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Research Article

Peroxisomes in the apocrine sweat glands of the human axilla and their putative role in pheromone production

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Abstract. The products of the human apocrine axillary glands contain volatile steroids which act as pheromones. The steroidal structure of these pheromones implies that the axillary glands should be able to synthesize cholesterol which is the essential precursor of these molecules. Since important steps in cholesterol synthesis are localized within peroxisomes, we investigated the occurrence and the putative role of peroxisomes in the axillary glands at protein and mRNA levels by immunocytochemistry, Western blotting, and RT-PCR. Numerous peroxisomes were localized in the cells of the apocrine glands by immunocytochemistry, and the presence of catalase was confirmed by Western blotting and RT-PCR. Additionally, RT-PCR revealed the presence of mRNAs of two peroxisome-associated enzymes of the cholesterol biosynthetic pathway, mevalonate kinase and farnesyl diphosphate synthase. The results suggest that the peroxisomes in the human apocrine axillary glands may play a pivotal role in the biosynthesis of pheromones.

Key words. Peroxisome; pheromone; axillary apocrine gland; cholesterol biosynthesis.

The existence of human pheromones has excited the imagination of both scientists and the public in recent years, stimulating substantial research in this field. Pheromones are secreted by the apocrine sweat glands which are found mainly in the axilla and, to a lesser extent, in other regions of the skin, such as the mammary areola, the labia majora, and the circumanal region. Human pheromones have been reported to excite the residual human vomeronasal organ [1–3], to activate specific regions within the brain [4], to affect social behavior [3, 5], to regulate ovulation [6], and to modulate, in a gender-specific way, physiological parameters such as serum levels of testosterone, luteinizing hormone, and follicle-stimulating hormone as well as respiration and cardiac frequency [7, 8]. Chemically, the currently known human pheromones are volatile steroid molecules such as, e.g., the 16-androstenes, 5α-androst-16-en-3β-ol or 5α-androst-16-en-3-one [3–5]. Accordingly, the biosynthesis of pheromones should be strongly associated with the biosynthesis of cholesterol, the essential substrate for steroid hormone synthesis. Recent evidence suggests that many of the enzymes of the cholesterol synthesis pathway exist mainly in the peroxisomes [9–12]. In particular, the enzymes mevalonate kinase (MVK) and farnesyl diphosphate synthase (FPPS) seem to be localized predominantly, if not exclusively, in this organelle [13–15]. Additional evidence for the essential role of peroxisomes in cholesterol biosynthesis is provided by the deficiency of enzymes of cholesterol synthesis in patients with defects in peroxisome biogenesis [16, 17].

To date, neither the presence of peroxisomes nor their function in the apocrine axillary glands have been the object of any research or even discussion. Their crucial role in steroidogenesis, however, strongly suggests their presence in those glands. The present study was designed to investigate the suspected occurrence and the putative role

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of peroxisomes in the human apocrine axillary glands. To this end, we used Western blotting and RT-PCR in combination with light and electron microscope immunocytochemistry to study the expression of peroxisomal enzymes both at the protein and mRNA level.

Materials and methods

Tissue preparation

Sections of axillary skin were obtained from different institutions of the University of Basel. Two samples were from the Institute of Anatomy, one from the Kantonsspital, and two from the Institut für Rechtsmedizin.

Immediately after removal, one portion of each sample was frozen in liquid nitrogen for subsequent biochemical analyses. For light microscope immunohistochemical studies, samples were fixed in Carnoy's fixative containing 60% ethanol, 30% chloroform, and 10% glacial acetic acid [18]. Tissue slices were dehydrated in graded series of ethanol and embedded in paraffin (Paraplast Plus; Monoject Scientific, Athy, Ireland) at 57 °C. For electron microscope immunocytochemistry, samples were treated with a fixative containing 4% formaldehyde, 0.25% glutaraldehyde in 0.1 M Pipes buffer, pH 7.4, followed by embedding in LR-white (London Resin Co. U.K.).

