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Functional significance of non-neuronal acetylcholine in skin epithelia

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ABSTRACT

In recent years, the physiological role of non-neuronal acetylcholine (ACh) and its receptors (AChR) in epidermal physiology has been under intense investigation. However, little is known about the role of the non-neuronal cholinergic system in inflammatory skin diseases. We chose the clinically nicotine-dependent skin disease hidradenitis suppurativa (HS) as model to study the influence of long term nicotine ingestion on epidermal morphology and AChR expression. HS is a chronic inflammatory, disabling disease of unknown pathogenesis emerging from the pilosebaceous unit of the intertriginous areas. In order to correlate our findings to specific nicotine effects, we used the organotypical coculture system (OTC) and raised artificial epidermis in the presence of nicotine. After 12 days in culture control OTC showed a mature epithelium, while nicotine treated OTCs were significantly thicker. Using immunofluorescence analysis, nicotine treated OTCs produced significantly stronger immunoreactivity (IR) for the a3, M3 and M5 AChR antisera than control. In contrast, the a7 nAChR antiserum showed a slightly reduced IR in the granular layer and the a9 nAChR IR retracted to the lower suprabasal layers. In HS epidermis we found the strongest IR for all AChR around the follicular infundibulum while in the sinus epithelia it was only weak. In contrast to the nicotine treated OTC, the a7 nAChR IR in the hyperplastic HS epidermis was clearly extended to all living layers. Altogether we provide first hints for a causative role of the non-neuronal cholinergic system in the pathogenesis of HS by promoting infundibular epithelial hyperplasia and thus follicular plugging.
INTRODUCTION
Proliferation and terminal differentiation, constant renewal and repair of keratinocytes are required to provide a highly complex, semi-permeable barrier, the epidermis, protecting the human body from physical and chemical damage, infection, dehydration and heat loss (Madison, 2003). The non-neuronal cholinergic system was recently recognized to play a fundamental part in the regulatory orchestra of epidermal physiology. It is firmly established that human keratinocytes synthesize and degrade acetylcholine (ACh) that acts via muscarinic (mAChR) and nicotinic receptors (nAChR) in either an autocrine or paracrine manner on the surrounding cells like fibroblasts or melanocytes (reviewed in Kurzen and Schallreuter, 2004). Hitherto, five molecular subtypes of mAChR have been identified in keratinocytes, melanocytes and fibroblasts, while the number of pharmacologically distinct pentameric nAChR composed of the a3, a5, a7, a9, a10, b1, b2 and b4 nAChR subunits is hard to define due to varying subunit combinations and an apparently highly variable expression pattern of the nAChR in the epidermis, especially of the heterooligomeric species of the a3*-type (reviewed in Kurzen and Schallreuter, 2004). Depending on their subunit composition, the nAChR show different affinities to ACh, choline and other cholinergic compounds like nicotine (Xiao et al., 1998; Nguyen et al., 2001; Verbitsky et al., 2000; Arredondo et al., 2002; Grando et al., 2006).

We examined the functional impact of the observed AChR distribution in the epidermis in a current study (Kurzen et al., 2006) using organotypic cocultures (OTC) as an in vitro skin equivalent system. In that system, blocking of all AChR by combined treatment with mecamylamine and atropine or treatment with strychnine (which blocks a9 nAChR) for 7-14 days resulted in complete inhibition of epidermal differentiation and proliferation. Blockage of nAChR with mecamylamine led to a less pronounced delay in epidermal differentiation and proliferation than blockage of muscarinic mAChR with atropine, evidenced by reduced epithelial thickness and expression of terminal differentiation markers such as CK2e, CK10 or ZO1. In addition, prominent acantholysis could be observed in the basal and lower suprabasal layers in mecamylamine-, atropine- and strychnine-treated cultures, accompanied by a decreased expression of desmosomal-, adherens junction- and tight junction proteins. This globally reduced cell adhesion led to cell death via intrinsic activation of apoptosis. In contrast, stimulation of AChR - nAChR more than mAChR - with cholinergic drugs resulted in a significantly thickened epithelium, accompanied by an increase of intercellular lipid content in the corneal layer. In that study, we could demonstrate that ACh is crucial for the development of a stratified epidermis-like epithelium in vitro.
The goal of the present investigation was to examine the influence of the cholinergic agonist nicotine on AChR expression in human skin. An organotypically cultured epidermis equivalent (OTC) treated with the cholinergic agonist nicotine was correlated with the in vivo situation in non-smoker’s and smoker’s skin. In addition we used previously characterized epithelia of the chronic inflammatory skin disease hidradenitis suppurativa (HS) a.k.a. acne inversa, as a model (Kurzen et al., 1999). In HS, the epidermis is under the influence of nicotine, since more than 80% of patients are active smokers (Jansen et al., 2001). In healthy smokers, nicotine reaches concentrations of 500 nM / L in the serum and about 150 nM / L in axillary sweat. In the intertriginous areas, where the HS lesions usually develop occlusive application may even produce higher local concentrations (Kintz et al., 1998; Alkondon et al., 2000). Thus, we wanted to find out whether AChR expression patterns change under chronic nicotine influence in a chronic inflammatory environment.

