Histamine transport and metabolism are deranged in salivary glands in Sjögren’s syndrome

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Abstract

Objective. To study histamine transport and metabolism of salivary gland (SG) epithelial cells in healthy controls and SS patients.

Methods. Enzymes and transporters involved in histamine metabolism were analysed in cultured human submandibular salivary gland (HSG) epithelial cells and tissue sections using quantitative real-time PCR and immunostaining. HSG cells were used to study [3H]histamine uptake [(±1-methyl-4-phenylpyridinium (MPP)] and efflux by liquid scintillation counting.

Results. mRNA levels of L-histidine decarboxylase (HDC) and histamine-N-methyltransferase (HNMT) were similar in the control and SS glands, but diamine oxidase was not expressed at all. Organic cation transporter 3 (OCT3) in healthy SG was localized in the acinar and ductal cells, whereas OCT2 was restricted to the myoepithelial cells. Both transporters were significantly decreased in SS at mRNA and protein levels. OCT3-mRNA levels in HSG cells were significantly higher than those of the other studied transporters. Uptake of [3H]histamine was inhibited by MPP in a time-dependent manner, whereas [3H]histamine-preloaded HSG cells released it.

Conclusion. Ductal epithelial cells are non-professional histamine-producing cells able to release histamine via OCTs at the resting state up to ~100 nM, enough to excite H3R/H4R+ epithelial cells, but not H1R, which requires burst release from mast cells. At the stimulated phase, 50–60 μM histamine passes from the interstitial fluid through the acinar cells to saliva, whereas uptake by ductal cells leads to intracellular degradation by HNMT. OCT3/histamine/H4R-mediated cell maintenance and down-regulation of high histamine levels fail in SS SGs.

Key words: Sjögren’s syndrome, salivary glands, histamine receptor, histamine, organic cation transporters.

Introduction

Histamine is a widely distributed biogenic amine that is predominantly synthesized by mast cells, basophils and enterochromaffin-like cells, which produce histamine at a high rate, store it in granules and release it in a regulated manner in bursts so that very high local histamine concentrations are momentarily reached [1]. The novel pre-synaptic H3 receptor (H3R) is best known for its ability to regulate neurotransmitter release, whereas H4 receptor (H4R) linked to immune responses and autoimmune diseases is predominantly expressed in antigen-presenting dendritic cells [2–4] and T and B lymphocytes [5, 6].

The novel histamine receptors have almost 10 000-fold higher affinity for histamine than conventional histamine
receptors (e.g. $pK_a$ is 8.3 for $H_4R$ and 4.2 for $H_1R$) [7, 8]. Resting plasma histamine 3–8 nM and salivary histamine 3–112 nM can stimulate the novel histamine receptors, almost half maximally. Burst release is required to reach, for example, concentrations of 50–60 μM to stimulate conventional low-affinity histamine receptors. Therefore, during the resting or maintenance phase, low histamine concentrations act at high-affinity receptors, whereas regulated burst release is required to engage the conventional histamine receptors.

$H_4R$ is found in healthy human salivary gland (SG) epithelial cells but is highly decreased in SS [9]. It was therefore analysed if SG epithelial cells contain any non-professional histamine-producing cells, with a potential to produce histamine and via some basal or constitutive release/transport mechanism maintain low histamine concentrations, which are only able to regulate target cells equipped with the novel, high-affinity $H_3R$ or $H_4R$ in an autocrine and paracrine mode, but too low to activate the classical low-affinity receptors. After local mast cell-mediated burst release, the high histamine concentration acts on $H_1R$/$H_2R$, which needs to be down-regulated to return to the resting phase. Therefore the histamine degrading potential of SGs was also analysed. Due to the involvement of $H_1R$ in immune responses and in SS [9], the histamine synthesizing, transporting and metabolizing capacity was also assessed in SS, an autoimmune epitheliitis [10], because this has potential relevance for the resting phase, when the non-professional cells and novel high-affinity histamine receptors play a major role, and for the activation phase, when the professional histamine-releasing cells and the conventional low-affinity histamine receptors play a major role.