Antibodies, SDS-PAGE and immunoblotting

Polyclonal antibodies against the peroxisomal enzymes catalase (CAT), and acyl-CoA oxidase (AOX) were used. The antibodies were a generous gift of Prof. Dr. A. Völkl, Institute of Anatomy and Cell Biology, University of Heidelberg. Their specificity was assessed as described previously [19].

For Western blotting, tissues were homogenized in a homogenization buffer (250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% ethanol, pH 7.4) using an Ultra-Turrax (IKA Labortechnik, Staufen, Germany) and equal amounts of protein were subjected to SDS-PAGE. After electrotransfer of the polypeptides onto nitrocellulose, the sheets were incubated overnight with the primary antibody at a concentration of 1 µg protein/ml. After repeated washing, a peroxidase-conjugated goat anti-rabbit antibody (Sigma, Munich, Germany) was added for 1 h at room temperature. The immunoreactive bands were visualized by enhanced chemoluminescence (ECL; Amersham International Little Chalfont, U.K.) according to the manufacturer's protocol.

Morphological localization of peroxisomes

For light microscopy, paraffin sections were incubated for immunohistochemistry using antibodies against CAT and AOX. The bound antibodies were visualized by a biotinylated secondary antibody followed by incubation with an avidin-peroxidase conjugate (ExtrAvidin peroxidase staining kit; Sigma) and aminoethylcarbazol as substrate [20]. The sections were counterstained with hematoxylin. For electron microscopy, ultrathin sections of LR-white-embedded tissue were incubated with an antibody to CAT using the protein A-gold procedure with 12-nm gold particles [21].

RNA isolation

Total RNA was isolated with the RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentration was determined using the A_{260}/A_{280} ratio. RNA purity and integrity were checked by running a formaldehyde gel (1% agarose).

Reverse transcription-polymerase chain reaction

RT-PCR was performed employing the Qiagen One-Step RT-PCR Kit. Two hundred nanograms of total RNA were used as template; RT was performed for 30 min at 50 °C, followed by 30 thermal cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The first cycle was preceded by denaturation at 95 °C for 15 min, and the last cycle was followed by extension at 72 °C for 10 min. The PCRs had to be optimized for the particular primers to be used. As a negative control, the RNA (equal amount) was added after RT.

PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide to visualize DNA bands.

Primers for CAT were 5′CAT CGC CAC ATG AAT GGA TA-3′ and 5′-CCA ACT GGG ATG AGA GGG TA-3′, yielding a 316-bp fragment; they represent the sense sequence 693–712 and the antisense sequence 1009–990, respectively (GenBank NM 001752).

