Research Article

Evidence for Endogenous Neurosteroid Production in the Mammalian Olfactory Mucosa: Immunocytochemical Localization of Cytochrome P450 Side-Chain Cleavage Enzyme

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Abstract

Steroid hormones and their metabolizing enzymes have previously been identified in the olfactory mucosa. Enzymes of the cytochrome P450 family that are thought to play a role in the metabolism or activation of airborne toxins have also been identified in mammalian olfactory mucosae. In the synthesis of steroid hormone, cholesterol is transported to the mitochondria where the side-chain cleavage enzyme (cytochrome P450scc) converts cholesterol into pregnenolone. Conversion of cholesterol to pregnenolone is an obligate step in steroid hormone production. The specific aim of this study is to identify cytochrome P450scc in the mammalian olfactory mucosae. Using polyclonal antibodies to cytochrome P450scc, we found immunoreactivity for cytochrome P450scc in the rat olfactory mucosa. Within the olfactory epithelium, the supranuclear region of sustentacular cells was immunoreactive for cytochrome P450scc. Olfactory neurons, basal cells, olfactory nerve axons and acinar cells of Bowman's glands were unstained. In positive control tissue (adrenal glands), staining for cytochrome P450scc was seen in all layers of the adrenal cortex. The localization of cytochrome P450scc to sustentacular cells is consistent with a functional role for mitochondrial cytochrome P450scc in the production of olfactory mucosa-specific neurosteroids. These endogenous neurosteroids are most likely involved in the modulation of olfactory function.

Introduction

Steroid hormones have been isolated in a number of areas of the mammalian brain [1-5]. Indeed, steroids such as pregnenolone and dehydroepiandrosterone (DHEA) and their sulfate and lipoidal esters in nervous tissue are in concentrations much greater than that found in blood plasma [5,6]. The steroids found in nervous tissues have been named neurosteroids [7]. Some of these nervous system associated steroids come from exogenous sources located outside of the nervous system, such as the adrenal glands or gonads. However, it has been shown that cells of the nervous system (glial cells and neurons) are capable of making their own endogenous neurosteroids [8].

Data from several studies have revealed that a number of enzymes involved in steroid hormone formation are found in both neuroglial cells and neurons [9-11]. In addition, circulating steroids from exogenous sources such as progesterone, androgens and 11 deoxycorticosterone (DOC) can be converted to neuroactive steroids, such as allopregnanolone, androstanediol or 3α 5α tetrahydroDOC in the brain by the enzymes 5α reductase and 3α hydroxysteroid dehydrogenase (HSD) [5,12].

Cholesterol that is required for steroid hormone production is available from two major sources: (1) through de novo synthesis from acetyl coenzyme A [13,14]; or (2) from plasma lipoproteins, i.e., LDL [15]. The way in which sterol is supplied to the cell appears to be related, at least in part, to the steroidogenic activity of the tissue. For example, luteinized ovaries generally depend on lipoproteins as the main source of cholesterol used in steroidogenesis [16-18].

In the synthesis of steroid hormone, cholesterol undergoes a series of enzymatic reactions catalyzed by both cytochrome P450 and non-P450 enzymes. The initial step in all steroidogenic pathways involves the transport of cholesterol to the inner mitochondrial membrane by a carrier protein called steroidogenic acute regulatory protein (StAR) [19]. This protein has been isolated in many steroid producing cells and tissues [19,20], including glial cells and neurons [21,22]. Once delivered to the mitochondria, cholesterol is converted to pregnenolone by cholesterol desmolase, a cytochrome P450 enzyme (CYP11A) called cholesterol side chain cleavage, often designated cytochrome P450scc. Cytochrome P450scc belongs to a large family of cytochrome P450 oxidases, all of which have a molecular weight of about 50,000 kDa and a single heme group. Conversion

of cholesterol to pregnenolone by cytochrome P450scc is an obligate rate-limiting step in steroid hormone production by all steroidogenic cells. After formation of pregnenolone in the mitochondria, this steroid is transported to the smooth endoplasmic reticulum, where it can be converted to progesterone by the enzyme 3-β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone can then be converted into deoxycorticosterone by cytochrome P450C21. Through a series of reactions, pregnenolone can also be converted to form androgens, such as testosterone or through the action of the aromatase enzyme form estrogens [23].

