Denervation of rat adrenal glands markedly increases preproenkephalin mRNA
(opioid peptides/regulation of gene expression)

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ABSTRACT The effect of denervation on the expression of rat adrenal preproenkephalin has been examined. Following splanchnicectomy there was a several-fold increase in the steady-state levels of preproenkephalin mRNA, which became maximal after 24–48 hr (>10-fold). These results indicate that the previously observed increase in rat adrenal enkephalin-containing peptides following denervation occurs entirely by a pretranslational mechanism. The increase in preproenkephalin mRNA was accompanied by a 50–75% decrease in rat adrenal poly(A)+ RNA. Neural input thus exerts a profound trophic influence on preproenkephalin gene expression and RNA metabolism in rat adrenals.

The adrenal medulla has been used extensively in studies of the neural regulation of catecholamine biosynthesis (1). However, the mechanisms regulating preproenkephalin biosynthesis in the adrenal medulla are less well characterized. Previous studies have demonstrated that denervation of the rat adrenal gland leads to a marked increase in the number of enkephalin-positive chromaffin cells (2) and the glandular content of enkephalin-containing (EC) peptides (3, 4). The time course for the increase in EC peptides as well as their molecular weight distribution suggested that denervation induces an increase in rat adrenal preproenkephalin biosynthesis. Intact preproenkephalin has in fact been identified as the predominant EC peptide that accumulates in rat adrenals following denervation (5). However, in the absence of turnover studies one cannot entirely rule out other explanations for this increase such as a selective decrease in the rate of opioid peptide secretion or degradation. In the present study we demonstrate that following denervation there is a >10-fold increase in the preproenkephalin mRNA content of rat adrenals, thus establishing a pretranslational mechanism for this response. The significance of these findings is discussed with respect to the neural regulation of adrenal preproenkephalin gene expression in the rat.

MATERIALS AND METHODS

Adrenal glands were removed from male Sprague–Dawley rats (150–200 g) by Taconic Farms (Germantown, NY) at various time points after unilateral (left-side) splanchnicectomy and delivered to us on dry ice.

Intact and denervated glands at each time point were pooled separately and poly(A)+ RNA was isolated by using the guanidinium thiocyanate/CsCl gradient procedure (6) followed by oligo(dT) column chromatography (7). Recovery of 32P-labeled globin mRNA under these conditions was >75%. RNA transfer analysis of poly(A)+ RNA fractions was carried out according to Thomas (8) by using Baby Blots (Bethesda Research Laboratories). Preproenkephalin mRNA was quantified by RNA blotting (9) using a Minifold apparatus (Schleicher & Schuell). Rat brain poly(A)+ RNA was used as a standard. Quantitation of autoradiograms (Kodak XAR-5 film) was carried out by using a soft laser scanning densitometer (LKB) and a Minigrator (Spectra Physics, Piscataway, NJ). Densities were converted to picograms of hybridized preproenkephalin cDNA by scintillation spectrosopy of rat brain poly(A)+ RNA dots.

Two different 32P-labeled preproenkephalin DNA probes were used in these experiments. A synthetic 30-nucleotide-long oligodeoxyribonucleotide that is complementary to a portion of both the human and bovine preproenkephalin mRNA sequences (10) was labeled at its 5′ end by using polynucleotide kinase (P-L Biochemicals) and [γ-32P]ATP (Amersham; >5000 Ci/mmol; 1 Ci = 37 GBq). Human preproenkephalin cDNA (918 base pairs) was isolated from the plasmid pHPE-9 (a gift from M. Comb and E. Herbert) (11) by digestion with HincII, EcoRI, and EcoRV (New England Biolabs) and purified on agarose gels followed by centrifugation on a sucrose gradient. The cDNA was labeled by nick-translation by using [α-32P]dCTP (Amersham; 3000 Ci/mmol) to a specific activity of 1–4 × 106 cpm/μg.

Total RNA and poly(A)+ RNA were measured by UV absorption (1 A260/ml = 40 μg). DNA was measured in guanidinium thiocyanate extracts by using the dianimobenozoic acid fluorescence assay (12).