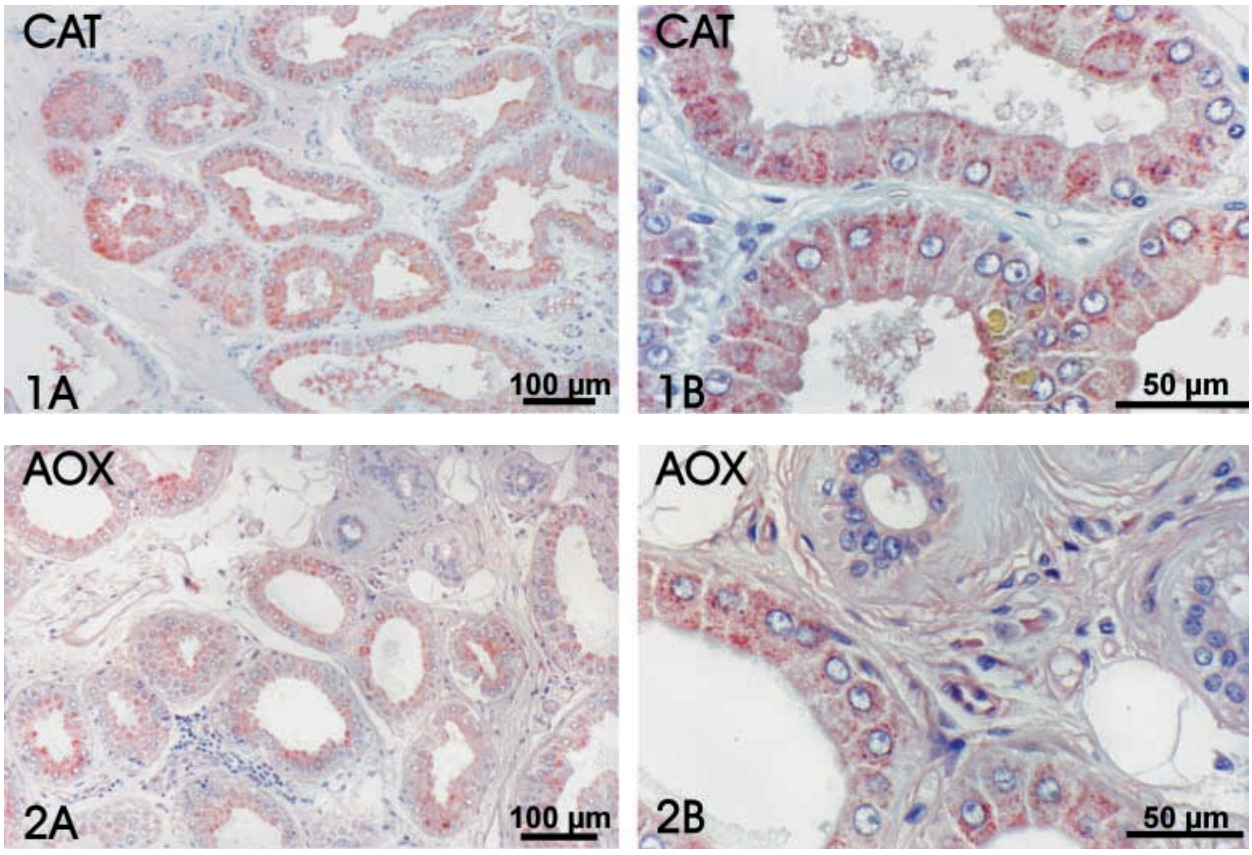
The primers 5′-CTC TCC AGA TCC TGC TGA CC-3′ and 5′-AGC TCT TCC AGC ACG AGG TA-3′ served to amplify a 205-bp-fragment of MVK, corresponding to nucleotides 762–782 (sense) and 966–946 (antisense), respectively (GenBank NM 000431).

The 199-bp-fragment of FPPS was amplified using the sense primer 5′-CCA ATG CCA AGA AGA TCC TG-3′ (nucleotides 989–1008) and the antisense primer 5′-CTG CCC GTA ATT TTC CTT CA-3′ (nucleotides 1188–1169) (GenBank XM 001352).

Results

Morphological observations

With both antibodies, to CAT and to AOX, positive immunoreactivity was obtained (figs. 1, 2). The secretory cells of the apocrine sweat glands contained numerous positively stained peroxisomes distributed throughout the cell. In some areas, immunoreactivity accumulated in the apical cell regions. Generally, the staining was restricted



Figures 1, 2. Immunocytochemical localization of peroxisomes in the apocrine sweat glands of human axilla. Light micrographs from sections incubated with an antibody to catalase (1) and acyl-CoA oxidase (2). Overview (*A*) and detail (*B*). Note the specific (red) stained peroxisomes in the epithelium of the apocrine glands. Sometimes, large (yellowish) secretory vesicles can be observed near the apical site of the epithelium of the apocrine glands (fig. 1B). In figure 2 selective staining in the apocrine glands is shown, with no staining of merocrine glands or excretory ducts.

to the epithelial cells of the apocrine sweat glands, and no labeling was detected within myoepithelial cells or in the epithelium of the merocrine glands or excretory ducts (fig. 2).

In fresh and appropriately fixed material, the ultrastructure of the secretory cells was well preserved (fig. 3). Nuclei, secretory vesicles, endoplasmic reticulum, mitochondria, and peroxisomes could be easily identified, and the apices of the secretory cells showed microvilli which varied in number and length. Immunocytochemical staining for CAT was localized in the matrix of peroxisomes with only negligible background. The peroxisomes showed a more or less circular shape and a profile diameter of about 300–600 nm. The number and localization of peroxisomes was in line with the light microscopy observations.