The purpose of the study is to investigate the distribution of cytochrome P450scc in the rat olfactory mucosa by using immunohistochemistry in order to identify the potential steroidogenic capacity of the olfactory mucosa. Using immunohistochemical techniques, we report the cellular distribution of cytochrome P450scc in the olfactory mucosa of the rat.

Materials and Methods

Tissue isolation and processing

Tissues from CD rats (Charles River Laboratories, Inc., Wilmington, MA), 18-21 days old, were fixed by vascular perfusion with 4% paraformaldehyde in 0.1 M acetate buffer pH 6 followed by 4% paraformaldehyde in 0.1 M borate buffer pH 11. The olfactory mucosa (directly anterior to the olfactory bulb on the roof of the nasal cavity) was dissected away from the bony tissues of the nasal chambers. The tissues were fixed by immersion overnight at 4°C. Following fixation, tissues were rinsed in phosphate buffered saline, pH 7.4. The tissues were then infiltrated with chilled phosphate-buffered, graded (10%-20%-30%) sucrose solutions, embedded in O.C.T. embedding medium (Miles, Elkhart, IN) and sectioned at 6-20 μm with a cryostat.

Immunohistochemistry

Cryostat sections were rehydrated with 0.01 M phosphate-buffered saline at pH 7.4 (PBS), for 15 min at room temperature. For immunofluorescence, some of the tissues were quenched for autofluorescences by incubating with 0.025% cresyl violet for 1 min. For immunoperoxidase studies, the tissues were quenched with 0.3% H_2O_2 in absolute methanol to destroy endogenous peroxidase activity. The sections were then incubated overnight in primary antibody at 4°C. The primary antibodies [two rabbit polyclonal antibodies raised against peptide sequences of rat cytochrome P450scc protein (either amino acids 421-441 or amino acids 509-526; Chemicon, Temecula, CA) were diluted (1:200 - 1:1000) with PBS containing 1% normal goat serum and 0.1 Triton X-100. For immunofluorescence detection, following incubation with the primary antibodies, cryostat sections were incubated with a fluorescein-labeled goat anti-rabbit IgG (Zymed Immunochemical Co., Burlingame, CA), rinsed in PBS and mounted in a PBS solution containing 0.1% p-phenylenediamine and 70% glycerol. For immunoperoxidase detection, sections exposed to the primary antibodies were incubated with a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), followed by incubation with reagents of a peroxidase-label ABC kit (Vector Laboratories, Burlingame, CA). Peroxidase activity was localized using diaminobenzidine (DAB) and hydrogen peroxide (both from Zymed Immunochemical Co., Los Angeles, CA) as substrates. The

peroxidase reaction was terminated by three rinses in PBS, and slides were mounted in aqueous mounting medium (Sigma Chemical Co., St. Louis, MO). Slides were examined and photographed by bright field or epifluorescence using a Nikon microscope. Steroidogenic tissues from the adrenal glands were used as positive controls. As a negative control, the primary antibodies were omitted from the incubation medium.

Western blot analysis

To characterize the specificity of the cytochrome P450scc antibodies, we used western blot analysis with rat adrenal gland, olfactory mucosa and brain extracts. The rat tissues were homogenized in a buffer containing 2% SDS, 10% glycerol and 0.0625 M Tris HCl, pH 6.8 and protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN). The resulting lysates were boiled for 5min, centrifuged and protein concentration was determined with assay reagent BCA (Pierce Biotechnology, Inc., Rockford, IL). The proteins (30 μg of extract/lane) were separated on an SDS/PAGE (12.5% acrylamide gel) in a Biorad Mini-protease II dual slab-cell apparatus (BioRad Laboratories, Richmond, CA). The samples were transferred onto a nitrocellulose membrane overnight in 25 mM Tris, 0.2 mM glycine, 20% methanol and 0.01% SDS. Membranes were blocked for 1 hr. in Tris-buffered saline (TBS) containing 8% non-fat milk powder and were probed with rabbit antibodies directed against amino acids 421-441 or amino acids 509-526 of cytochrome P450scc(see above), diluted in 5% BSA in TBS. The membranes were then washed in TBS and 0.2% Tween-20 and incubated with peroxidase goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) diluted 1:1000 for 1 h at room temperature. The peroxidase reactivity was visualized using 0.5 mg/ml of diaminobenzidine in TBS and 1 μl/ml of 30% H_2O_2.

