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Tissue-Specific Expression of Rat IL-18 Gene and Response to Adrenocorticotropic Hormone Treatment

Shuei Sugama, Yoonseong Kim, Harriet Baker, Cristina Tinti, Hocheol Kim, Tong H. Joh, and Bruno Conti

IL-18 is a pleiotropic cytokine also proposed to have a role in modulating immune function during stress. Initially found in immune cells, IL-18 mRNA is detectable in several tissues including the cells of the zona reticularis and the zona fasciculata of the adrenal cortex, where its levels are elevated by acute stress or adrenocorticotropic hormone treatment. In the present study, we compared the expression of IL-18 in the adrenal cortex with that of spleen and duodenum, two other IL-18-positive tissues. In situ hybridization showed that, in contrast to the adrenal cortex, in spleen and duodenum IL-18 is primarily localized to immune cells. In duodenum, IL-18 mRNA was also detectable in epithelial cells. Northern blot demonstrated that while the adrenal gland synthesized IL-18 mRNA of 1.1 kb, spleen and duodenum produced a 0.9-kb transcript. RT-PCR, sequencing, Western blot, primer extension, and rapid amplification of cDNA end analysis demonstrated that the three tissues synthesize IL-18 mRNAs containing the same coding region and produce the same IL-18 peptide, but differ in the length of their 5′-untranslated region, indicating tissue-specific usage of the promoter region. Finally, in contrast to the adrenal gland, adrenocorticotropic hormone treatment did not increase the levels of IL-18 mRNA in spleen and duodenum. These results demonstrate tissue-specific expression and promoter usage of IL-18 gene and suggest that the adrenal cortex and not immune cells may be the source of IL-18 during stress. The Journal of Immunology, 2000, 165: 6287–6292.

The hypothalamic-pituitary-adrenal (HPA) axis is a vital homeostatic system involved in the regulation of neuroendocrine functions (1). Stress activates the HPA axis by inducing the production of the corticotropic releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus in the brain. In turn, CRH stimulates the anterior lobe of the pituitary gland to synthesize the adrenocorticotropic hormone (ACTH), which is released into the bloodstream. ACTH acts on the adrenal glands, where it stimulates the cells of the cortex to synthesize and release glucocorticoids (GC). Finally, GC inhibit the synthesis of CRH and ACTH, providing a negative autoregulatory feedback. Because GC are powerful pharmacological antiinflammatory and immunosuppressive agents, and given the clinical evidence linking stress and diseases, the HPA axis is also believed to be a mediator of immunomodulation during stress.

IL-18, initially called IFN-γ-inducing factor, is a proinflammatory cytokine and a powerful stimulator of the cell-mediated immune response, with inflammatory associated tissue damage, and antimicrobial and antitumor activity (2–8). IL-18 was originally isolated from Kupffer cells of mice challenged with LPS to induce toxic shock (2) and was also cloned from rat adrenal gland (9). Significantly, in the adrenal gland IL-18 is produced in the zona reticularis and zona fasciculata of the adrenal cortex, the same cells that synthesize GC (9). In addition, ACTH treatment stimulated the production of IL-18 mRNA and protein in the adrenal cortex (10). These data indicated that the CNS may modulate the synthesis of IL-18 in the adrenal gland through the HPA axis and suggested the possibility that IL-18 may have a role in maintaining the homeostasis of the immune system during acute stress (10). The adrenal cortex is not the only source of IL-18. It has also been demonstrated in a variety of other cell types and tissues, including cultured astrocytes and microglia derived from the CNS as well as monocytes/macrophages, keratinocytes, and osteoblasts (11–14). The possible contribution of these IL-18-producing tissues to the stress response has never been investigated, specifically in immune cells that were reported to have functional ACTH receptors (15, 16).

The first step in the control of IL-18 production is at the transcriptional level. Previous analysis demonstrated that the mouse IL-18 gene is composed of seven exons (16). In mouse, two promoter regions located upstream of the untranslated exons, 1 and 2, respectively, modulate the basal and the inducible production of mouse IL-18 mRNA (17, 18). In addition, our previous data showed the existence of two isoforms, IL-18 and IL-18α, in rat adrenal gland. Thus, both differential promoter usage and/or alternative splicing might be involved in the regulation of IL-18 transcription in rat.

