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Mammary histidine decarboxylase vulnerability to enzyme antisense oligonucleotides: Histamine and polyamine systems cross-talk

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Summary. Histamine system is suggested to have a role in mammary gland growth regulation, differentiation and functioning during pregnancy and lactation. Histidine decarboxylase activity undergoes significant changes during pregnancy and lactation. Pregnancy associated elevation of HDC activity and mRNA transcript in mouse mammary gland was successfully affected by enzyme antisense oligonucleotides treatment. The enzyme activity of resting mammae was unaffected as it lacked inducible pool of HDC. The short-term mammary histamine shortage evoked influenced the mRNA expression of histamine receptors (H_1 and H_2) and ornithine decarboxylase during pregnancy. There were essentially no morphological changes in the mammary gland upon the treatment, however, adipocytes neighbouring alveolar structures were more pronounced. These findings further substantiate the role of histamine in mammary gland physiology and emphasise presence of common motifs of biogenic amines and polyamine metabolism as well as mutual interferences implicating observed "cross-talk" phenomenon.

Keywords: Mouse mammary gland – Antisense oligonucleotides – L-Histidine decarboxylase – Ornithine decarboxylase – L-Aromatic acids decarboxylase – Histamine – Histamine receptors

Introduction

The biogenic amines and polyamines-histamine, dopamine, serotonin, putrescine, spermidine and spermine are involved in human physiology (smooth muscle contraction, gastric acid secretion, cell growth, neurotransmission and inflammation) as well as pathologies for example carcinogenesis, tumor invasion, Alzheimer, Parkinson, schizophrenia, anorexia, obesity and others (Medina et al., 2003). The amine actions are mostly mediated through the activation of membrane-associated G protein coupled to their respective receptors. However, the compounds also play their pleiotropic roles inside a cell maintaining macromolecular synthesis and cell proliferation rates.

Histamine and polyamine systems are both thought to be involved in normal and neoplastic tissue growth as well as in differentiation processes (Medina et al., 1999). In the mammary gland histamine is suggested to play a role in regulation of pregnancy associated growth and differentiation and functioning during lactation whereas spermidine is linked to the milk production (Maslinski et al., 1993; Russell and McVicker, 1972). The available data suggest in mammae two pools of histamine are present mastocyte- and epithelial cell related ones (Wagner et al., 2001, 2002). The coexistence of histamine producing cells and the tissue histamine receptors distribution (Wagner et al., 2003a) are in line with the amine importance in mammary evolution and function (Fig. 1). Histamine synthesis in the mammary gland proceeds by L-histidine decarboxylation, a reaction which is catalyzed by specific enzyme, L-histidine decarboxylase (HDC, EC 41.1.22). L-amino acids decarboxylase (L-AADC, EC 4.1.1.28) also present in mammae and highly specific to substrates: L-DOPA and 5-hydroxytrotophan is regarded as a minor contributor.

In order to get better insight into functional relevance of histamine synthesis in pregnancy associated growth of mammary gland we persued study at inducing short-term histamine deficiency in mouse by applying specific HDC antisense oligonucleotides.

Materials and methods

Animals

Virgin female mice (Mus musculus) (BALB/C, 20 g body wt) were used. Animal procedures complied with the Polish legislation concerning experiments on animals and were approved by the local care committee.

Pregnancy dating

Female mice were housed with males of proven fertility for three days accompanied by daily microscopic examination of vaginal smears. The

A

Enzyme/ Receptor	mRNA expression* (%) / enzyme activity** (%) Mouse mammary gland		
	Resting	11th day of pregnancy	17-19th day of pregnancy
HDC		200*	57* / 74**
ODC		118*	419*
L-AADC[1]	100%	nt	71**
H_1		168*	244*
H_2		bd	94*

[1] activity measured against L-histidine; *nt*, not tested; *bd*, below detection