Earlier only mast cell-derived high micromolar histamine levels and the conventional low-affinity $H_1R$ and $H_2R$ were considered to play in histamine-mediated physiology in healthy individuals and in pathophysiology in patients with SS. However, more recently a novel high-affinity histamine receptor, $H_4R$, responsive to nanomolar concentrations of histamine was described [11]. Furthermore, non-professional histamine-producing cells containing low-activity histamine-synthesizing 74 kDa HDC enzyme, able to produce and modulate such low nanomolar concentrations, were also described. These non-professional histamine-producing cells include antigen-presenting dendritic cells and lymphocytes, which may play a role in focal sialadenitis in SS [12, 13]. This inspired us to study whether such non-professional histamine-producing cells, able to stimulate their $H_4R$-provided acinar target cells at nanomolar concentrations, are also present in human SGs.

Non-professional histamine-producing cells and $H_4R$ are such new discoveries that their contribution to the SG physiology and pathology is still unclear. It is very interesting that the former finding of a deficiency of $H_4R$ in SS [9] is now found to be coupled with a deficiency of the equilibrative uniporter-type histamine transporters, which subjects the SGs in SS to a severe combined lack of the ligand and its receptor.

Materials and methods

Labial salivary glands

The ethics committee of the Hospital District of Helsinki and Uusimaa approved the study (19/E5/03) and all subjects gave their informed consent. Five to 10 labial salivary glands (LSGs) were collected from each subject for diagnostic purposes [9]. For real-time quantitative PCR and immunofluorescence, LSGs were snap-frozen in liquid nitrogen and stored at −80°C. For immunohistochemical analysis, LSGs were fixed in formalin and embedded in paraffin. The diagnosis of primary SS was based on the inclusion and exclusion criteria as defined in the American-European Consensus Group criteria [14]. The SS patient group consisted of one man and seven women, with a mean ± S.D. age of 53.9 ± 6.2 years, resting salivary flow of 0.6 ± 0.4 ml/15 min and focus score of 1.6 ± 0.3. SS patients were positive for at least four of the six classification criteria, one of which was focal sialadenitis in all patients. In addition, two of eight patients were SS-A and/or SS-B positive. All exclusions were performed according to the American-European Consensus Group criteria. Healthy controls included one man and five women, with a mean ± S.D. age of 47.1 ± 5.8 years and a resting salivary flow of 0.9 ± 0.3 ml/15 min. All controls had a focus score <1, and none of them fulfilled the SS classification criteria.

HSG cell culture

HSG cells were cultured as described [9] in DMEM/F-12 containing 2.5 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum.

Real-time quantitative reverse-transcription PCR

Total tissue RNA was isolated using the High Pure RNA Tissue Kit (Roche, Basel, Switzerland). Complementary DNA was synthesized using the SuperScript First Strand cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA) [9]. Total HSG cell RNA was extracted using the MirVana miRNA Isolation Kit (Ambion/Life Technologies, Grand Island, NY, USA) and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Life Technologies).

For human L-histidine decarboxylase (HDC), histamine-N-methyl transferase (HNMT) and diamine oxidase (DAO) transcripts, quantitative RT-PCR was performed using the iCycler iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the IQ SYBR Green Supermix Kit (Bio-Rad). Sequences of the primers were as follows: HDC: forward primer 5′-TTG ATT GCC CTG CTG GCA GC-3′, reverse primer 5′-TGC ACA GAC AAA GAC GGG CAC C-3′; HNMT: forward primer 5′-TGG CAT CTT CCA TGA GGA GCT T-3′, reverse primer 5′-AAA ATC CCA AAG CAG GTC AT-3′; DAO: forward primer 5′-GCT ACC GTC TCC CCT AT-3′, reverse primer 5′-CCC AGG CCA GCA GGT CCT A-3′, and β-actin housekeeping gene: forward primer 5′-TCA CCC ACA CTG TGC CCA TCT A-3′, reverse primer...
5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3'. Sequences were searched using the NCBI Entrez Search system and sequence similarities using the NCBI Blastn program. Expression levels of OCT1/SLC22A1, OCT2/SLC22A2 and OCT3/SLC22A3 were quantified as described [15, 16]. PMAT/SLC29A4 expression was quantified using a commercial kit. Messenger RNA expression was measured using TaqMan technology on a 7900HT Real-time PCR system (Life Technologies).