To further investigate the role of IL-18 in response to stress, the present study compared the tissue localization and the ACTH-mediated induction of IL-18 mRNA in adrenal cortex with that in spleen and intestine, two other peripheral sites of IL-18 synthesis that also participate in the stress response. The data demonstrate tissue-specific expression of IL-18 mRNA and ACTH inducibility, suggesting differential promoter usage in endocrine and immune cells.
Materials and Methods

Animal and treatment

All procedures were approved by the Institutional Animal Care and Use Committee of Cornell University Medical College. Male Sprague Dawley rats (300–400 g) were purchased from Charles River (Wilmington, MA). For ACTH treatment, animals received s.c. injection of 8 UPS units (80 mg) ACTH (ACTHHar gel; Rhone-Poulenc Rorer Pharmaceuticals, Collegeville, PA) or saline (control). Access to food and water was ad libitum and the light/dark cycle was maintained at 12/12 h. Animals were sacrificed 4 h after ACTH treatment. Tissues were rapidly dissected, frozen in liquid nitrogen, and stored at −80°C for further analysis. Plasma ACTH and corticosterone levels were determined by RIA using ACTH and corticosterone kits (ICN Biomedicals, Costa Mesa, CA).

In situ hybridization

In situ hybridization was performed as previously described (19). In brief, animals were deeply anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with saline containing 0.5% sodium nitrate and 10 ml heparin sulfate, followed by cold formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The tissues were postfixed in the same fixative for 1 h and stored in 30% sucrose overnight. Free floating sections (40 μm for adrenal and 60 μm for spleen and duodenum) were obtained on a freezing microtome and placed in vials containing 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 50 mM DTT. Tissues were prehydrated in 50% formamide, 10% dextran, 2× SSC, 1× Denhardt’s solution, 10 mM DTT, and 0.5 mg/ml sonicated and denatured salmon sperm DNA. Denatured [32P]dATP-labeled cDNA IL-18 probe was added to the vial (107 cpm/ml/vial) (9), and hybridization was conducted overnight at 48°C. The sections were washed in serial dilutions of SSC at 48°C starting with 2× SSC and ending with 0.1× SSC. After a 15-min wash in 0.05 M phosphate buffer, sections were mounted and dehydrated. For determination of optimal developing time, slides were exposed to Kodak BioMax MS films (Kodak, Rochester, NY). To obtain cellular resolution, sections were fixed in Kodak NTB-2 emulsion and exposed at 4°C for 2 wk. After developing in Kodak D-19 at 16°C, sections were fixed in Kodak fixer, counterstained with cresyl violet, dehydrated, and coverslipped.

Northern blot analysis

Total RNA was extracted using RNeasy kit (Qiagen, Santa Clarita, CA) following manufacturer’s procedure. Northern blot analysis was performed as previously described (20). Briefly, 20 μg total RNA were electrophoresed in a 1% agarose gel, transferred to Hybond-N membrane (Amer sham, Arlington Heights, IL), and hybridized with [32P]dCTP-labeled 18-nucleotide cDNA IL-18 probe. The same filter was washed and reprobed with cyclophilin probe, which was used as an internal standard. Signals were quantified by densitometry.

RT-PCR and sequence analysis

Total RNA was extracted by RNeasy Mini kit (Qiagen) from adrenal gland, spleen, and duodenum following manufacturer’s instructions. Two micrograms of total RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of oligo(dT)15. The cDNA obtained was amplified with specific 3′ primer (5′-ATG GCT GCC ATG TCA GAA GA-3′) and specific 5′ primer (5′-TTG TTA AGC AGT TTA AAT ATC ATG CCG CCT GAG CC-3′) for rat IL-18. The amplification was performed in 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The last extension step at 72°C was prolonged for 5 min. The amplified cDNA was sequenced after subcloning into pcT-TOPO vector (Invitrogen, Carlsbad, CA). DNA sequencing was performed using a Sequenase Version 2.0 DNA Sequencing Kit (Amersham).