MATERIALS AND METHODS

Tissues
After informed consent according to the declaration of Helsinki and approval by the local ethical committee, normal human adult skin from the margins of routine surgical procedures and skin specimens from patients with HS were obtained after routine surgical therapy. Immediately after removal, samples were frozen in isopentane cooled in liquid nitrogen and stored at – 80 °C. Fresh samples were used to isolate normal epidermal keratinocytes and human dermal fibroblasts.

Organotypic cocultures (OTC)
Organotypic cocultures were prepared as described previously (Kurzen et al., 2006). Lyophilized collagen type I (IBFB Leipzig, Germany) was resolubilised at 4 mg / ml in 0.1 % acetic acid and kept at 4°C. Human dermal fibroblasts and normal epidermal keratinocytes were isolated from skin specimens as described (Stark et al., 2004a). The fibroblasts were added to the gel to give 105 cells / ml in the collagen gel. 2.5 ml of the collagen / fibroblasts solution was poured onto filter inserts (3 µm pore size, Falcon) of a 6 well plate (BioCoat, Becton-Dickinson, Heidelberg. Germany). Thereafter, the normal epidermal keratinocytes were plated inside glass rings at a density of 106 cells per well. After 2 days the keratinocytes were raised to the air-liquid interface. The air lift procedure is defined as day 0 of OTC. OTCs were grown in FAD-Medium with 10 % FCS and 50 µM ascorbic acid. At the end of the experiment, the epidermal-collagen specimens were snap frozen in liquid nitrogen and stored.
at – 80 °C. In a single experiment, OTCs of one condition were always performed twice. All cell culture experiments were repeated at least twice. +/- Nicotine (1 mM, Sigma) was added to the OTCs on the day of the air-lift.

**Determination of epithelial thickness**
The epithelial thickness obtained was determined by measuring the thickness of the malpighian layer at 2 representative points of each specimen, so that for each condition, at least 6 different values were obtained. Epithelial thickness of normal epidermis and HS epidermis was determined from 5 different healthy and diseased donors. In the case of HS epidermis, either 5 points of each specimen were picked randomly or epidermal thickness at the tips of the papilla and at thickest points of the rete ridges were determined separately at 5 different points. Statistical analysis was performed using the Wilcoxon sum-of-ranks test (available on www.statpages.net).

**Immunofluorescence staining**
For immunofluorescence analysis, 6 µm cryostat sections of human skin, OTCs or HS specimens were used air dried and unfixed. Immunofluorescence reactions were performed at least 3 times on different specimens according to standard procedures (Kurzen et al., 2004). After blocking with 5 % non-fat dry milk and 1 % BSA in Tris-buffered saline, the primary antibodies were applied to the specimens for 1 h at room temperature or for 12 h at 4 °C. To detect the different AChR subunits, we used our own guinea-pig antisera against a3 nAChR (GP5b), a5 nAChR (GP1a), a7 nAChR (GP9a), a9 nAChR (GPT1), M1 mAChR (GP20a), M3 mAChR (GP19b), M5 mAChR (GP12b) all characterized in Kurzen et al., 2004. The following secondary antibodies were applied for one hour at room temperature: biotin-coupled, preabsorbed goat anti-guinea pig antisera obtained from Dianova (Hamburg, Germany). Antibody binding was visualized with Cy-2 or Cy-3 coupled streptavidin (Dianova). Negative controls were performed by replacing the primary antibody with PBS or an irrelevant, species–matched antibody. Washes (3 x 10 min) in PBS or TBST were performed after each step. The staining was observed and visualized with a Leitz DMRB immunofluorescence microscope.
RESULTS

