Experimental Animals

Original

Cholinergic- rather than adrenergic-induced sweating play a role in developing and developed rat eccrine sweat glands

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Abstract: Both cholinergic and adrenergic stimulation can induce sweat secretion in human eccrine sweat glands, but whether cholinergic and adrenergic stimulation play same roles in rat eccrine sweat glands is still controversial. To explore the innervations, and adrenergic- and cholinergic-induced secretory response in developing and developed rat eccrine sweat glands, rat hind footpads from embryonic day (E) 15.5–20.5, postanal day (P) 1–14, P21 and adult were fixed, embedded, sectioned and subjected to immunofluorescence staining for general fiber marker protein gene product 9.5 (PGP 9.5), adrenergic fiber marker tyrosine hydroxylase (TH) and cholinergic fiber marker vasoactive intestinal peptide (VIP), and cholinergic- and adrenergic-induced sweat secretion was detected at P1–P21 and adult rats by starch-iodine test. The results showed that eccrine sweat gland placodes of SD rats were first appeared at E19.5, and the expression of PGP 9.5 was detected surrounding the sweat gland placodes at E19.5, TH at P7, and VIP at P11. Pilocarpine-induced sweat secretion was first detected at P16 in hind footpads by starch-iodine test. There was no measurable sweating when stimulated by alpha- or beta-adrenergic agonists at all the examined time points. We conclude that rat eccrine sweat glands, just as human eccrine sweat glands, co-express adrenergic and cholinergic fibers, but different from human eccrine sweat glands, cholinergic- rather than adrenergic-induced sweating plays a role in the developing and developed rat eccrine sweat glands.

Key words: adrenergic nerves, cholinergic nerves, eccrine sweat glands, Sprague-Dawley rats, sweat secretion

Introduction

In humans, skin and its appendages, including eccrine sweat glands, hair follicles, and sebaceous glands, play important functions to protect our body from harmful things in the outside world such as the hot, sun rays, germs and toxic substances [1, 2]. Eccrine sweat gland is one of the important appendages and is distributed almost all the skin [1]. The main function of eccrine sweat gland is to regulate body temperature through sweat secretion [1–3]. Human eccrine sweat glands are innervated by large numbers of cholinergic fibers and a few adrenergic fibers [1, 4, 5]. Both in vivo and in vitro studies demonstrated that cholinergic agonist methacholine, alpha-adrenergic agonist phenylephrine, and beta-adrenergic agonist isoproterenol all can induce sweat secretion of human eccrine sweat glands [6–8].

In patients with hypohidrosis or anhydrosis, by inherit or skin injury or nerve damage, their eccrine sweat glands are no longer functioning properly, which cause overheating of their body, and lead to heat cramps, heat exhaustion or even heatstroke [2, 9]. Therefore, it is
necessary to repair the damaged eccrine sweat glands and their nerves, and regenerate the lost ones. To study on the repair and regeneration of the eccrine sweat glands and dwell on the underlying mechanisms, laboratory animals are often used. Among the laboratory animals, rat and mouse, are the widely used rodents. Different from humans, the eccrine sweat glands of rat/mouse, are concentrated in footpads, and their primary functions are to increase friction and enhance grip rather than cool body temperature [10–13]. However, the innervation of rat/mouse sweat glands, and whether cholinergic and adrenergic stimulation both play roles in rat/mouse eccrine sweat glands are still controversial [14–16]. To explore the question, the innervations and adrenergic- and cholinergic-induced secretory response in developing and developed rat eccrine sweat glands were investigated.

Materials and Methods

Rat experiments

Virgin female and male Sprague-Dawley (SD) rats, weighing 180–200 g, were obtained from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and maintained on a 12 h light/dark cycle with food and tap water ad libitum. The room was maintained at 22 ± 1°C with low humidity. One week later, females were caged with males (ratio 4:1) at night. Pregnancy was confirmed by the formation of a copulatory plug and the first morning after getting pregnant was designated as embryonic day (E) 0.5. Once the female rats were demonstrated to be pregnant, they were caged separately. Parturition typically occurred at E21.5 days of gestation. Rat hind footpads from E15.5–20.5, postanal day (P) 1–14, P21 and adult were collected, conventionally fixed in 4% paraformaldehyde, embedded in paraffin, and cut for immunofluorescence staining. The number and sex of the rats used was shown in Table 1. Sweat test was performed by iodine-starch method before footpads were collected in postnatal rats. At each time point, at least three rats from different litters were tested or collected, and the specific number and sex of the rats used was showed in Table 1. The number of rats and procedures used in this study were in accordance with the guidelines for Ethical Conduct in the Care and Use of Animals, and all animal study protocols were approved by the Hubei University of Medicine-Animal Care and Use Committee (The approval number: 2020-007).