Western blot analysis

Goat anti-rat IL-18 polyclonal Ab was purchased from Research Diagnostics (Flanders, NJ). Protein extracts were obtained by lysing cells in RIPA buffer (1% Nonident P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 30 ml/ml aprotinin, and 1 mM sodium orthovanadate). The lysate was centrifuged and the supernatant was used for electrophoresis or stored at −80°C for further analysis. Samples were electrophoresed on a 12% SDS-PAGE denaturing gel using a mini protein apparatus (Bio-Rad Laboratories, Hercules, CA) and electrotransferred on Hybond ECL (Amersham) nitrocellulose membrane. The membrane was blocked by incubation in TBS containing 5% nonfat dry milk and 0.1% Tween 20. After a 3-h incubation with primary Ab, the filter was washed for 5 min three times in TBS/0.1% Tween 20 before addition of the specific HRP-conjugated secondary Ab. The filter was incubated for 1 h and washed three times in TBS/0.1% Tween 20. Binding was detected by chemiluminescence using the ECL plus system, and the signal was revealed by autoradiography on Kodak BioMax MS films.

Primer extension study

Primer extension study was conducted using the Primer Extension System Kit (Promega, Madison, WI) on 20 μg of rat adrenal gland, spleen, and duodenum total RNA using end-labeled IL-18-specific primer (5′-GCC TTC TTT TCGT CAT CGG GCC AGC-3′), complementary to exon 3. The reverse-transcriptase product was electrophoresed on a denaturing polyacrylamide gel and exposed to a phosphor image analyzer film (FujiFilm, Stamford, CT) for 24 h.

Rapid amplification of 5′-cDNA ends (5′-RACE)

The 5′ end of the rat IL-18 cDNA was cloned by the 5′-RACE method (21) using the SMARTII RACE kit (Clontech Laboratories, Palo Alto, CA). The 5′-RACE procedure was conducted according to the manufacturer’s instructions. Briefly, an oligonucleotide, 5′-cDNA synthesis primer (5′-AAG CAG TGG TAA CAA CGG AGG AGG TAC-3′), was used as a primer to synthesize first-strand cDNA from 1 μg of poly(A) from adrenal gland, spleen, and duodenum in the presence of SuperScript II reverse transcriptase (Life Technologies, Grand Island, NY). SMART II oligo was attached to the resulting cDNA by SuperScript II reverse transcriptase. The 5′-region of cDNA was amplified by PCR using a rat IL-18 gene-specific primer (5′-GTA GTG TTA AGC AGT TTA AAT ATC ATG CCG CCT GAG CC-3′) and universal primer (5′-CTA ATG CTA CGG CTC ACT ATG CTA GGG CGA GTG TTA ACA ACG CAG AGT-3′). The PCR conditions of the universal primer were as follows: 32 cycles of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C. PCR products were isolated from the gel and purified using a gel extraction kit (Qiagen). The PCR products were subcloned into pcT-TOPO vector (Invitrogen) and subjected to DNA sequence analysis.

Statistics

Results are expressed as the mean ± SE of the density relative to the control value. Differences in density of detected IL-18 were evaluated using the Student t test, with a p of <0.05 considered to indicate a statistically significant difference.

Results

Tissue localization and distribution of IL-18 mRNA

The localization of IL-18 mRNA in adrenal gland, spleen, and duodenum was investigated in situ hybridization analysis (Fig. 1). In adrenal gland, as previously shown (9), the expression of IL-18 mRNA was evenly distributed in the zona reticularis and the zona fasciculata. Signal did not occur in either the zona glomerulosa or the adrenal medulla (Fig. 1). In spleen, IL-18 mRNA was localized in the red pulp and was absent in the white pulp (Fig. 1). In duodenum, IL-18 mRNA was localized to the crypt epithelial cells and was absent in the villus epithelial cells (Fig. 1). The localization of IL-18 mRNA in the duodenum was consistent with the expression of IL-18 mRNA in the rat intestine (22). In the adrenal gland, IL-18 mRNA was localized to the zona reticularis and the zona fasciculata. Signal did not occur in either the zona glomerulosa or the adrenal medulla. The localization of IL-18 mRNA in the adrenal gland was consistent with the expression of IL-18 mRNA in the rat adrenal gland (23).