Influence of nicotine on epidermal morphology and AChR expression in vitro

In order to determine whether nicotine is capable of inducing epidermal hyperplasia, we measured the epidermal thickness of the OTCs after 7 days and 12 days in culture with and without nicotine present. We found no significant difference between controls on day 7 and 12 (mean 78 and 68 µm), while at both time points epithelial thickness was significantly increased by nicotine (mean 100 and 128 µm), p < 0.0001. Normal smoker’s skin showed a mean of 47 µm while HS epidermis was significantly thicker with 149 µm mean, p<0.001 if HS epidermis was measured randomly. Since in HS epidermis there was a psoriasiform hyperplasia, we separately assessed the thickness at the tips of the papillae (mean 69µm) and at longest extension of the rete ridges (230µm) (Fig. 1).

AChR expression in OTCs

In immature control cultures on day 7 (Fig. 2a-a’’, 3a-a’’), antisera against those AChR typical for basal cells, namely a3, a9 and M5 AChR produced a strong staining. a3 and M5 AChR immunoreactivity (IR) were restricted to the basal cells and a9 AChR IR showed a wide distribution over the all living layers. Interestingly, the M3 AChR antiserum displayed only a faint staining of the basal side of the basal cell row. The AChR subunits typical for suprabasal cells, namely a7 and M1 remained negative in the immature OTCs. In mature OTCs after 12 days in culture (Fig. 2b-b’’, 3b-b’’), the a3 and M3 AChR IR remained restricted to the basal cells, while a9 AChR IR extended in a fishnet-like pattern to all living layers. a7- and M1 AChR IR became prominent in the granular layer and M5 AChR IR was slightly stronger than in normal epidermis and extended to the spinous layers. In nicotine treated cultures (Fig. 2c-c’’, 3c-c’’) the pattern changed: a3-AChR IR extended to all layers, showing in part a cytoplasmic and/or membranous staining pattern. In contrast, a7 AChR IR was reduced to a thin granular layer and a9 AChR IR remained restricted to the basal and spinous layers, also showing cytoplasmic staining in addition to membranous staining. M1 AChR IR was weaker than in control OTCs, restricted to the upper spinous and granular layer. M3 AChR IR was significantly increased, showing a bright staining of all layers. M5 AChR IR was slightly stronger than in control OTCs, distributed over all living layers.
AChR expression in normal smoker’s skin and in HS epithelia

In the present study AChR expression in skin specimens taken from the margins of routine surgical procedures from non-smoking (Fig. 4a-a” 5a-a” ) and smoking patients (Fig. 4b-b”, 5b-b”) between 30 and 70 years, including different body sites, showed only minor variations using our previously characterized antisera. In both, a3 AChR IR was bright and restricted to epidermal basal layer, a5 AChR IR was predominant in basal cells but also detectable in the spinous and granular layer in a membranous pattern. M1 and a7 AChR IR were predominant in the granular and upper spinous layers, slightly weaker in smoker’s than in non-smoker’s skin. M3 and M5 -AChR IR were bright and restricted to the basal and lower suprabasal layers.

In HS specimens, the epidermis showed a psoriasiform hyperplasia, with the strongest IR for all AChR periinfundibularly (Fig. 4c-c”, 5c-c”). a3- and M3- and M5 AChR IR was strongest in the basal layer, but also showed a diffuse cytoplasmic staining in the suprabasal layers. a5 AChR IR was stronger than in normal smoker’s skin, detectable in all living layers. a7-, a9- and M1 AChR IR was prominent in all suprabasal layers in a membranous fishnet like pattern.