RESULTS

To determine whether the changes in rat adrenal EC peptides following splanchnic nerve sectioning reflected an increase in preproenkephalin mRNA, rat poly(A)+ RNA fractions from rats that had been unilaterally denervated for 24 hr were submitted to RNA transfer analysis (Fig. 1). By using the synthetic 32P-labeled oligodeoxyribonucleotide probe, no preproenkephalin mRNA was detectable in the poly(A)+ RNA fraction from innervated adrenal glands. This is undoubtedly due to the relatively low abundance of preproenkephalin mRNA normally present in rat adrenal glands as well as to the use of a nonhomologous DNA probe. However, in poly(A)+ RNA fractions from denervated rat adrenals preproenkephalin mRNA was markedly increased and therefore readily detectable (Fig. 1).

The time course for the denervation-induced increase in preproenkephalin mRNA was examined by using an RNA blotting procedure. As early as 12 hr after denervation an increase in preproenkephalin mRNA was already evident (Fig. 2). Preproenkephalin mRNA continued to increase with time, reaching maximal values 24–48 hr after denervation and declining thereafter. A comparison of the effects of denervation on the gene product preproenkephalin and its corresponding mRNA are shown in Table 1. As can be seen, the appearance of the specific mRNA preceded that of the gene product, preproenkephalin, and started to decline while preproenkephalin was still increasing.

Abbreviation: EC, enkephalin-containing.

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The nonhomologous (human) cDNA probe used in these experiments did not permit accurate measurement of the small amounts of preproenkephalin mRNA in innervated rat adrenals. The observed values for innervated glands (Table I) thus may only represent upper limits. However, the increase in preproenkephalin mRNA on denervation was so great that experimental variations in the control innervated glands are not a factor. It is worth noting that the content of preproenkephalin mRNA expressed per individual adrenal gland as well as per microgram of rat adrenal poly(A)+ RNA increased following splanchnicectomy (Table I). The effect of denervation on preproenkephalin mRNA is therefore selective and not due to a general increase in the population of adrenal poly(A)+ RNA. In fact, the amounts of total adrenal RNA as well as poly(A)+ RNA actually decreased by as much as 75% after denervation (Table I). The decreases in RNA content followed a time course essentially identical to that seen for the increase in preproenkephalin mRNA. The extent to which this reflects changes in medullary versus cortical RNA content has not been determined. However, the DNA content of rat adrenal glands remained unchanged by denervation (data not shown). The observed decreases in RNA content therefore represent changes in RNA metabolism and are not indicative of cell death.

**DISCUSSION**

The present studies clearly demonstrate that splanchnic nerve sectioning causes a marked increase in the steady-state levels of rat adrenal preproenkephalin mRNA. The magnitude of this increase (>10-fold) is comparable to that observed for adrenal proenkephalin, indicating that the effects of denervation result from a pretranslational mechanism. Whether this involves an increase in the rate of transcription of the preproenkephalin gene or is the result of changes in one or more posttranscriptional events (e.g., mRNA stabilization or processing) remains to be determined.

### Table 1. Effect of denervation on rat adrenal nucleic acids and EC peptides

<table>
<thead>
<tr>
<th>Time, hr</th>
<th>Total RNA, µg</th>
<th>Poly(A)+ RNA, µg</th>
<th>Proenkephalin mRNA, pg</th>
<th>EC peptides, pmol/mg of protein</th>
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<tr>
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<td>Innervation 56</td>
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<td></td>
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<td>5</td>
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<tr>
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<td>0.3</td>
<td>5</td>
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<td>Denervation 46</td>
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</table>

Data are not corrected for recovery. RNA values are expressed per adrenal gland.

*Values refer to picograms of nick-translated human proenkephalin cDNA hybridized per adrenal gland. One microgram of rat brain poly (A)+ RNA standard bound 1.8 pg of cDNA.