FIGURE 1. Dark field photoemulsion autoradiogram of in situ hybridization of IL-18 mRNA signal in the adrenal gland of ACTH-treated animal (left) and bright field cresyl violet staining of the same section showing the histology of the adrenal gland. IL-18 signals appear as white grains in dark field and is uniformly distributed in the zona reticularis (zr) and the zona fasciculata (zf) of the adrenal cortex. No signal was present in the zona glomerulosa (zag) or the adrenal medulla. Asterisk indicates the localization of the same blood vessels in the two photomicrographies. Bar is 150 μm.
localized in immune cells scattered throughout the organ with the exception of the follicles (Fig. 2A). In duodenum, IL-18 mRNA was predominantly expressed in cells of the gut-associated lymphoid tissue of the lamina propria. Lower levels of IL-18 mRNA also occurred in intestinal epithelium (Fig. 2B).

The scattered pattern of IL-18 localization exhibited by immune cells in spleen and in the lamina propria of duodenum was also found in stomach (data not shown), where it was possible to analyze a representative IL-18-positive cell at high magnification (Fig. 2, C–F). Based on nuclear morphology, this cell was identified as a granulocyte. These results indicate that the adrenal gland is a source of IL-18 distinct from immune cells.

Molecular size of IL-18 mRNA and protein

Northern blot analysis of IL-18 mRNA performed on total RNA extracted from the adrenal gland, spleen, and duodenum of healthy rats showed one single band in all tissues. The data also showed that the size of adrenal gland IL-18 mRNA is 1.1 kb, in contrast to spleen and duodenum, which synthesize a 0.9-kb IL-18 mRNA (Fig. 3A).

To investigate whether the difference in the length of the mRNA reflects the production of different tissue-specific peptides, RT-PCR, sequencing, and Western blot analysis were performed. The primers used for the RT-PCR cover the entire coding region of the known rat IL-18 sequence from the start codon to the 3′-untranslated region. The results showed that the IL-18 mRNA synthesized in the adrenal gland, spleen, and duodenum contains coding region of the same size (Fig. 3B). Sequencing analysis of the PCR product confirmed that the same 582-base composition encodes for the same 194-aa peptide (data not shown). This conclusion was also demonstrated by Western blot analysis showing that all tissues produced immunoreactive peptides with the same molecular size of 22 kDa corresponding to the reported precursor IL-18 protein (22) (Fig. 3C).

Primer extension and RACE analysis of IL-18 cDNA from different tissues

The analysis of the IL-18 mRNAs was extended to the 5′ region upstream of the start codon by primer extension and RACE, and to the 3′-untranslated region by RACE. Primer extension confirmed that the adrenal cortex synthesizes IL-18 mRNA containing 5′-untranslated regions longer than those observed in spleen and duodenum, which share the same transcription start sites (Fig. 4). The adrenal gland showed one major transcription start site at position −264 and at least five more upstream start sites (−303, −337, −388, −407, and −449). In contrast, spleen and duodenum showed a major transcription start site at position −149 and four additional common upstream start sites (−158, −170, −179, and −196). The duodenum also showed a start site at position −176, and the spleen also has a possible transcription start site at position −264. The pattern of primer extension revealed that adrenal gland and immune cells synthesize IL-18 mRNAs from different transcription start sites. Furthermore, RACE analysis was performed on six adrenal gland, four spleen, and two duodenal clones. The longest isolated clones do not represent the longest transcripts seen
in primer extension analysis, and the complete sequence of rat IL-18 gene is not known. Yet sequence alignment with the mouse IL-18 gene and 5'-cDNA ends revealed a high degree of homology (>88%) (Fig. 5). Specifically, the 5'-untranslated region of rat spleen and duodenal cDNA is homologous to mouse exon 2, while that of adrenal gland is homologous to exon 2, exon 1, and beyond.

