Endogenous Ouabain
Upregulation of Steroidogenic Genes in Hypertensive Hypothalamus but Not Adrenal

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**Background**—Mammalian tissues contain a presumed endogenous Na\(^+\), K\(^+\)-Na\(^+\)-ATPase inhibitor that binds reversibly to the Na\(^+\) pump with high affinity and specificity. The inhibitor has been linked to the pathogenesis of experimental volume-expanded and human essential hypertension. This compound has been isolated from mammalian hypothalamus and appears to be an isomer of the plant-derived cardiac glycoside ouabain, if not ouabain itself. The objective of this study was to test the hypothesis that a biosynthetic pathway exists in mammalian tissues to produce a steroid derivative closely related to plant cardiac glycosides.

**Methods and Results**—Using bioinformatics and genomic techniques, Milan hypertensive rat tissues were studied because this strain has a 10-fold increase in hypothalamic ouabain-like compound that is linked to the pathogenesis of the hypertension. A putative steroid biosynthetic pathway was constructed and candidate genes encoding enzymes in this pathway were identified from sequence databases. Differential expression of selected genes in the pathway was studied by microarray analysis and quantitative polymerase chain reaction, with functional validation by gene silencing using small interfering RNAs. Marked upregulation of genes coding for P450 side chain cleavage and \(\Delta^5\)-3\(\beta\)-hydroxysteroid dehydrogenase/\(\Delta^5\)-4-isomerase enzymes in hypertensive hypothalamus but not adrenal was found, compared with normotensive Milan rats. Knockdown of the latter gene decreased production of ouabain-like factor from neural tissue.

**Conclusions**—Our findings support the possibility that a unique steroid biosynthetic circuit exists in Milan rat brain, functioning independently from adrenal, which could account for the overproduction of the hypothalamic ouabain-like compound in this species. *(Circulation. 2005;112:1301-1308.)*

**Key Words:** brain • genes • hormones • hypertension • inhibitors

Because the cardiac glycoside binding site of the Na\(^+\), K\(^+\)-Na\(^+\)-ATPase has been evolutionarily conserved, and because both cardenolide-like (ouabain, digoxin) and bufodienolide-like (marinobufagenin) compounds have been isolated from mammalian tissue sources, the notion arose that mammalian analogs to the cardiac glycosides might exist. Over the past 2 decades, multiple investigators have reported extraction of Na\(^+\) pump-inhibitory activity from body fluid and tissue sources, and this inhibitor has been linked to the pathogenesis of experimental volume-expanded and human essential hypertension.\(^1,2\) Structural analysis by our group indicated that one ouabain-like compound (OLC), isolated from bovine hypothalamus (hypothalamic inhibitory factor, HIF), appears to be ouabain itself or a closely related isomer.\(^3,4\)

Some controversy has surrounded the origin of OLC: endogenous versus acquired from the environment. Thus, the field has progressed slowly in part because no synthetic pathway has been described. Basic work indicates that mammalian (rat and mouse) adrenal tissue can synthesize digitalis-like bioactivity by using mammalian steroid pathway precursors,\(^5-7\) and both physiological and pharmacological stimuli have been shown to influence the release or synthesis of OLC from forebrain (hypothalamus) and adrenal tissues.\(^8-10\) Recently, Komiyama and coworkers\(^11\) reported that an adrenal medullary-derived cell line (PC12) releases an OLC into the culture supernatant and baseline released amounts increase in a dose-dependent manner as a function of adding progesterone to the culture media. PC12 cells are a well-characterized, single-cell clonal line, derived from a transplantable rat adrenal pheochromocytoma.\(^12\) They are frequently used as a model in studies of neural cells and secretion. The OLC released was shown by liquid chromatography–mass spectrometry to have a molecular mass identical to ouabain. Because nuclear magnetic resonance analysis was not done, the authors could not say whether the stereochemistry of this OLC

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is that of ouabain or an isomer. It is known that certain neurosteroids can be synthesized in brain tissue either de novo from cholesterol or from other steroid precursors, and their presence in the nervous system has been shown to be independent of adrenal gland production.13 However, a pathway for mammalian synthesis of cardiac glycoside analogues is, as yet, incomplete.