Prestained molecular weight markers (Sigma-Aldrich Corp. St. Louis, MO) were run in parallel to estimate protein sizes. The reported molecular weight of cytochrome P450scc is 49 kDa [24].

The experiments involving the use of animals in this study were approved by the Animal Investigation Committee of Western University of Health Sciences.

Results

The olfactory mucosa of the rodent consists of a pseudostratified epithelium composed of three major cell types, sustentacular cells, basal cells and olfactory receptor cells overlying a lamina propria containing connective tissue elements, blood vessels, nerve axons of olfactory receptor cells and olfactory Bowman's glands. We used a polyclonal antibody generated against amino acids 421-441 (carboxyl terminal) of the rat cytochrome P450scc to identify the cellular location of this enzyme in the rat olfactory mucosae. By the use of both immunoperoxidase (Figure 1) and immunofluorescence (fluorescein or FITC; Figure 2) techniques in the rat olfactory mucosae, we found that the enzyme was found preferentially localized to the apical region of the olfactory epithelium. The observed distribution was consistent with localization to the supranuclear regions of the sustentacular cell. A similar distribution was found when a second antibody specific for amino acids 509-526 (carboxyl terminal) of rat cytochrome P450scc enzyme was used (Figure 3). In the case of both antibodies used, no appreciable staining was seen in other areas of the olfactory mucosa (see Figure 2 and 4). When a non-immune rabbit serum was used as a control, there was no noticeable staining (in this example,
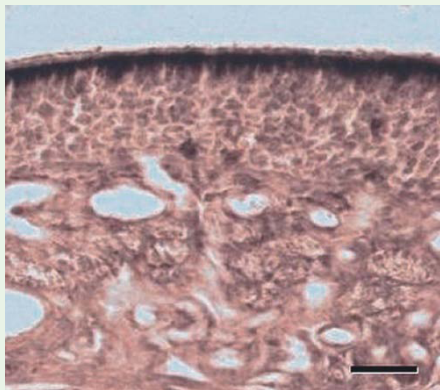
Figure 1: Immunoperoxidase localization of cytochrome P450scc in rat olfactory mucosa. Immunoreactivity is indicated by a dark area in the apical region of the olfactory mucosa. Counter staining of nuclei by hematoxylin is seen in the deeper areas of the olfactory mucosa. Bar = 50 µm.
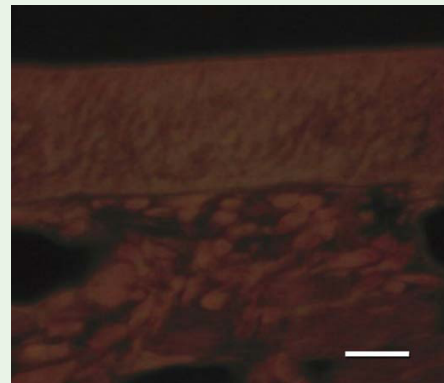

Figure 4: Control FITC staining of olfactory mucosa using a nonimmune rabbit serum. Note the absence of any fluorescent signal. Bar = 50 µm.
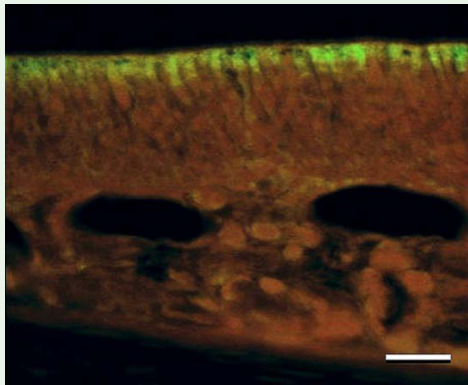

Figure 2: Immunofluorescent (FITC) localization of cytochrome P450scc in rat olfactory mucosa using antibody to amino acids 421-441. Immunoreactivity is indicated by a light (fluorescent) area corresponding to the apical region of the olfactory mucosa identified by immunoperoxidase. Bar = 50 µm.
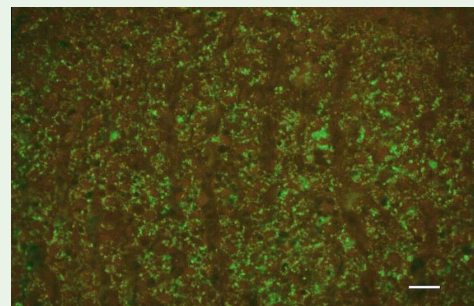