The 5' end of sequenced duodenal cDNA corresponds to the junction between exons 1 and 2 of mouse, and the 5' end cloned from spleen is only 17 bp shorter. Primer extension also indicated that the longest spleen and duodenal transcript start sites are near the boundary between exons 1 and 2.

The 3' regions of cDNAs from all tissues were exactly identical and the same as the previously published sequence (data not shown) (9).

**Tissue-specific expression of IL-18 mRNA in response to ACTH**

Because immune cells were reported to have functional ACTH receptors (15, 16), we tested the hypothesis that ACTH injection also increased the levels of IL-18 in spleen and duodenum. The treatment elevated the levels of plasma ACTH (mean control, 136 ± 48 pg/ml; mean ACTH, 980 ± 88 pg; p < 0.01) and of plasma corticosterone (mean control, 124 ± 35 ng/ml; mean ACTH, 491 ± 127 ng/ml; p < 0.01). As previously shown (10), ACTH treatment increased the levels of IL-18 mRNA in adrenal gland (3-fold), but had no significant effect on the levels of the transcript in spleen and duodenum (Fig. 6). Compared with the controls, ACTH treatment did not have any effect on the molecular size of IL-18 mRNA in any of the tissues.

**Discussion**

The present results showed that 1) the adrenal gland and immune cells are distinct sources of IL-18; 2) tissue-specific expression of IL-18 gene may be achieved by differential usage of promoter region; and 3) the adrenal gland, and not immune cells, may be the source of IL-18 during stress.

The hypothesis that the CNS may influence immune function is supported by findings that the brain, the endocrine, and the immune systems form integrated network (23–25). However, the effects of the interaction of these systems on immunity remain unclear and often controversial. For instance, although stress has long

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**FIGURE 4.** Transcription start sites (tss) of rat IL-18 mRNA. Primer extension study was conducted to determine the tss using total RNAs from adrenal gland (A), spleen (B), and duodenum (C). The numbers beside each bar indicate the relative positions of the tss from the translation start codon (ATG).

**FIGURE 5.** *Top,* Organization of mouse IL-18 promoter region; P1, promoter 1; P2, promoter 2. *Bottom,* Sequence of IL-18 mRNAs 5'-untranslated region. Sequence alignment of the 5'-untranslated regions of IL-18 cDNAs from adrenal gland (AG), spleen (SPL), and duodenum (DDM) obtained by RACE analysis and that of mouse. Regions of homology are indicated by double dots (). The boundaries of exons 1, 2, and 3 of mouse IL-18 cDNA are indicated. Numbering is progressive from the translation start codon (ATG) to the 5' end.

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been regarded as being immunosuppressive, it has also been reported to have immunoenhancing effects (26). Although experimental models indicated that acute stress may be immunoenhancing and chronic stress may be immunosuppressive, the elements and mechanisms involved have not been clearly understood.

In previous studies, we demonstrated that the immunostimulatory and proinflammatory cytokine IL-18 can be synthesized in the zona reticularis and the zona fasciculata of the rat adrenal cortex and that its synthesis may be stimulated by acute stress or ACTH treatment (9, 10). Hence, we hypothesized that this cytokine may play a role in maintaining the homeostasis of the immune system during stress, possibly together with GC, also induced in the zona reticularis and zona fasciculata by ACTH (10).

Because IL-18 is not exclusively produced in the adrenal gland, the question arose of whether stress also stimulated its production in other tissues (12–14). In situ hybridization demonstrated that the source of IL-18 mRNA in the adrenal gland is specific to the cells of adrenal cortex and differs from that in the spleen and intestine, where IL-18 is primarily produced by immune cells. These observations are in agreement with our previous studies and with reports describing the localization of IL-18 in immune cells in the gut-associated lymphoid tissue as well as epithelial cells in intestine (7, 27, 28).

The histological evidence that the cellular source of IL-18 in adrenal gland differs from that in spleen and intestine was also demonstrated by the finding that these tissues synthesize IL-18 mRNAs of different size. The hypothesis that the difference observed could reflect the production of two distinct isoforms of IL-18, possibly IL-18α and the previously described IL-18β, was excluded by sequencing analysis of the open reading frame and by Western blot, revealing that all three tissues synthesize the same peptide.