In draining sinus epithelia of HS (Fig. 4d-d”, 5d-d”) that have been characterized previously by one of us (HK, Kurzen et al., 1999), AChR expression was grossly reduced. Only a3- and M3 AChR IR were present in the basal cells, while in the suprabasal cells a mostly cytoplasmic weak staining of a7, a9 and M5 AChR antisera was detectable.

DISCUSSION

While the presence and function of the non-neuronal cholinergic system in human skin has been characterized by many different studies in the last decade, the role of acetylcholine and its receptors in skin diseases remains largely terra incognita (Kurzen, 2004). Apart from phenotype and function of non-neuronal AChR in human skin, HS has been in the focus of our interest. This chronic inflammatory disabling disease is thought to emerge from the pilosebaceous unit of the intertriginous areas and is hence called acne inversa in the European literature (Sellheyer and Krahl, 2005). The pathogenesis of HS has remained elusive, while different triggering factors have been described, amongst which tobacco smoking, sweating, obesity and colonization with staphylococcus aureus seem to be the most important. Almost 90% of HS patients are active smokers which makes HS, together with e.g. pustular palmoplantar psoriasis, a tobacco-related skin disease (Jansen et al., 2001). Based on this observation, we set up the hypothesis that nicotine as main toxin in cigarette smoke might exert its disease provoking capacity through direct action upon nicotinic AChR present in HS
lesions. In the present study, we show that non-neuronal AChR are present in HS epidermis, apparently accentuated around the pilosebaceous infundibulum. Interestingly, in the sinus tracts, there was only a very faint AChR expression paralleling that in immature OTCs. In a previous study we have shown that HS sinus tract epithelia show similarities to hair follicle outer root sheath epithelia and are under the influence of a varying inflammatory infiltrate. Since we did not find any obvious AChR expression variabilities in the sinus tract epithelia, we did not pursue a correlation to the previously described different epithelial entities (type I-III) of the tracts (Kurzen et al., 1999). Even though we have to consider the influence of inflammatory cytokines on epidermal morphology (Gniadecki, 1998), it is a noteworthy observation that in our OTC system, nicotine is the strongest inducer of epidermal hyperplasia (see also Kurzen et al., 2006) and at the same time, nicotinic AChR show a particularly strong expression at the place where HS is supposed to originate from: the infundibulum of the pilosebaceous unit. In addition to our quantitative analysis, we observed the most pronounced epidermal hyperplasia around the infundibula, which is well in line with the above mentioned chain of arguments. Even though epidermal hyperplasia or acneiform lesions have not been reported after transdermally applied nicotine patches, this may be due to the time required to induce epidermal hyperplasia or follicular plugging in vivo (Smith et al., 1992). Previously, chronic nicotine treatment has been described to induce persistent functional inactivation but numerical up-regulation of all nAChR subtypes (Ke et al., 1998). Our OTC results are well in line with this observation. We found a stronger IR for almost all AChR examined in the nicotine treated OTCs and for several AChR subunits in smoker’s epidermis compared to non-smoker’s epidermis. Only, the a7 AChR IR appeared reduced in nicotine treated OTCs and did not significantly change in normal smoker’s epidermis as compared to non-smoker’s epidermis. However, a7 AChR IR was particularly strong with an extended pattern in HS epidermis. On the one hand this may be interpreted as nicotine induced AChR up-regulation and on the other hand may point the way to an important role or even disease specific regulation of this homooligomer in HS. However, further studies in other skin diseases have to be performed in order to judge the specificity of this result. The same holds true for the a5 AChR subunit. While in normal non-smoker’s epidermis, we had previously found a distinct pattern, restricted to the basal layer and a specialized single-cell layer in the stratum granulosum, now in smoker’s and HS epidermis we found a rather strong expression in all living layers, paralleling that of the a3 AChR subunit. Previously, the a5 AChR subunit has been suggested to confer a higher ACh-susceptibility to a3ß2 and a3ß4 heterooligomers (Gerzanich et al., 1998).
Altogether we present data which again point towards a highly complex regulation of AChR expression in epidermal physiology and pathophysiology. Our data are well in line with a central role of nicotine and hence the non-neuronal cholinergic system in the development of epidermal infundibular hyperplasia and hence follicular plugging in HS which may be central in provoking flares of this acne-like disease (Sellheyer and Krahl, 2005).
REFERENCES