B

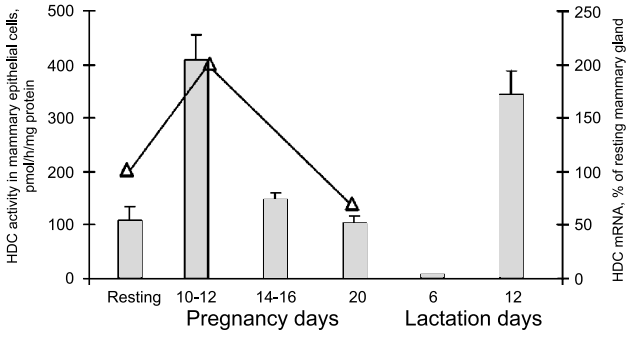

Fig. 1. **A** Enzymes involved in the biosynthesis of biogenic amines and histamine receptors in mammary gland in relation to functional development of mammae. **B** Left axis: L-Histidine decarboxylase (*HDC*) activity in cultured mammary epithelial cells derived from female mice mammary glands of different physiological stages. Right axis: mammary gland development related HDC mRNA transcript level (. . . Δ . . .)

presence of spermatozoa and/or vaginal plug was regarded as the first day of pregnancy. Pregnancy day was further verified at autopsy by embryo development stage.

Oligonucleotides

An antisense 29-mer phosphorothioate oligodeoxynucleotide ([S]ODN), relative to the translation initiation codon of mouse L-histidine decarboxylase mRNA (nucleotides 62 to 90, 5′-C ACA GGG CTC CAT CAT CTC CCT TGG ATT G-3′) was designed using the computational simulation and mfold 3.1 software (Zuker et al., 1999) based on the sequence data of HDC cDNA (Accession X57437). Sense [S]ODN identical to the targeted HDC mRNA sequence served as a specificity control. [S]ODNs used were Na^+-salts and purified by mean of HPLC.

The efficacy of the antisense probe was verified in previous optimalization experiments *in vitro* (Wagner et al., 2003b).

[S]ODNs administration

2 h prior to each experiment miniosmotic pumps (Alzet 1007D; Alza, Palo Alto, CA, USA) were loaded with [S]ODNs dissolved in sterile 0.9% NaCl and stored in saline until implantation. Virgin and pregnant (10–12th pregnancy day) mice were anesthetized and miniosmotic pumps with either antisense or sense [S]ODNs were inserted subcutaneously into a paraspinal pocket. The pumps were set to deliver 12 nmoles/24 h (5.25 mg/body wt./24 h) of [S]ODNs for 7 days. Following a week [S]ODNs infusion mice were decapitated and abdominal glands dissected.

L-Histidine decarboxylase-, L-aromatic acids decarboxylase-, histamine assays

HDC and L-AADC activities were assayed essentially as described previously (Wagner et al., 2003a) except enzymes activities were measured against L-histidine in the presence of appropriate enzyme inhibitor i.e. alpha-methylDOPA (L-AADC inhibitor, final conc. 200 μM) or (S)-alpha-fluoromethylhistidine (HDC inhibitor, 100 μM), respectively. Formed histamine was estimated by radioenzymatic method and the enzymic activity was expressed in pmol histamine synthesized per hour of incubation per mg protein.

For estimation of the endogenous histamine concentration the tissue homogenate was spun at 1000 g and obtained supernatant deproteinized (100°C, 10 min). Histamine concentration was measured as in enzyme activity assays, radiometrically and expressed in nmol/mg protein.

Evaluation of the transcripts levels of L-histidine decarboxylase, ornithine decarboxylase (ODC), H_1 and H_2 receptors