Western blotting

Western blotting (fig. 4) showed a 58-kDa immunoreactive band in protein preparations of the axillary glands which corresponds to the size of human CAT [22].

RT-PCR

RT-PCR analyses of RNA transcripts of CAT, MVK, and FPPS are shown in figure 5. In line with the detection of CAT protein by Western blotting, the results indicated the occurrence of CAT mRNA in the axillary samples (fig. 5A). Moreover, RT-PCR revealed the presence of mRNAs for MVK (fig. 5B) and FPPS (fig. 5C).

Discussion

The occurrence of steroidal pheromones in the products of the apocrine glands of the human axilla is well established [3, 6, 23, 24]. To date however, the production site of these pheromones has never been considered in detail. There are two possibilities: the axillary glands could themselves produce the pheromones de novo, alternatively, they could take up these substances or their precursors from the blood stream. Cholesterol and various steroid hormones are known to be present in the blood, and experiments injecting radioactive steroids have

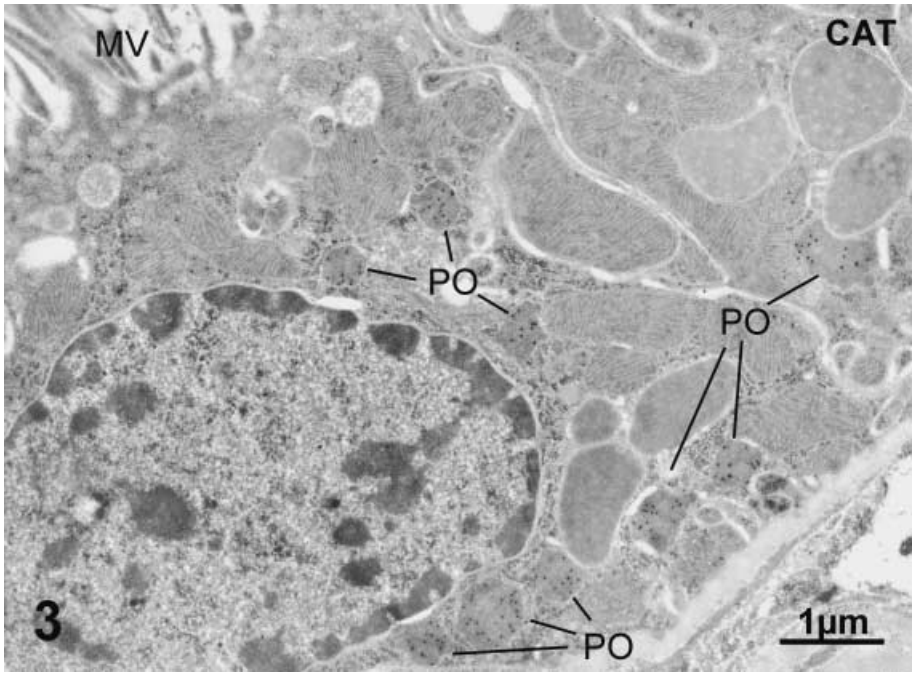


Figure 3. Electron micrograph of epithelium from a human apocrine axillary gland incubated for immunocytochemical localization of catalase. The peroxisomes (PO) show massive gold immunolabeling and are localized in all parts of epithelial cells, thus confirming the light microscopy results (MV, microvilli at the apical surface of the epithelium).

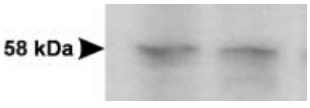


Figure 4. Immunoblot from two different preparations of human apocrine sweat glands incubated with an antibody to catalase. The immunocomplexes were visualized by the ECL technique (for details see Materials and methods). Note the specific molecular weight of human catalase at 58 kDa.