LEGENDS TO THE FIGURES

Fig.1 Nicotine induces epithelial hyperplasia
OTCs cultured for 7 or 12 days in the absence and presence of 1mM nicotine. At both time points nicotine induces significant epidermal hyperplasia (* = p< 0.05, ** = p< 0.0001). Also in vivo, in HS epidermis, there is a significant epidermal hyperplasia, evidenced by an increase in epidermal thickness (p< 0.0001). In HS epidermis measured randomly, there was a large standard deviation of values, which was due to the psoriasiform elongation of rete ridges that have been determined separately (mean 230µm) while the epithelium at the tips of the papillae remained relatively thin (mean 69µm).

Fig.2 Expression of selected nAChR in OTCs
Immature OTCs (a-a''), mature OTCs (b-b'') and nicotine treated OTCs (c-c'') stained with antisera against human a3 nAChR (a, b, c), a7 nAChR (a', b', c') and a9 nAChR (a'', b'', c''). Note the prominent expression of a3 nAChR in nicotine treated cultures (a'') while a7 nAChR IR seems to most prominent in mature control OTCs (b'). Note absence of a7 nAChR in immature OTCs (b). Original magnification 40x.

Fig.3 Expression of selected mAChR in OTCs
Immature OTCs (a-a''), mature OTCs (b-b'') and nicotine treated OTCs (c-c'') stained with antisera against human M1 mAChR (a, b, c), M3 mAChR (a', b', c') and M5 mAChR (a'', b'', c''). While M1 mAChR IR and M5 mAChR IR are absent in immature OTCs (a, b), the M5 mAChR is strongly expressed in the basal cell row (c). In mature OTCs M1 mAChR IR is predominantly cytoplasmic and can be detected in the upper spinous and granular layer. M3 mAChR IR (a', b') is restricted to the basal cells while M5 mAChR IR (c') is extended to all cell layers. In nicotine treated cultures M1 mAChR IR (a'') seems to be localized to both cytoplasm and cell membrane and there is an obvious increase in M3 mAChR IR (b'') as compared to control (b'). M5 mAChR IR remains unchanged compared to control OTCs. Original magnification 40x.

Fig.4 Expression of selected nAChR normal skin and HS
Normal human non-smoker’s epidermis (a-a''), smoker’s epidermis (b-b''), HS epidermis (c-c'', d-d'') was stained with antisera against human a3 nAChR (a-d), a5 nAChR (a'-d') and a7
nAChR (a’’-d’’). Note a3 AChR IR restricted to epidermal basal layer (a, b) while a5 AChR IR is predominant in basal cells but also detectable in the spinous and granular layer (a’, b’). a7 AChR IR is predominant in the granular and upper spinous layers, slightly weaker in smoker’s (b’’) than in non-smoker’s skin (a’’). Note the additional and prominent staining of suprabasal cells at the HS infundibulum for all AChR shown (c-c’’) while in the sinus tracts (d-d’’) there is only a faint immunoreactivity. Original magnification 40x.

**Fig.5 Expression of selected mAChR normal skin and HS**

Normal human non-smoker’s epidermis (a-a’’), smoker’s epidermis (b-b’’), HS epidermis (c-c’’, d-d’’)) was stained with antisera against human M₁ mAChR (a-d), M₅ mAChR (a’-d’) and M₅ mAChR (a’’-d’’). M₁ AChR IR was predominant in the granular and upper spinous layers, slightly weaker in smoker’s (b) than in non-smoker’s skin (a). M₃ and M₅ AChR IR were bright and restricted to the basal and lower suprabasal layers especially in smoker’s epidermis (b’, b’’) while in non-smoker’s skin (a’, a’’) the IR was weaker and more evenly distributed over all epidermal layers. Note the extended IR for all AChR shown at the infundibulum (c-c’’) while in the sinus tract epithelium (d-d’’) there is only a weak reactivity of the M₃ mAChR antiserum in the basal cells. Original magnification 40x.