The levels of transcripts of histidine decarboxylase, ornithine decarboxylase, H_1 and H_2 receptors were evaluated by using commercially available DNA microarray-GE Array Kit (SuperArray Inc., USA). In brief, RNA was extracted from abdominal mammary gland tissue with TRI reagent (Sigma Chemical Co., St. Louis, MO, USA) and proceeded to one run reverse transcription (M-MLV RT 400 U, Rnase inhibitor 40 U, 1 mM dATP, dGTP, dTTP, 10 μM dCTP, all reagents Promega Corporation, USA) and cDNA labeling (50 μCi [α-^{32}P]dCTP, 10 mCi/mmol, Amersham Pharmacia Biotech, UK) at 42°C for 25 min. Genes cDNA spotted on membranes were hybridized with labeled probe and denaturated salmon testes DNA (100 μg/ml, Sigma Chemical Co., St. Louis, MO, USA) in hybridization buffer overnight at 68°C. Washed membranes were enclosed in polyethylene bags and exposed to Storage Phosphor Screen (Kodak Eastman, Rochester, NY, USA) for 5 days. Densytometrical analysis was performed with Typhoon 8600 Variable Mode Imager and ImageQuant software (Molecular Dynamics, USA).

Histochemistry

Tissue morphology was examined by light microscopy after routine hematoxylin-eosin staining of 15 μm paraffin sections.

Data analysis

The data are presented as mean ± SEM for n experiments. Comparisons among groups were carried out using analysis of variance (ANOVA) and *post hoc* Newman-Keuls test.

Results

HDC-, L-AADC activity and histamine concentration in mammary gland after HDC antisense oligonucleotide treatment

Treatment of pregnant mice with antisense [S]ODN resulted in reduced HDC activity in mammary gland as measured at 17–19th day of pregnancy and compared to sense oligonucleotides-treated and non-treated control counterparts. The enzyme activity was decreased down to 42% of that seen in the control group (Fig. 2A). Non-specific inhibitory effects of sense oligonucleotides