Because hypothalamus appears to be an enriched source for the hypothalamic OLC (namely HIF), we addressed the issue of endogenous biosynthesis in the hypothalamus by using molecular and genomic approaches. Tissues from Milan hypertensive strain rat hypothalamus contain 7 to 10 times more extractable OLC than the Milan normotensive strain (MNS) control rats,15 and a primary role for OLC is proposed in the pathogenesis of renal tubular Na⁺ transport abnormalities and the development of hypertension in this strain.16 Importantly, tissue contents of OLC in Milan rats were previously shown to be unaffected by rat chows containing varying amounts of extractable Na⁺ transport inhibitors.17

We report a bioinformatics analysis to deduce an OLC biosynthetic pathway. Several of the relevant human genes for crucial steps in the pathway could be identified, and these candidate genes along with their identified rat orthologues are identified by the number key, common names, gene symbol, and Locus Link numbers (Figure 1).

Putative Biosynthetic Pathway for Ouabain-Like Compound in Rat Brain

Having established from the literature that (1) mammalian adrenal cells synthesize a compound with structural and bioactivity characteristics of an OLC, (2) digitalis plants can produce cardiac glycosides by using mammalian steroid precursors, and (3) steroid synthetic pathways exist in brain tissue, we used a bioinformatics approach to develop a putative biosynthetic pathway (Figure 1). First, we examined the literature on steroid hormone and cardiac glycoside synthesis to come up with enzyme candidates. These could be broken down into 3 categories: (1) known human enzymes that are very likely to be involved in the pathway (eg, p450 side chain cleavage); (2) unknown human enzymes that should exist if OLC is built up in a step-wise fashion (eg, 11α-hydroxylase); there is a known 11β-hydroxylase, but no known 11α-hydroxylase; and (3) enzymes that could perform the necessary steps but are not known to exist in the animal kingdom. Protein and nucleotide sequences of known enzymes were obtained from NCBI. Known sequences were then subjected to BLAST analysis, looking for similar enzymes, perhaps with similar steroid binding domains (eg, BLAST 11β-hydroxylase to find 11α-hydroxylase). Both nucleotide and protein BLAST were used. We continued to run BLAST on results from initial BLAST: protein-protein (blastp), nucleotide-nucleotide (blastn). Blastx (translated nucleotide-protein) and tblastx (translated nucleotide-translated nucleotide) were also used.18 The Omega software program (Accelrys) was used to find regions of homology between known enzymes to determine the steroid binding domain. Conserved regions were then subjected to BLAST to generate more enzyme candidates. This approach allowed us to generate a list of candidate genes by using LocusLink.

Animals and Isolation of RNA

Adult MHS rats and their normotensive (MNS) controls were used. MHS and MNS rats were obtained from the internal stock colony (Prassis Sigma tau, Settimo Milanese, Italy). Rats were maintained under a controlled temperature of 22°C and relative humidity of 55±10%, with a 12-hour light/dark cycle. Rats were fed a standard diet (Altromin, Rieper) with free access to water. Systolic blood pressure (SBP) and heart rate were recorded weekly at the tail by plethysmography (BP recorder, U. Basile). SBP increases in MHS over MNS starting from 4 to 5 weeks of age. At 3 months, SBP is significantly higher in MHS (168±0.9 mm Hg; mean±SEM) compared with MNS (142±1.0 mm Hg). Seven MHS and 7 MNS rats were euthanized at 5 months (according to the animal care guidelines at Prassis Sigma tau, Settimo Milanese, Italy). Immediately after, the hypothalamus was removed by making vertical incisions at the optic chiasm and just anterior to the mammillary bodies to a depth of 2 mm, with removal of intervening tissue. Whole adrenal glands were dissected free of the kidney; no attempt was made to separate cortical and medullary portions. Tissues were weighed and frozen in liquid nitrogen. RNA was extracted by using Trizol reagent (Life Technologies) or RNeasy (Qiagen) according to the manufacturers’ recommendations, with representative gels showing clean bands of 18S and 28S RNA, characteristic of high-quality, undegraded RNA.

Methods

Microarray Analysis

Using CodeLink UniSet Rat expression bioarray technology (30 mer, single-dye technology, 9900 rat oligonucleotide gene probes [Amersham Biosciences]), biotin-labeled cRNA targets were prepared from hypothalamic and adrenal total RNA from age-matched male MHS and MNS rats, hybridized to the array and scanned following the manufacturer’s specifications (Motorola Document 080045 to 00 Rev. 1). Scans were performed on a Perkin-Elmer HT 5000, using ScanArray Express software (Perkin-Elmer). Images were analyzed with CodeLink Analysis version 2.1.17 (Amersham Biosciences). Uregulated candidate...
sequences were BLAST analyzed to confirm identity. Negative control genes (bacterial) provided by the manufacturer established the threshold to conclude actual expression of studied genes in the respective tissues.