Transport studies
HSG cells (5 x 10^5/well) were seeded into 24-well cell culture plates and grown for 24 h. All uptake studies were carried out at 37°C as described [17]. Cells were washed with uptake buffer (130 mM NaCl, 25 mM hydroxyethylpiperazine ethanesulfonic acid, 1.2 mM KH2PO4, 4.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgSO4, 5.6 mM glucose, pH 7.4) [18] at 37°C. Uptake was initiated by replacing this with uptake buffer supplemented with 100 nM [3H]histamine (392 GBq/mM; PerkinElmer, Waltham, MA, USA), without and with 2 mM 1-methyl-4-phenylpyridinium (MPP, inhibits organic cation transporters). Uptake was stopped by three washes in ice-cold uptake buffer. Cells were lysed in 0.2% SDS and radioactivity was measured by liquid scintillation counting (Hidex 300SL TDCR, Turku, Finland).

For efflux studies, cells were pre-incubated for 60 min at 37°C in uptake buffer containing 100 nM [3H]histamine and washed three times with ice-cold uptake buffer. Efflux was initiated by addition of uptake buffer (without [3H]histamine) at 37°C. At indicated time points, the radioactivity ([3H]histamine) in the cell culture medium and cells was measured.

Immunohistochemical staining
For 4-μm tissue sections, antigen retrieval was performed using a microwave oven. Slides were rinsed in tap water and incubated in (i) 1% H2O2 for 10 min and washed 3 x 5 min in 10 mM phosphate buffer 140 mM saline (PBS, pH 7.4); (ii) 10% normal goat or horse serum for 1 h at 22°C; (iii) monoclonal mouse anti-human HDC IgG produced against the 492–506 fragment of the enzyme located in the carboxyterminal domain of the most active 53 kDa enzyme isoform (1:350; provided by F.S.-J. and J.L.U.), 1:500 diluted monoclonal affinity active 53 kDa enzyme isoform (1:350; provided by a Leica DM6000 microscope and DFC365FX camera (Leica Microsystems, Wetzlar, Germany). Specificities of the OCT2 and OCT3 antisera were confirmed by using antigen absorption controls.

Results
Histamine-metabolizing enzymes in LSG
HDC and HNMT mRNA were present in healthy human LSGs, but DAO mRNA was absent. These mRNA values were not different from those measured in LSGs in SS (Fig. 1A). Immunostaining localized HDC and HNMT immunoreactivity in LSGs to epithelial cells in the intralobular excretory salivary ducts in a similar pattern in both healthy individuals and SS patients. In addition, mast cells were strongly HDC immunoreactive (data not shown). DAO immunostaining was negative, although the positive sample controls, tissue sections of human kidney, were strongly positive (data not shown). Negative-staining controls excluded false-positive staining (Fig. 1B).

Histamine transporters in LSG
Expression levels of OCT transporters and ENT4/PMAT were measured because they have been implicated in histamine transport [20, 21]. OCT3 mRNA was present at levels >100-fold higher than OCT1, OCT2 and ENT4/PMAT mRNA, which were barely detectable in healthy and SS SGs. Healthy SGs showed higher expression of OCT3 than SS SGs (P = 0.0286, Fig. 2A).