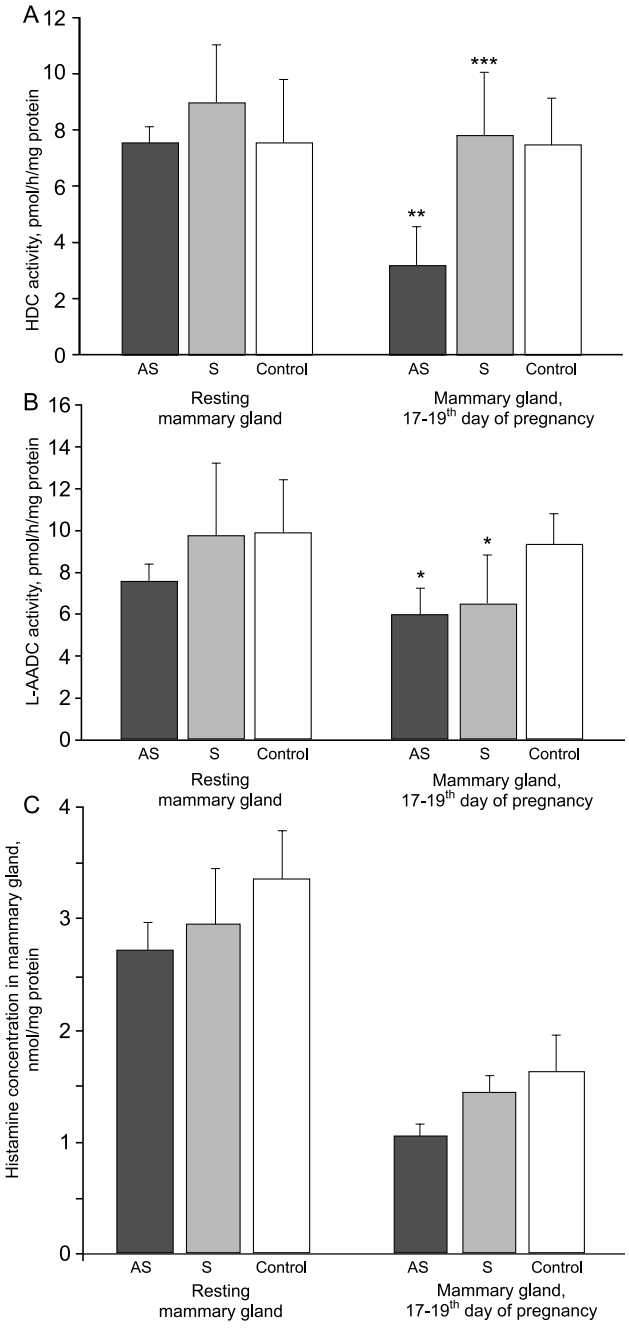

Fig. 2. HDC (**A**), L-AADC (**B**) activities and histamine concentration (**C**) in mammary gland after antisense/sense oligonucleotides treatment of pregnant and virgin mice. *AS*, antisense oligonucleotide; *S*, sense oligonucleotide; *Control*, non-treated animals. Values represent the mean ± SEM of 4–7 determinations. $^{*}p<0.05$, $^{**}p<0.001$ vs respective Controls; $^{***}p<0.001$ vs respective AS treated counterpart

on HDC activity of pregnancy mammary gland were not found.

On the other hand, antisense oligonucleotides did not exert any inhibitory effect on HDC activity in mammary gland of virgin mice. In contrast, in mammary glands of virgin mice applied HDC sense [S]ODN increased the enzyme activity by 20%.

Unexpected effects of the probes relevant to aromatic L-amino acids decarboxylase (sharing less than 50% of homology) were observed in pregnancy mammary glands (Fig. 2B). Both antisense and sense [S]ODNs exerted inhibitory effects on L-AADC activity in pregnancy mammary gland decreasing it down to 64% and 67% of control group, respectively. Decrease of L-AADC activity by 23% after treatment with antisense [S]ODNs was found in mammary gland of virgin mice.

In mammary gland of pregnant mice (Fig. 2C) the treatment with antisense [S]ODNs was associated with 36% decrease versus 12% decrease of the tissue histamine concentration found after application of sense [S]ODNs. In mammary gland of virgin mice treated with either antisense or sense [S]ODNs the decrease in histamine concentration by 19% and 13%, respectively, was recorded.

HDC, ornithine decarboxylase (ODC), H_1 and H_2 receptors mRNA expression levels in pregnant mice treated with [S]ODNs

Densytometric analysis showed decrease in tissue HDC, ODC and H_1 transcripts but not H_2 receptor mRNA upon antisense [S]ODN treatment (Table 1). Thus, HDC, ODC and H_1 receptor mRNA levels were reduced by 32%, 14% and 19%, respectively and H_2 receptor mRNA was increased (9%). While HDC transcript level in mammary glands of pregnant mice treated with sense [S]ODN was not altered, ODC and H_1/H_2 mRNAs were either up-regulated or down-regulated, respectively.

Histological examination

Morphological analysis of the mammary glands showed no gross changes in the structure of epithelial cells and

Table 1. Quantitation of the HDC, ODC, H_1 and H_2 mRNA levels in mammary gland of antisense (AS) and sense (S) oligonucleotide treated mice by densitometric analysis, normalized to GAPDH mRNA expression and expressed as % of control (not treated animals); $n \leq 3$

Enzyme/receptor	mRNA expression (%)		
	AS	S	Control
HDC	68	104	
ODC	86	147	100
H_1	81	bd	
H_2	109	76	