Figure 5: Immunofluorescentlocalization of cytochrome P450scc in rat adrenal gland using antibody to amino acids 509-526. Immunoreactivity appears as punctate regions within the cells located in the zona fasciculata shown here. A similar pattern was seen using the antibody to amino acids 421-441. Bar = 50 µm.
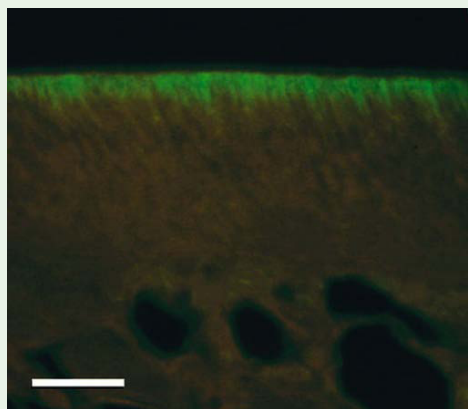

Figure 3: Immunofluorescent localization of cytochrome P450scc in rat olfactory mucosa using antibody to amino acids 509-526. Immunoreactivity is indicated by a light (fluorescent) area corresponding to the apical region of the olfactory mucosa identified by antibody to amino acids 421-441. Bar = 50 µm.
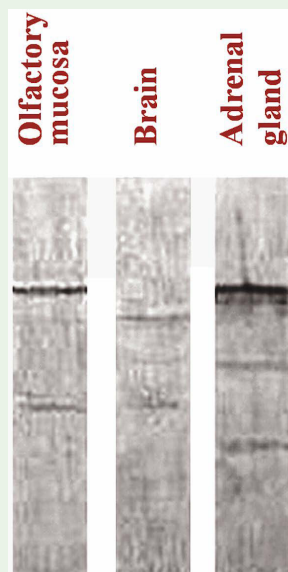

Figure 6: Immunoblot (Western) identification of cytochrome P450scc in rat olfactory, brain and adrenal glands using antibody to amino acids 509-526. A major immunoreactive band is seen at 49 kDa in the olfactory and adrenal gland. A slightly lower molecular weight band is seen in brain tissue.

immunofluorescent) of structures in the olfactory mucosa (Figure 4), indicating that the immunoreactivity was not due to random binding of the secondary reagent but was specific for cytochrome P450scc. As a positive control, a known steroidogenic tissue (adrenal gland) was used. In the rat adrenal glands, both primary antibodies were localized to the cells located in the adrenal cortex (zona glomerulosa, fasciculata and reticularis). The rows of cells in the zona fasciculata can be seen in Figure 5.

Western blot analysis using the polyclonal antibody to amino acids 509-526 (Figure 6) of cytochrome P450scc on olfactory and adrenal tissue showed a major reactive band at 49 kDa. This corresponded to the expected molecular weight of cytochrome P450scc [24]. The major band seen in brain tissue was a slightly less molecular weight (~45 kDa), this decreased molecular weight of P450scc in brain tissue is consistent with other studies [2].

Discussion

In this study, we report on the identification of cytochrome P450scc in the mammalian olfactory mucosa. The enzyme was localized to the apical region of the mucosa that indicates either an association with mitochondria located in the supranuclear region of sustentacular cells or the dendritic ending of olfactory receptor neurons. In contrast to a recent report [25], we did not detect immunoreactive staining for P450sccs in acinar cells of Bowman's glands.

In several areas of the central nervous system, cytochrome P450scc, other steroidogenic enzymes and StAR protein have been shown to be associated with glial cells and/or neurons [2,11,21,22,26,27]. Our findings in the olfactory mucosa are consistent with the CNS, since sustentacular cells have glial-like supportive functions for olfactory neurons. The localization of cytochrome P450scc to the apical region of the olfactory epithelium indicates that the endogenous neurosteroid produced may be released from sustentacular cells directly into the surrounding olfactory receptive field (dendrites). The olfactory odorant receptor proteins are members of the seven membrane spanning proteins that are g-protein-linked and related to visual receptor proteins [28]. It is well documented that neuroactive steroid action is mediated through a non-genomic mechanism involving neurotransmitter proteins, some of which are g-protein linked [5]. In the olfactory system, the neurosteroid produced by olfactory epithelial cells may directly modulate the functions of the g-protein-linked olfactory receptor proteins located on the dendritic endings of the olfactory receptor neurons.