Immunostaining disclosed OCT2 at the protein level in myoepithelial cells, which partly surround the acini and the intercalated and striated ducts. OCT2 staining was much stronger in the myoepithelial cells in LSGs in healthy controls than in patients with SS. OCT3 was immunolocalized to the cell membranes of the acinar and ductal epithelial cells in healthy LSGs, being more strongly expressed at the basolateral than at the apical domain. In contrast, OCT3 immunostaining was very weak or even totally absent in the acinar and ductal epithelial cells in LSGs in SS, although the DAPI-stained blue nuclei disclosed that these cells were organized to tubuloacinar structures (Fig. 2B).
In vitro [³H]histamine transport

In quantitative RT-PCR experiments, HSG cells expressed OCT3 mRNA at a level >10-fold higher than OCT1 and OCT2, and >2-fold higher than ENT4/PMAT, suggesting a leading role of OCT3 in histamine transport in HSG cells (Fig. 3A).

Cultured HSG cells showed time-dependent [³H]histamine uptake from the loading buffer containing 100 nM radiolabelled histamine and this uptake was inhibited by MPP (Fig. 3B). Conversely, HSG cells preloaded with 100 nM [³H]histamine released it to a histamine-free medium in a time-dependent manner (Fig. 3C and D). Experiments performed in 260 mM glucose showed that [³H]histamine uptake and efflux were sodium independent.

Discussion

The positively charged monoamine histamine is synthesized from L-histidine by the endoplasmic 53 kDa HDC in

Fig. 1 Expression of major enzymes related to histamine metabolism in the LSGs.
Fig. 2 Expression of major histamine transporters in the LSGs.

(A) mRNA levels of organic cation transporters (OCT1, OCT2, OCT3) and plasma membrane monoamine transporter (ENT4/PMAT) in salivary glands from healthy individuals (H) and SS patients. OCT3 showed the highest expression among the transporters in the salivary glands. SS samples expressed OCT3 at lower levels compared with healthy controls. (B) Double immunofluorescence staining revealed co-localization of OCT2 and smooth muscle α-actin in the myoepithelial cells (arrows in the OCT2 healthy panel). Double immunofluorescence staining showed strong basolateral (arrows in the inserts) and weak apical plasma membrane localization (arrowheads) of OCT3, counterstained with human smooth muscle α-actin. SS samples showed much weaker expression of OCT2 and an almost total lack of OCT3 (arrows in the inserts). Scale bars: 50 μm.
professional histamine-synthesizing cells into storage granules, from which it can be rapidly released to reach momentarily high local concentrations as a result of a burst release [1]. These concentrations are high enough to stimulate the conventional low-affinity H1R and H2R and apparently reach 50–150 μM [7]. Such professional cells include mast cells, basophils and enterochromaffin-like cells.

Recently attention has been paid to non-professional histamine-producing cells. They utilize the cytoplasmic 74 kDa HDC isoform to synthesize histamine, but at a ~100–1000-fold lower rate than the professional cells [1]. This histamine is not stored after synthesis but is released into the cytoplasm of the cell, from where it seems to pass into the extracellular space. Because histamine is a monovalent cation at the physiological pH, it needs transporters to cross the plasma membrane. OCT3 is the prime histamine transporter [22] and was highly expressed at the mRNA level in SGs [23] and seems to be responsible for histamine transport across the membrane of the non-professional histamine-producing cells. Our mRNA analyses confirm that OCT3 is the major OCT transporter expressed in human SGs, while ENT4/PMAT [21] was virtually not expressed. Immunolocalization showed a predominantly basolateral localization of OCT3 in the salivary epithelial cells, supporting our hypothesis that histamine produced within the cells can be released via OCT3. In common with other OCTs, OCT3 forms an electrogenic facilitative diffusion system that translocates organic cations in both directions across the plasma membrane [20]. Once outside the cell, the histamine generated by the non-professional histamine-producing cells binds effectively to its high-affinity receptors. It is concluded that the non-professional histamine-producing cells maintain the basal level histamine in tissues and body fluids. Indeed, the resting plasma concentration of histamine is 0.3–1.0 ng/ml (2.7–9 nM) [24] and in saliva is 0.31–12.4 ng/ml (2.8–112.7 nM;
1 nM = 0.11 ng/ml) [25]. Amounts in saliva are enough to stimulate the high-affinity H3R (pKi 7.8) and H4R (pKi 8.3) half-maximally, but are too low to affect the conventional low-affinity H1R and H2R, which require ~10,000-fold higher concentrations for activation [7]. This is the first study to show, using monoclonal anti-human HDC antibodies, that normal human LSG excretory duct cells contain HDC in excretory ductal cells and have histamine-synthesizing potential. This extends observations on the presence of HDC in the excretory duct cells in rat submandibular glands [26], but contradicts the universal and indiscriminate presence of HDC in all submandibular gland epithelial cells as was seen in staining with polyclonal anti-rat HDC [27], which might result from cross-reactivity or false-positive staining.