Quantitative (Real-Time) Polymerase Chain Reaction Analysis

Real-time polymerase chain reaction (RT-PCR) was performed with the use of an Applied Biosystems Real-Time Quantitative PCR System 7700, according to the manufacturer’s protocols. Primers were designed with Primer Express (Applied Biosystems), following the manufacturer’s guidelines. Amplicons were approximately 100 bp in length and were amplified by using primers with no significant secondary stem loop or homodimer structures. In addition, all primer sequences were screened with BLAST against the rat genomic database to ensure that the primers designed were specific for the gene transcript of interest. RT-PCR primer pairs are (5’ to 3’): NM_017286 (CAGGA CCTGG GCTCA ACTAT G and AGAGA

Figure 1. Hypothetical biosynthetic pathway for conversion of cholesterol to endogenous cardiac glycosides. Relevant human genes coding for pathway enzymes are identified by Locus Link numbers and gene symbol. Rat orthologue genes in bold were found upregulated by rat microarray analysis. Rat orthologues italicized and underlined were not available on the CodeLink microarrays. Structures show plant ouabain and candidate precursor molecule.
CACCACCCCTAATGCT, NM_017265 (CAGAC TAGGAGCAGACAA AT and ATTAGGGAAGAAAGCTTGTG GACTAG). For each experimental sample, 2 μg of total RNA was reverse-transcribed with Multiscribe (Applied Biosystems) and random hexamers as primer. After quantification, 10 ng cDNA from each tissue sample was amplified by using primer pairs for the gene of interest plus an internal control reaction with primers for GAPDH or β-actin. The RT-PCR was carried out (95°C, 10 minutes; 68°C, 2 minutes; 72°C, 10 minutes; 34 cycles), and the data were processed with Sequence Detector (Applied Biosystems). RT-PCR products were checked for appropriate size by agarose gel electrophoresis.

**Statistical Analysis**

Relative gene expression was determined by subtraction of the GAPDH or β-actin cycle number to obtain ΔCt and calculating the power of the ΔΔCt. Therefore, HSD and SCC gene expression levels are relative to the control genes. Probability values were determined with the use of a paired Student t test.

**RNA Interference**

We designed the siRNAs for these experiments by using several well-described principles.19,20 The siRNA duplexes are 21 nucleotides in length, with a 2-nucleotide 3’ overhang and a GC content of 50% or less. The target sequences are located at least 50 nucleotides after the start codon. All sequences were checked by BLAST to ensure that our gene of interest was exclusively targeted. All siRNAs were purchased (Xeragon). The oligonucleotide sequence found to effectively silence were purchased (Xeragon). The oligonucleotide sequence found to effectively silence were purchased (Xeragon). The oligonucleotide sequence found to effectively silence were purchased (Xeragon). The oligonucleotide sequence found to effectively silence were purchased (Xeragon). The oligonucleotide sequence found to effectively silence were purchased (Xeragon). The oligonucleotide sequence found to effectively silence were purchased (Xeragon). The oligonucleotide sequence found to effectively silence were purchased (Xeragon).

**Results**

**Microarray Analysis: Side Chain Cleavage and Hydroxysteroid Dehydrogenase Isomerase Genes Are Differentially Expressed in Hypertensive Hypothalamus but Not Adrenal**

Working from the hypothetical biosynthetic pathway outlined in Figure 1, rat orthologues to the human candidate genes were identified. To test for the presence of target genes in our samples, we compared RNA from 2 hypertensive and 2 normotensive animals by using microarray analysis. Genes coding for 7 of the enzymes were present on Amersham Codelink rat gene chips. All these genes were expressed in hypothalamic and adrenal tissues. Genes for 5 pathway enzymes showed no differential expression, whereas genes coding for the P450 side chain cleavage (P450scc, gene NM_017286) and Δ5-3β-hydroxysteroid dehydrogenase isomerase (Δ5-3β-HSD, NM_017265) enzymes showed 3.8- and 5.2-fold increased expression, respectively, in the hypertensive as compared with normotensive rat hypothalamus (Table). These enzymes are proximal in the proposed biosynthetic pathway with P450scc catalyzing the conversion of cholesterol to pregnenolone, and Δ5-3β-HSD, pregnenolone to progesterone (Figure 1).