bd, below detection

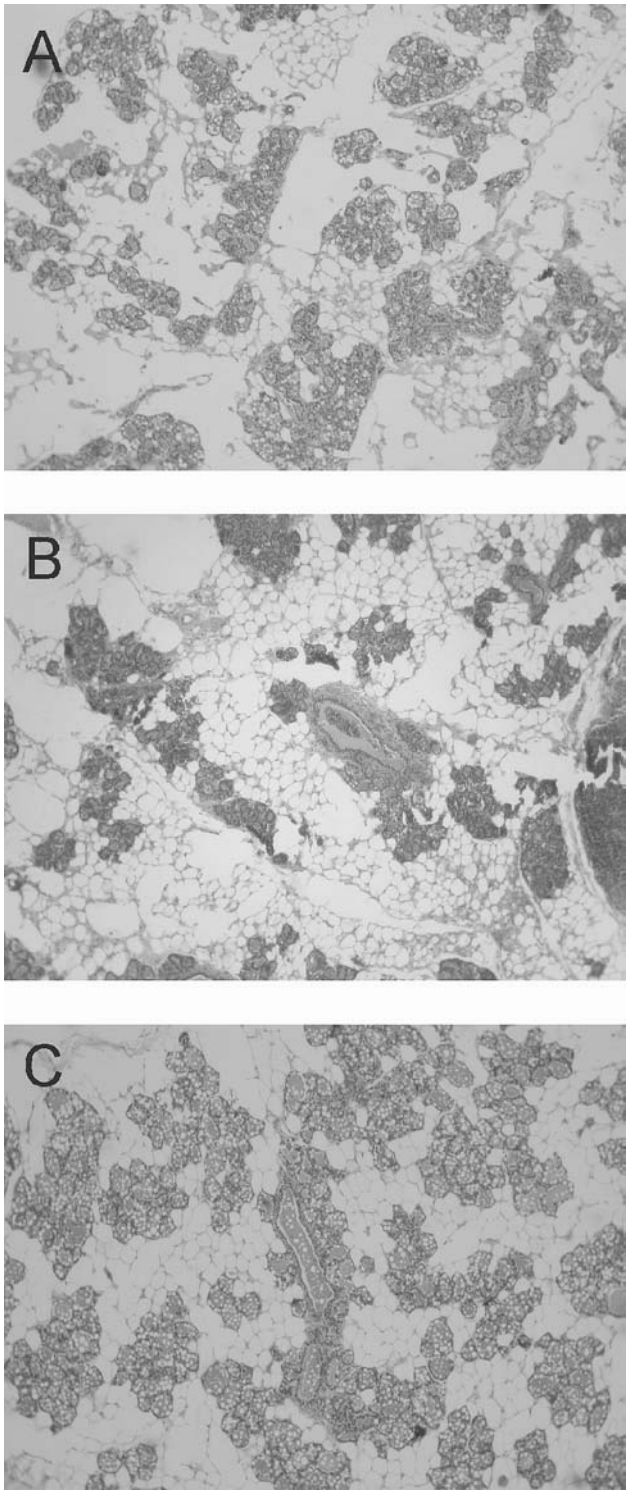

Fig. 3. Hematoxylin-eosin stained sections of mammary gland of pregnant mice non-treated (**A**), treated with sense oligonucleotides (**B**) and treated with antisense oligonucleotides (**C**). Magnification: 100×

alveolar tree between the treated and untreated animals. However, in mammary gland of pregnant mice treated with antisense [S]ODNs the presence of the high-fat containing adipocytes in the interstitial tissue was more pronounced than in corresponding control counterparts (Fig. 3C).

Hematoxylin and eosin staining of resting mammary gland of mice treated with either antisense or sense [S]ODNs revealed the infiltration of lymphocytes along blood vessels and both lymphocytes and histocytes in the vicinity of alveolar and ductual structures.

Discussion

Previous biochemical and immunohistochemical studies revealed the presence of histamine of epithelial origin in mammary gland and showed that epithelial cells possess specific histidine decarboxylase as well as bear histamine receptor H_1. Epithelial histamine was suggested as a co-mediator in mammae growth and differentiation control (Maslinski et al., 1993; Wagner et al., 2003a). To evaluate the functional relevance of histamine synthesis during pregnancy associated outgrowth of mammary gland we designed specific antisense oligonucleotides against HDC mRNA expressing only peripheral action and not crossing Blood Brain Barrier (Agrawal et al., 1991) in contrast to commonly applied suicide HDC inhibitor, (S)α-fluoromethylhistidine (Skratt et al., 1994). We have taken into account that brain histamine affects hypothalamus-pituitary-adrenal axis neuroendocrine activity thus, hypothalamic histaminergic neurons might contribute to (in)direct stimulation and modulation (via postsynaptic H_1, H_2 and presynaptic H_3 receptors) of mammotropic hormones release (prolactin, oxytocin and cortisol) having impact on mammae growth and function (Bugajski et al., 2000).