| Time points | Number | Sex |
|-------------|--------|-----|
| E15.5       | 6 from 3 different litters, 2 in each litter | NA |
| E16.5       | 6 from 3 different litters, 2 in each litter | NA |
| E17.5       | 6 from 3 different litters, 2 in each litter | NA |
| E18.5       | 6 from 3 different litters, 2 in each litter | NA |
| E19.5       | 6 from 3 different litters, 2 in each litter | NA |
| E20.5       | 6 from 3 different litters, 2 in each litter | NA |
| P1          | 3 from 3 different litters, 2 in each litter | 3 3 |
| P2          | 3 from 3 different litters, 2 in each litter | 3 3 |
| P3          | 3 from 3 different litters, 2 in each litter | 3 3 |
| P4          | 3 from 3 different litters, 2 in each litter | 3 3 |
| P5          | 3 from 3 different litters, 2 in each litter | 3 3 |
| P6          | 3 from 3 different litters, 2 in each litter | 3 3 |
| P7          | 3 from 3 different litters | 2 1 |
| P8          | 3 from 3 different litters | 2 1 |
| P9          | 3 from 3 different litters | 2 1 |
| P10         | 3 from 3 different litters | 2 1 |
| P11         | 3 from 3 different litters | 2 1 |
| P12         | 3 from 3 different litters | 2 1 |
| P13         | 3 from 3 different litters | 2 1 |
| P14         | 3 from 3 different litters | 2 1 |
| P21         | 3 from 3 different litters | 2 1 |
| Adult       | 3 from 3 different litters | 2 1 |

NA, not applicable.
min, then slowly cooled to room temperature for antigen
retrieves, and incubated with 10% normal goat serum
(C0265, Beyotime, Jiangsu, China) in 37°C PBS for 30
min to block non-specific sites. Subsequently, the sec-
tions were incubated respectively with the following
primary antibodies: mouse anti-PgP9.5 (ab8189, 1:100
dilution, Abcam, Cambridge, MA, USA), rabbit anti-TH
(T8700, 1:1,000 dilution, Sigma, St. Louis, MO, USA)
and rabbit anti-VIP (1:1,000 dilution, 20077, Immuno-
star, Hudson, WI, USA), at 4°C overnight, followed by
incubation with HRP-labeled goat anti-rabbit secondary
antibody (A0208, Beyotime) or HRP-labeled goat anti-
mouse secondary antibody (A0216, Beyotime) for 1h at
room temperature, and then incubation with fluorescein
isothiocyanate (FITC)-labeled tyramide (1:50) in a 1×
amplification solution (Perkin Elmer, Wellesley, MA,
USA) for 10 min in the dark at room temperature. Fi-
ally, sections were counterstained with 5 µg/ml 4′,
6-diamidino-2-phenylindole (Dapi, Beyotime) for 10
min at room temperature in the dark and mounted with
antifade mounting medium (Beyotime). normal rabbit
or mouse isotype IgG instead of the primary antibody
was used as the negative control. Sections were washed
three times with PBS between steps.

Sweat tests

The iodine-starch sweat test was carried out in P1-21,
and adult rats as follows. Iodine/alcohol solution at 2%
was applied to rat palmar surface of the restrained rats.
After the alcohol evaporated, a suspension of 1 g starch/1
ml castor oil was applied. Subsequently, cholinergic
agonist pilocarpine (0.2 mg/kg body weight) [17], and
adrenergic agonists adrenaline hydrochloride, isoprena-
line hydrochloride or noradrenaline bitartrate, at the
doses of 0.01 mg/kg body weight, 0.1 mg/kg body
weight, 0.2 mg/kg body weight, or 1 mg/kg body weight,
were injected intraperitoneally to individual rat and
sweating was recorded [16]. Rats injected with 0.9%
saline intraperitoneally was used as negative controls.
Fine black dots appeared on footpads and at the toe tips
indicating actively sweating.

Results

Eccrine gland placodes were not found at E15.5–18.5
(Figs. 1 A and B) [18]. Epidermal basal cells in footpads
begin to invaginate into the dermis at E19.5, indicating
the formation of sweat bud placodes (Fig. 1C) [18]. With
time progressed, cells at the tip of the sweat gland buds
grew downwards and differentiated into duct cells (Figs.

Fig. 1. Immunofluorescence staining for protein gene product 9.5 (PGP 9.5) at different development time point of rat eccrine sweat
glands. Expression of PGP 9.5 in rat eccrine sweat glands at E17.5 (A), E18.5 (B), E19.5 (C), E20.5 (D), P1-P14 (E–R), P21 (S),
adult (T) and negative control (U). From the time of rat sweat bud placodes formation at E19.5 to adulthood, PGP 9.5 was de-
tected surrounding the eccrine sweat glands (C–T). Normal mouse isotype IgG instead of the primary antibodies was used as the
negative control, and there was no positive staining in negative control (U). The eccrine gland structures were labeled by aster-
isk. Scale bar is 20 µm.
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1D–F) [18]. As the straight duct proceeded, it formed an early coiled gland from its tip at about postnatal day 3 (P3) [Fig. 1G] [18].

