/H11002/H11002 mice appear not to be attributed to the transcriptional activation of steroidogenic enzymes at an extraordinary rate, exceeding those of fasted wild mice. Another possible explanation for this discrepancy is that the secretion of corticosterone into the blood might be affected by the bis gene deletion. In addition to ACTH stimulation, it has been proposed that the secretion of corticosterone might be modulated by adrenocortical innervations as well as the intra-adrenal circuit or by paracrine control by the adrenal medulla (17). Thus, the absence of Bis might disrupt the fine-tuning of this system on the release of corticosterone, although direct evidence for this is currently unavailable.

Fig. 4. Determination of susceptibilities of thymocytes from wild and bis/H11002/H11002 mice to apoptotic stimuli. A: the thymocytes were isolated from wild and bis/H11002/H11002 mice and cultured with indicated concentrations of dexamethasone (DEX) or VP-16 for 8 h. Cells were then processed for flow cytometric analysis of the annexin V-FITC and propium iodide (PI) staining. A representative dot blot analysis of the annexin V vs. PI staining out of 4 experiments with 0.1 M of DEX was shown. B: after incubation with DEX or VP-16, the percentage of annexin V+/PI− (early apoptosis) and annexin V+/PI+ (late apoptosis) was calculated by flow cytometric analysis using CellQuest software (Becton Dickinson) and represented as white and gray bars, respectively. Results are represented as means ± SE (n = 4). C: Western analysis for caspase-3 in wild and bis-null thymocytes after 4 h of incubation shows similar patterns and increasing density of active cleaved form of caspase-3, depending on the concentration of DEX. A representative assay from 3 independent experiments with similar results was shown. α-Tubulin expressions were provided as a loading control.

Fig. 5. Effect of starvation on the induction of corticosterone and alterations of the thymus and the adrenal gland in wild littermates. A: wild littermates were fasted for 72-h periods between days 11 and 14 after birth, and 3 parameters, corticosterone, glucose, and body weight, were determined for each mouse (n = 6). **P < 0.01, ***P < 0.001. B, bottom: the effect of starvation on the induction of apoptosis in the thymus was shown by a TUNEL assay. B, top: H & E staining reveals atrophy of thymic cortex in fasted mice. The scale bars represent 100 μm. C: the quantization of mRNA for steroidogenesis in adrenal gland from fasted and control-fed mice was performed as in Fig. 1C. *P < 0.05, **P < 0.01.
Collectively, in bis⁻/⁻ mice, corticosterone levels were markedly higher than that for the simply fasted wild littermates, subsequently leading to severe loss in thymic cortex. These results raise the possibility that Bis might be involved in the regulation of steroidogenesis or release under physiological conditions or upon stress. However, the molecular mechanism by which Bis affects corticosterone levels remains to be addressed in future studies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