We have succeeded in diminishing activity of HDC by antisense [S]ODN treatment in mammary gland of pregnant mice (Fig. 2A, Table 1). 7th-day infusion of antisense oligonucleotide-HDC0062 evoked 32% drop down of HDC transcript which was directly followed by 58% HDC activity decrease and resulted in lowered histamine concentration in mammary gland (by 36%) as compared to non-treated animals (Fig. 2C). These data suggest that the successful treatment affected inducible pool of mRNA HDC present in pregnant and absent in virgin mice. Inducible pool of HDC is characterized by great amount of mRNA-target for antisense [S]ODN and fast turnover of enzyme protein (Medina et al., 2003) – features, which are favourable for efficient action of antisense oligonucleotide.

Reduced HDC activity and histamine concentration in mammae of antisense [S]ODN treated pregnant mice

coincided with reduced level of H_1 mRNA. It was previously shown that skin H_1 receptors are very sensitive to endogenous histamine concentration (Fitzsimons et al., 2001). HDC knockout mice fed histamine-free food showed totally abolished H_1 skin receptors providing evidence for possible similar phenomenon of histamine shortage induced H_1 receptor decrease in skin-originated mammary gland. On the other hand, short-term mammary histamine shortage caused up-regulation of H_2 receptor suggesting compensatory feedback of mammary gland and tissue histamine sensitivity increase. Histamine dependent mechanism of histamine H_1 and H_2 receptors expression may be considered. Either histamine receptor was affected by sense [S]ODN indicating some side-effects of phosphorothioate oligonucleotides.

Although there were no gross changes in morphology under reduction of histamine synthesis, fat rich cells were more pronounced in the antisense oligonucleotides treated mice. Interestingly enough, lipolysis in adipocytes has been shown to be regulated by histamine via H_2 receptors (Carpene et al., 2001). We speculate that histamine synthesis manipulation might affect adipocyte maturation or lipolysis-lipogenesis balance.

In case of ODC, antisense [S]ODN treatment down-regulated enzyme mRNA expression by 14%, reverse effect induced infusion of sense [S]ODN increasing ODC transcript level by 47%. It seems that, polyamine system activity may (in)directly respond to mammary histamine system manipulations indicating so-called ''cross-talk'' phenomenon of histamine and polyamine systems being involved in tissue growth. Both, histamine and polyamine systems share their roles in cell differentiation and mitotic activity of normal and neoplastic cells (Medina et al., 1999) and compete for the same crucial substrate: S-adenosyl-L-methionine, which is a donor of aminopropyl group for spermidine/spermine synthesis and a high-energy methyl group donor in N-methyltransferase mediated histamine catabolism. Thus, S-adenosyl-L-methionine availability could be a limiting factor and play an important role in biogenic amine metabolism regulation in ''amine-handling'' cells. It is also worth mentioning that oxido-reduction reactions catalyzed by enzymes: diamine oxidase [DAO, not present in mammae, Maslinski et al. (1993)] and aldehyde dehydrogenase are also involved in routs of histamine and putrescine polyamine precursor degradation.

Seen L-AADC activity decrease in [S]ODNs treated pregnant mice account for unexpected, non-specific action of oligonucleotides with enzyme protein (Levin, 1999) rather than enzyme – specific effects related to amino acids decarboxylases interplay. Thus, we could not verified thesis of L-AADC activity increase toward L-histidine decarboxylation after HDC inhibition.

In summary, provided results suggest that HDC antisense oligonucleotide treatment was successful and affected inducible pool of histamine of epithelial origin HDC, associated with mammary gland morphogenesis of pregnant mice but not virgin ones. Histamine synthesis shift led to adaptive response of H_2 histamine receptor expression and corresponded with changes in stromal interstitial cells appearance. Data add more evidence for histamine and polyamine systems interactions – ''cross talk'' and further substantiate role of H_1 histamine receptor in pregnancy associated mammary development.

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