**FIG. 2.** Time course for the effect of unilateral denervation on rat adrenal preproenkephalin mRNA. Poly(A)+ RNA (12 µg) from innervated (I) and denervated (D) rat adrenal glands, rat liver (RL), and rat brain (RB) was denatured by heating to 60°C for 15 min in the presence of 7.4% formaldehyde/0.9 M NaCl/0.09 M sodium citrate (denaturing buffer). Three-fold serial dilutions of each sample were made up in denaturing buffer and applied in a volume of 0.5 ml to nitrocellulose (8.0, 2.7, and 0.9 µg of adrenal samples and 8.0-0.1 µg of liver and brain samples, from left to right). Wells were rinsed with 0.5 ml of denaturing buffer, and the filter was then baked, prehybridized, and hybridized with nick-translated human proenkephalin cDNA (10⁷ cpm) at 42°C as outlined in the legend to Fig. 1. Numbers refer to the hours following denervation after which adrenal glands were removed. This experiment was repeated four times.

**FIG. 1.** Effect of splanchnicectomy on rat adrenal preproenkephalin mRNA. Innervated and denervated adrenal glands (0.3-0.4 g) were obtained from rats that had been unilaterally denervated for 24 hr. Poly(A)+ RNA was prepared and the entire sample from each group of glands [30 µg from innervated adrenals (I) and 6 µg from denervated adrenals (D)] was subjected to electrophoresis on 1% agarose gels containing 10 mM methylmercury hydroxide. Six micrograms of rat brain poly(A)+ RNA (B) was included as a standard. After transfer onto nitrocellulose, the filters were baked and then prehybridized for 4-12 hr at 30°C in 0.7 M NaCl/0.07 M sodium citrate/0.05 M sodium phosphate, pH 7.0/0.02% bovine serum albumin/0.02% Ficol/0.02% polyvinylpyrrolidone/0.1 mg of salmon sperm DNA per ml/50% formamide. Hybridization with 32P-labeled synthetic oligodeoxyribonucleotide (2 x 10⁶ cpm) was carried out at 30°C for 12-18 hr in prehybridization buffer containing 10% dextran sulfate. The filter was washed four times in 0.3 M NaCl/0.03 M sodium citrate/0.1% NaDodSO₄ at room temperature for 10 min each, followed by four washes at 50°C in 15 mM NaCl/1.5 mM sodium citrate/0.1% NaDodSO₄ for 30 min. An autoradiogram was exposed after an overnight incubation at -70°C. Similar results were obtained in five different experiments.
Another unanswered question concerns the cellular mechanisms that are responsible for this response. One possibility is that denervation causes an induction of preproenkephalin gene expression by stimulating the release of acetylcholine from splanchnic nerve endings. Tyrosine hydroxylase has been shown to undergo trans-synaptic induction in response to increased splanchnic nerve activity (13, 14). However, the fact that catecholamines do not increase following denervation of rat adrenal glands (15) suggests that a trans-synaptic mechanism does not explain our findings. An alternative explanation is that splanchnic innervation of the rat adrenal medulla exerts an inhibitory trophic influence on preproenkephalin gene expression and that denervation leads to derepression. The expression of the skeletal muscle proteins apolipoprotein A1 (16) and tropomyosin and troponin (17) also appears to be under innervation-dependent repression that is reversible by denervation. Analogous mechanisms may operate in rat adrenal chromaffin cells to regulate preproenkephalin gene expression. Based on such a model, the transient nature of the increase in preproenkephalin mRNA following denervation is somewhat unexpected. However, this could conceivably represent tissue reinnervation in these experiments. Innervation-dependent repression may also explain the unusually small amounts of EC peptides in the rat adrenal gland as compared to other species. The factor(s) responsible for this putative repression remain to be determined. Recent studies (18) suggest that acetylcholine may play such a role in the rat adrenal gland but other repressive trophic factors must still be considered. The large decreases in RNA content following splanchnic nerve sectioning indicate that neural input also has a dramatic effect on rat adrenal RNA metabolism. Similar decreases in both total and poly(A)+ RNA populations have been observed in skeletal muscle after denervation (15). The effects of denervation on adrenal EC peptides and RNA metabolism in other species and on other innervated rat tissues that express proenkephalin must be determined.

We have recently succeeded in cloning rat preproenkephalin cDNA, which is a much more sensitive probe for rat preproenkephalin mRNA than the nonhomologous cDNAs used here (10). This should further improve our ability to study the regulation of rat preproenkephalin gene expression.