In addition, neurosteroids in the CNS have been shown to direct or influence dendritic and axonal growth in neurons [29-31]. It is, therefore, conceivable that the neurosteroids produced by the olfactory epithelial cells may be involved in directing dendritic growth during the continual replacement of receptor neuron from differentiating basal cells. The olfactory neurosteroids may also be transported to the olfactory receptor neuron's axonal endings in the olfactory bulb. Thus, influencing the growth and proper connection of the axon during the renewal of olfactory neuron from basal cells.

Although, a localization of cytochrome P450scc to the olfactory receptor neurons cannot be totally ruled out, it seems more likely that the distribution of cytochrome P450scc observed in this study is consistent with the supranuclear region of sustentacular cells. This suggestion is supported by a recent study [25] where they reported a lack of immunofluorescent staining for P450scc in olfactory receptor cells. As is the case for the steroid-producing cells of the ovary, it appears that in some areas of the brain glial cells and neurons act in a cooperative manner to synthesize their final steroid product [5] and, thus, form a steroidogenic unit. In the ovary, the granulosa and theca interna cells cooperate in the production of estrogen [23]. The thecal cells possess the cytochrome P450scc enzyme allowing them to convert cholesterol into pregnenolone. This activity by thecal cells is in response to binding of luteinizing hormone (LH) to receptors for LH located on the plasma membrane of thecal cells. The thecal cells possess other enzymes needed to convert pregnenolone into androgens. LH stimulates theca cells to produce androstenedione (and, to a lesser extent, testosterone), which are both androgens. However, they lack the aromatase enzyme needed to modify the androgen structure to that of estrogen. Androstenedione and testosterone produced by the thecal cells are released from the cells and diffuse to and enter the granulosa cells that possess the aromatase enzyme. Thus, these cells can convert androgens into estrogens (estradiol or estrone) by aromatase. In this way, the theca interna and granulose cells act as a steroid unit in the production of estrogen; one cell possesses enzymes the other lacks. In the olfactory mucosa it is possible that the sustentacular cells contain the cytochrome P450scc enzyme needed to produce the steroid precursor, pregnenolone. The pregnenolone may be taken into the olfactory neuron and either transported to the dendrite or axon endings, or converted to other neuroactive steroids by enzymes, such as 5α reductase and 3α HSD [5,12] that may be in the olfactory neuron. Further studies will be needed to find out whether these enzymes are located in the olfactory neurons or sustentacular cells or both.

As stated above, pregnenolone may be taken up by the olfactory neuron and transported to the axon endings in the olfactory bulb. This suggestion is supported by the reported localization of pregnenolone receptors in the olfactory bulb [32]. Pregnenolone may, therefore, act through a genomic mechanism involving a pregnenolone receptor or may act through a non-genomic mechanism by modulating the neurotransmitter receptors, such as glutamate receptors [33] on neurons located in the olfactory bulb. The presence of P450scc in the olfactory mucosa suggest a role of this cytochrome in steroid hormone synthesis, however, further investigations will be required to show that this is involved in olfactory pregnenolone biosynthesis.

Taken together, our findings suggest that olfactory epithelial cells are capable of synthesizing endogenous neurosteroids. However, due to the limitations of this light microscopic study, the exact intracellular localization of cytochrome P450scc within the olfactory epithelium remains unknown. Future biochemical and high resolution (immunoelectron microscope) studies will be required to determine the subcellular distribution of P450scc. In addition to the machinery to make neurosteroids, the cells of the olfactory mucosa possess steroid hormone receptors, including those for adrenocorticoids [34-37]. Thus, the neurosteroids (endogenous or exogenous) may act directly through a genomic mechanism involving the steroid hormone binding to its receptor. Alternately, the neurosteroids may act via a non-genomic mechanism by binding to either the olfactory odorant receptor or a g-linked neurotransmitter receptor. These endogenous neurosteroids produced within the olfactory mucosa, therefore, may

play an important role in modulation of olfactory neuron function either in the olfactory mucosa or at the olfactory bulb.

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