Because both hypothalamus and adrenal have been considered as sources for OLC, we analyzed adrenal RNA from the corresponding hypertensive and normotensive animals. The ratio of expression (MHS/MNS) of the P450scc and Δ5-3β-HSD genes was approximately 1.0 by the gene chip analysis, consistent with no differential expression (Table). Using quantitative PCR, we expanded the number of animals analyzed for differential expression of P450scc and Δ5-3β-
HSD, based on the microarray findings. Figure 2 shows data for hypothalamic mRNA levels from 6 normotensive and 6 hypertensive animals and adrenal mRNA levels from 7 normal and 7 hypertensive animals. The mean values for HSD in normal and hypertensive hypothalamic tissues were 0.0029 and 0.025, respectively, \( P < 0.005 \). The ratio of hypertensive/normal HSD gene expression is 8.8. The mean values for SCC in normal and hypertensive hypothalamic tissues were 0.0026 and 0.015, respectively, \( P < 0.05 \). The ratio of hypertensive/normal SCC gene expression is 5.7. Thus, HSD and SCC mRNA abundance was elevated in the hypothalamus of hypertensive rats (Figure 2A), confirming the microarray findings.

RNA from the corresponding normal and hypertensive adrenal tissues was also analyzed. HSD and SCC mRNA are present at similar levels in the normotensive and hypertensive adrenal tissues: Mean values for HSD in normal and hypertensive tissues were 0.0029 and 0.025, respectively, \( P < 0.005 \). The ratio of hypertensive/normal HSD gene expression is 8.8. The mean values for SCC in normal and hypertensive hypothalamic tissues were 0.0026 and 0.015, respectively, \( P < 0.05 \). The ratio of hypertensive/normal SCC gene expression is 5.7. Thus, HSD and SCC mRNA abundance was elevated in the hypothalamus of hypertensive rats (Figure 2A), confirming the microarray findings.

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RNA Interference in PC12 Cells

To use the PC12 cells in the RNA interference experiments, we needed to determine whether they expressed mRNA from the target genes of interest identified in Milan rat hypothalamus by microarray and PCR. The RNA was tested for the presence of HSD and SCC mRNA by quantitative PCR (see Methods) and was compared with Milan normotensive rat RNA. Both genes are expressed in PC12 cells (Figure 3). Furthermore, we observed a higher expression level for HSD mRNA in PC12 cells compared with MNS rat hypothalamus \( (P < 0.005) \).

**PC12 Cells Express HSD and SCC mRNA**

We chose to initiate the RNA interference studies (RNAi) focused on one gene, the HSD3B1 isoform of \( \Delta 5 \)-3\( \beta \)-HSD (NM_017265), because this gene was markedly overexpressed by RT-PCR analysis in MHS hypothalamus. We first confirmed the presence of both \( \Delta 5 \)-3\( \beta \)-HSD and P450scC genes in PC12 by our RT-PCR technique (Figure 3). We then designed 2 small interfering RNA (siRNA) duplexes. One
PC12 cells transfected with HSD110 have lower levels of Δ5-3β-hydroxysteroid dehydrogenase isomerase (NM_017265; HSD) mRNA than mock-transfected PC12 cells. RNA was collected 24 hours after transfection. RNA levels were measured by quantitative PCR, using primers specific for HSD and equal amounts of starting material. HSD siRNA transfected PC12 cells exhibit a change in OLC bioactivity that parallels the change in RNA expression. Complete inhibition of active Na""\textsuperscript{+}"" transport is caused by 1 mmol/L ouabain. Rb""\textsuperscript{+}"" uptake into the erythrocytes was 80% ouabain-sensitive. Results are mean ± SEM triplicate assay points for 6 RNAi experiments. *P<0.05 (RNA fold change), +P<0.0005 (inhibition of uptake) vs no siRNA.

**Discussion**

Using a bioinformatics approach, we generated a steroid biosynthetic pathway leading from cholesterol as precursor through intermediate hydroxylation steps consistent with generation of a steroid intermediate(s), which, after lactone ring addition and glycosylation, could result in generation of ouabain or its isomer (Figure 1). Rat orthologues of 7 potentially relevant human genes in the early steps for this pathway were identified and found to be available for microarray analysis on the CodeLink rat gene chips. All orthologue genes were expressed in both hypothalamic and adrenal tissues. Analysis of MHS and MNS RNA showed no differential expression of genes encoding 5 of the enzymes but revealed significant overexpression in hypertensive hypothalamus for genes coding for P450scc, 5α-reductase, and HSD110. HSD110 mRNA was more abundant in hypertensive hypothalamus relative to normotensive hypothalamus. Thus, individual variation within the hypertensive universe of candidate genes as an effective initial analytical technique.