Regarding OCTs, a normalized human RNA master plot disclosed high levels of OCT3 (SLC22A3) mRNA in non-defined human SG extracts [23] and now in the present work OCT3 and OCT2 have been located to acinar and myoepithelial cells, respectively.

Mast cells are increased in SS [28]. Professional histamine-producing cells can momentarily release enough histamine to activate the conventional low-affinity H1Rs and H2Rs. This is compatible with our finding of H1R on human vascular endothelial cells [29]. Stimulation of these cells via H1R increases cytosolic calcium activation of endothelial nitric oxide synthase (eNOS) [30] and production of NO. This leads to vasodilation and increased vasopermeability, seen in skin as the wheal and flare response (urticaria) upon mast cell activation. SGs did not contain extracellular histamine degrading DAO. It was therefore of interest that 100 nM radiolabelled histamine was effectively taken up by HSG cells from the extracellular medium. A 10,000-fold higher histamine concentration after burst release will probably also lead to rapid uptake of histamine by the OCT2/OCT3-equipped cells [31]. Down-regulation of the activated, high-histamine state is then mediated by the intracellular HNMT-mediated degradation [32, 33], which works optimally first at relatively high histamine concentrations ($K_m = 6-13 \mu M$) [24]. Thus a new role is suggested for the ductal OCT3+ cells, a role in the intracellular degradation of histamine released from mast cells in human SGs. Histamine is also taken up by mast cells via OCT3, but there the cytoplasmic histamine is taken up by the recovering storage granules via active VMAT2-mediated transport [1]. This conclusion is also supported by in vitro uptake studies, which show effective uptake of 100 nM histamine by cultured HSG cells. Together with immunostaining results, inhibition of this uptake in functional studies by MPP, a monovalent model cation and histamine analogue with a higher affinity for OCT3 than histamine ($K_m = 47 \mu M$ vs $220 \mu M$) [34] suggests a leading role for OCT3 in histamine uptake in human SGs, although these cells also express lower levels of ENT4/PMAT mRNA.

**Fig. 4** Different conditions of the salivary gland epithelial cells.

(A) A healthy salivary gland at a resting state equipped with intracellular histamine synthesizing HDC. Newly synthesized histamine is transported into the extracellular space via OCT3, where its concentration is high enough to activate histamine receptor 4 (H4R). (B) A healthy salivary gland after mast cell granulation in a stimulated state. Excess extracellular histamine is taken up by the salivary duct epithelial cells via OCT3 and degraded intracellularly by HNMT or passes via the basolateral and apical equilibrative uniporters (OCT3) to saliva. (C) In SS at the resting state, histamine is normally synthesized, but due to the lack of OCT3, it cannot pass outside the extracellular space, and even if it could, the deficiency of the high-affinity H4R restrains stimulation. In SS, at the stimulated high-histamine state after mast cell degranulation histamine cannot be effectively taken up by the cells for subsequent intracellular degradation or transcellular excretion (not shown).
In healthy SGs, OCT3 was localized in the basolateral and more weakly in the apical plasma membrane of the acinar and ductal epithelial cells, compatible with its polarization [15]. OCT2 was restricted to the smooth muscle α-actin containing myoepithelial cells. At the basal resting state, the strong basolateral OCT3 localization would allow histamine efflux from ductal epithelial cells and subsequent binding to acinar and ductal cell H4R [9] (Fig. 4A).