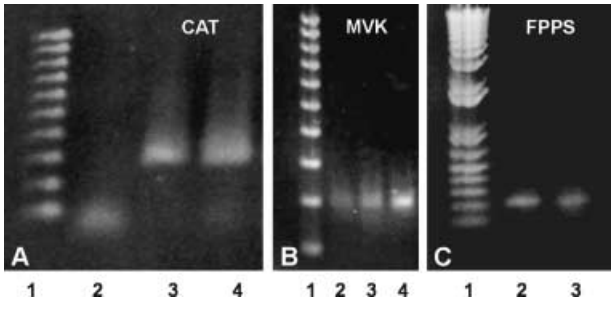


Figure 5. RT-PCR with primers for catalase (CAT), mevalonate kinase (MVK), and farnesyltransferase (FPPS). (*A*) Lane 1, 100-bp ladder; lane 2 – 4, RT-PCR with primers for CAT yielding a 316-bp fragment. Lane 2, negative control without RT reaction to exclude contamination by genomic DNA; lanes 3 and 4, RNA preparations from two different human axillae. (*B*) Lane 1, 100- bp ladder; lanes 2–4, RNA preparation from a human axilla, with three different concentrations of primer for the MVK 205-bp fragment. (*C*) Lane 1, 100-bp ladder; lanes 2 and 3, RNA preparations from two different human axillae.

shown that axillary glands are able to take up steroids from the blood and to excrete them [23]. Moreover, the occurrence of steroid-metabolizing enzymes in apocrine axillary glands [25, 26] implies that these glands are at least able to modify steroids. For de novo production of pheromones, however, the apocrine axillary glands should possess the ability to synthesize cholesterol which is the substrate for the synthesis of steroid hormones and, accordingly, of pheromones. The localization of essential parts of the cholesterol biosynthesis pathway in peroxisomes [9–13] implies that the presence of peroxisomes may be indispensable for steroid-producing cells. Indeed, peroxisomes have been commonly found in steroid hormone producing organs, such as the adrenal cortex [27], the ovary [28], and the Leydig cells of the testis [29, 30], emphasizing the importance of these organelles for steroid biosynthesis [31].

The present study has shown that the apocrine glands of the human axilla contain large numbers of peroxisomes. In contrast, peroxisomes were not detected in the merocrine sweat glands implying that they may be very rare or even totally absent in those glands. Together, these results emphasize a special function of these organelles in the apocrine glands, which is most probably connected with the synthesis of cholesterol, as the precursor of pheromones. This hypothesis is additionally supported by the presence of the mRNAs for MVK and FPPS, which are peroxisome-associated enzymes of cholesterol

biosynthesis. Accordingly, the results of the present study provide evidence that the apocrine axillary glands may be capable for de novo pheromone production.

Peroxisomes have been found in sebaceous glands with holocrine secretory activity and pheromonal function, such as the preputial glands and the Meibomian glands of the mouse [32, 33], the circumanal gland of the dog [34], and the uropygial glands of the mallard duck and other birds [35]. However, the pheromones of duck [36] and mouse [37] studied so far seem not to be related to human steroidal pheromones, suggesting that the possible role of peroxisomes in the synthesis of those pheromones may differ from their role in the human axilla. Nevertheless, peroxisomes may participate in pheromone production in these species, as shown by their involvement in the synthesis of the diesters of 3-hydroxy fatty acids in the duck uropygial gland, which exhibit pheromonal activity in this species [35, 36].

Generally, peroxisomes in mammals contain more than 50 different enzymes which play an important role in various metabolic pathways [38]. Peroxisomes house oxidases which produce hydrogen peroxide and CAT which degrades it [39]. Furthermore, in addition to their involvement in cholesterol biosynthesis, they are engaged in the β-oxidation of very long chain fatty acids [40] and prostaglandins [41], the formation of bile acids by oxidation of the cholesterol side chain [9, 42], and the synthesis of plasmalogens [30, 43–45]. The present study focused mainly on the putative role of peroxisomes in the synthesis of cholesterol as a precursor for pheromones in the apocrine axillary glands. The results obtained provide strong evidence that their involvement in pheromone synthesis may be one of the main functions of peroxisomes at this site. The broad spectrum of biochemical pathways, however, in which peroxisomes are involved suggests that their function in the apocrine axillary glands may not be restricted to this role and that the multiple features of these organelles have to be considered in future investigations.

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