We sought to confirm the microarray data by using quantitative (real-time) PCR analysis of the same RNA from hypertensive and normotensive tissues. Genes NM_017286 (P450scc) and NM_017265 (Δ5-3β-HSD) in MHS hypothalamus showed marked overexpression compared with MNS hypothalamus, confirming results of the microarray analysis (Figure 2). Overexpression in MHS is variable with some values overlapping with MNS hypothalamus. Individual blood pressure levels were measured in maturing animals (see Methods) but could not be correlated with individual hypothalamus OLC content (not measured). Thus, individual variation within the hypertensive
group could reflect differing degrees of blood pressure elevation among the hypertensive animals. Nevertheless, as a group, differential expression of genes governing the conversion of cholesterol to pregnenolone and pregnenolone to progesterone are confirmed by RT-PCR in hypertensive hypothalamus.

To establish the relevance of the transcript changes it is necessary to provide a functional validation that altering the abundance or function of the target gene(s) correlates with altered enzymatic activity or production of our final product, OLC. To address this crucial point, we used RNA interference to target the HSD3B1 gene in PC12 cells. We looked for evidence of production of Na\(^+\) transport inhibitory activity in these cells by purifying culture supernatants and testing purified product in bioassays and immunoassays for OLC, which are standard in our laboratory. Although it is possible that PC12 cells synthesize bioassays and immunoassays for OLC, which are standard in our

To our knowledge, there are no direct studies of specific gene expression related to the biosynthesis OLC in putative source tissues. The finding reported here of overexpression of p450scc- and β-HSD-associated genes is compatible with delivery of enhanced substrate intermediates to the corticosterone biosynthetic branch in MHS hypothalamus. This would support the observation that treatment of adrenal tissues with cyanoketone, which blocks Δ5-3β-HSD activity, was accompanied by decreased production of immunoreactive OLC by the cells as measured by radioimmunoassay. In addition, treatment of adrenal cells with aminoglutethimide (a specific inhibitor of p450scc activity) and cyanoketone resulted in no accumulating pregnenolone and less digitals-like compound activity.

The expression analysis reported here does not exclude the possibility that adrenal cortex is involved in OLC biosynthesis, though it suggests that adrenal enzyme levels are not altered in Milan hypertensive rats. MHS hypothalamic tissue shows a dramatically different pattern, with marked overexpression in hypertensive hypothalamus of 2 key genes in steroid biosynthesis. Modulation of one of these genes affects production of the OLC in neural tissue represented by PC12 cells. These findings support the possibility that a unique steroid biosynthetic circuit could exist in Milan rat hypothalamus, functioning independently from classic adrenal cortical pathways, to provide substrate for a branch leading to HIF production which would account for the increased extractable levels of this endogenous Na\(^+\), K\(^+\)-ATPase inhibitor now linked to the pathogenesis of hypertensive disease in this strain.

Finally, our findings for the HSD3B1 gene in rat tissues may be relevant to a recent report that polymorphisms in exon 4 of the human HSD3B1 gene are associated with elevated systolic and diastolic blood pressure in a cohort of essential hypertensive men.

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References

CLINICAL PERSPECTIVE

Since the discovery of endogenous analogues of opium, endorphins, and encephalins, it has been tempting to postulate that other plant alkaloids, for example, atropine and digitals, may mimic an intrinsic regulator of vertebrate physiology. Experimental observations suggested that there may be an endogenous analogue of digitalis—a potent inhibitor of membrane Na⁺, K⁺-ATPase ("sodium pump"). Thus, a steroid derivative was isolated from mammalian tissues with physiological characteristics remarkably parallel to cardiac glycosides, and structural analysis pointed to "endogenous digitalis" as an isomer of the plant glycoside ouabain. The endogenous OLC has been linked to the pathogenesis of human essential hypertension (salt-sensitive) and to the development of left ventricular hypertrophy. Little is known of OLC biosynthesis or how altered levels of pathway enzymes might affect OLC levels in humans. Using RNA from genetically essential hypertension (salt-sensitive) and to the development of left ventricular hypertrophy. Little is known of OLC biosynthesis or how altered levels of pathway enzymes might affect OLC levels in humans. Using RNA from genetically


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