The predominantly basolateral organization of OCT3 allows the handling of burst-released high histamine concentrations by acinar and ductal epithelial cells by two different mechanisms: intracellular, HNMT-mediated degradation in ductal cells (Fig. 4B) and salivary excretion via the apical OCT3, to which also an eventually apically located ENT4/PMAT could contribute. Even without transport to saliva, OCT3-mediated epithelial cell uptake could down-regulate extracellular histamine by sequestration independent of enzymatic degradation. The present findings suggest that histamine functions at the basal (constitutive) low-level histamine/high-affinity receptor interaction state [9] and at the stimulated high-level histamine/low-affinity receptor interaction state after regulated burst release. At their interface, down-regulation of the stimulated high-level histamine state via ductal cell-mediated, HNMT-dependent intracellular degradation and via acinar and ductal cell-mediated OCT3 (or ENT4/PMAT)-dependent excretion to saliva. Both the constitutive low and stimulated high histamine interactions seem to be severely disturbed in SS, due to the almost total lack of H4R and OCT3, respectively.

Bone marrow-derived leukocytes, such as antigen-presenting dendritic cells [12] and T and B lymphocytes [13, 35], belong to non-professional histamine-producing cells. This finding was first considered insignificant. This changed upon the detection of the high-affinity histamine receptors, found in both dendritic cells and lymphocytes [6, 36]. Therefore histamine may also have effects on local immune reactions and lymphocyte infiltrates in SS.

In conclusion, intralobular salivary excretory duct epithelial cells are non-professional histamine-producing cells. At the basal constitutive state, they seem able to release enough histamine via OCT3 to the extracellular fluid to stimulate H4R. If professional histamine-producing mast cells are locally activated, the conventional low-affinity H4R is also activated. At the same time, ductal, acinar and myoepithelial cells take up histamine via OCT2/3 for intracellular degradation, salivary excretion and/or intracellular sequestration. In SS, the basal maintenance phase seems to be disturbed due to the almost total lack of H4R in the acinar and ductal epithelial cells [9]. At the basal maintenance phase in SS, histamine produced by the non-professional ductal epithelial cells cannot be effectively released to stimulate salivary epithelial cells via H4Rs. In other words, at the resting phase impaired histamine release and lack of H4Rs both contribute to the same acinar/ductal cell maintenance and functional defect, therefore potentially contributing to SS development, maintenance and progression. In SS, the down-regulation of histamine after burst release seems to be disturbed due to a deficiency of OCT3 in acinar and ductal cells and of OCT2 in the myoepithelial cells. The monoamine transport system in SGs from SS patients is dramatically altered, identifying new drug targets in this autoimmune disease. Although the treatment of many other rheumatic diseases has greatly improved as a result of the introduction of biologics, there has been little progress in the treatment of SS except for some promise with rituximab treatment. Because low-level histamine/high-affinity H4R seems to be so important for the maintenance of the tubuloacinar glands and for the dendritic cell and lymphocyte-mediated immune responses against them, there is hope that some new synthetic small molecular weight H4R-modulating chemical drugs can in the near future provide a new paradigm for the treatment of SS targeting both resident structural and immigrant inflammatory cells.

**Rheumatology key messages**
- Non-professional histamine-producing cells were found in salivary glands in SS.
- Non-professional salivary epithelial cell histamine-N-methyltransferase also metabolizes histamine burst-released by mast cells.
- Impaired histamine transport in the salivary glands in SS may contribute to glandular pathology.

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