Primer extension, RACE analysis, and sequencing demonstrated instead that the transcripts differ in the length of their 5'-untranslated region and share complete sequence identity of the 3' and of the common 5'-untranslated region. Primer extension clearly showed that the adrenal gland and immune cells in spleen and intestine synthesize IL-18 mRNA using different unique transcription start sites. The adrenal gland showed multiple transcription start sites that were also evident in the Northern blot analysis as a broad band (1–1.2 kb) most likely constituting heterogeneous mRNA molecules unique to adrenal gland. In contrast, the spleen and intestine have a relatively narrow range of similar primer extension patterns and a narrow major band (0.9 kb) on Northern blot. The similarity of the primer extension patterns in spleen and duodenum is a further confirmation that in these two tissues IL-18 is produced by immune cells. However, small differences are present. For instance, in spleen primer extension study showed a faint band at −264 was also present in adrenal gland. Because Northern blot study does not suggest the generation of significant amount of spleen IL-18 mRNA from this start site in either saline- or ACTH-treated animals, the amount of transcript produced from this start site may not be significant. Another difference is a band at position −176 unique to duodenum, possibly derived from epithelial cells.

Sequence alignment of RACE clones demonstrated a high degree of homology between the rat 5'-untranslated region and that of mouse IL-18 comprising the murine untranslated exons 1 and 2. Although the sequence and the structure of rat IL-18 gene are not known, our data strongly suggest that its organization is similar to that of the mouse gene, whose transcription is modulated by two promoters, 1 and 2, located upstream to the untranslated exons 1 and 2, respectively. Regulation through these two promoters is controversial. For instance, in mouse cell lines, one study finds that promoter 2 drives constitutive transcription of the gene and promoter 1 is inducible. By using the same in vitro model, another group reported instead that both promoters are active in constitutive as well as in inducible expression (17, 18). In contrast, the present study never detected the presence of both mRNA forms in the same rat tissues in vivo. Although the experimental conditions used did not stimulate the production of IL-18 mRNA in immune cells, in the adrenal gland ACTH induced an elevation of IL-18 mRNA levels, but not in its size (10). Thus, while the adrenal gland uses the promoter region 1 for both basal and inducible activity, it seems to lack the ability to use the promoter region 2. This suggests the possibility that rat IL-18 gene is regulated by tissue-specific usage of promoter region 2 in immune cells and by promoter region 1 in adrenal cortex. Another interesting possibility is that the adrenal cortex does not synthesize IL-18 if not stimulated by ACTH, producing therefore solely inducible IL-18. Circadian fluctuation of endogenous ACTH levels, together with the supposed long t1/2 of IL-18 mRNA (17), might account for the levels of transcript detected in the control animal.

Leukocytes were reported to bear ACTH receptors that, like in the adrenal cortex, stimulate the intracellular synthesis of CAMP upon binding to the ligand (15). However, in contrast to the adrenal gland, ACTH treatment did not elevate IL-18 mRNA levels in spleen and duodenum. Because the presence of ACTH receptor specifically in the IL-18-positive cells in spleen and duodenum has not been reported, lack of induction could be due to the absence of ACTH receptors on these cells, of its function, or to a different transduction or transcription machinery. The amount of ACTH used in our experiment was chosen to be effective in inducing GC and proved to induce levels of plasma ACTH slightly higher than those observed under stress in physiological conditions. Yet, it is possible that higher dose of ACTH may actually stimulate IL-18 production in immune cells. The data suggest the HPA axis may stimulate the production of IL-18 in the adrenal cortex, not in immune cells.
The restricted response to ACTH and the peculiar usage of IL-18 promoter in the adrenal cortex indicate the existence of specific pathways involved in the modulation of IL-18 gene expression during stress. This may ultimately be an example of the molecular mechanisms underlying the brain-to-body communication. Because stress is known to influence the susceptibility to or the progression of illnesses such as cancer, infections, and autoimmune diseases, the modulation of IL-18 production in the adrenal gland might prove to have clinical relevance in these conditions.

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