The immunostaining results of different samples at the same time point were consistent, and no staining was observed in negative control (Figs. 1U, 2N and 3H). At E17.5 and E18.5, general sympathetic fiber marker PGP 9.5 was detected at dermal-epidermal junction (Figs. 1A and B). At E19.5, a time point of sweat bud placodes formation, general sympathetic fibers were detected surrounding the placodes (Fig. 1C). As morphogenesis proceeded, general sympathetic fibers embraced the developing and developed eccrine sweat glands (Figs. 1D–T). The expression of adrenergic fiber marker TH was undetectable until P7 in sweat glands of rat hind footpads (Figs. 2A–F), and pronounced from P10 on (Figs. 2G–M). VIP-positive fibers were visible, for the first time, at P11 in eccrine sweat glands of rat hind footpads (Fig. 3B), and the immunoreactivities of VIP were unequivocally detectable at P12 (Fig. 3C). From P12 on, VIP-positive fibers were appeared readily surrounding eccrine sweat glands (Figs. 3C–G).

Secretion response was detected by classic starch-iodine method. Control injections of 0.9% saline had no positive sweating dots (data not shown). Pilocarpine-induced sweat secretion was not detected in hind footpads until P16, indicating cholinergic-induced actively sweating (Figs. 4a–h and 5L). no black dots appeared on footpads after the injection of adrenaline hydrochloride, isoprenaline hydrochloride or noradrenaline bitartrate at varied dose, as well as at any tested time points, including adulthood, which suggested that alpha- and beta-adrenergic agonists could not induce active perspiration or adrenergic agonists play no role in sweat secretion in rats (Figs. 5A–K).

Discussion

Rat eccrine sweat glands were present exclusively in footpads. In the study, single immunofluorescence staining showed that the innervations of rat eccrine sweat glands varied with development. In early postnatal development, rat eccrine sweat glands expressed adrenergic phenotype fibers, but from late development to adulthood, rat eccrine sweat glands expressed both adrenergic and cholinergic fibers. When the sympathetic fibers are generated during development in laboratory rodents had been studied and debated extensively. In our study, rat sweat gland innervations initially displayed adrenergic properties at P7, and faint cholinergic activity was detected at P11 in developing hind footpads of rats. A study by Landis showed that the fibers innervating rat sweat glands possessed catecholamine immunofluorescence at P7, and pronounced acetylcholinesterase (AChE) and VIP by P14 [19]. The distribution of VIP and cholinergic nerves were the same, and cholinergic receptor inhibitors can inhibit the secretion response induced by VIP, so VIP was often used as a marker associated with cholinergic phenotype in neurons supplying sweat glands [19–21]. Another study by Schütz et al. showed that an adrenergic-specific TH was detectable at P1 and a cholinergic-specific vesicular acetylcholine transporter (VACHT) was visible at P3 in eccrine sweat glands of mouse forepaws [22]. Further studies showed that cho-
linergic fiber markers VACHT, choline acetyltransferase (ChAT) and calcitonin gene-related peptide (CGRP) appeared at different time points [23]. Discrete VACHT-immunoreactivities were present at P1, sparse ChAT immunoreactivity was detected at P8, and unequivocally detectable CGRP appeared at P14 in forepaw sweat glands of Wistar rats [23]. A study by Guidry et al. showed that TH-positive fibers were readily detected surrounding the developing sweat at P5, and weak choline transporter (CHT) expression appeared in the fibers innervating sweat gland anlagen at P7 in mouse and P10 in rat gland anlages of hind footpads [24]. Therefore, species, forepaws or hind paws, and different markers might account for the discrepancies of the initially detected time point of sweat gland innervations. Nonetheless, cholinergic fibers appeared slightly later than adrenergic fibers in the developing rat/mouse footpads, and the co-expression of adrenergic and cholinergic fibers in adult rat/mouse eccrine sweat glands were consistent in most studies [4, 16, 19, 22–25].

Subsequently, we examined sweat response of adult rats to alpha- and beta-adrenergic agonists, including adrenaline hydrochloride, isoprenaline hydrochloride and noradrenaline bitartrate, at the doses varying from 0.01 mg/kg body weight to 1 mg/kg body weight, can induce perspiration on rat footpads by starch-iodine test (A–K). The presentative pictures of beta-adrenergic agonist isoprenaline hydrochloride (0.01 mg/kg body weight) -induced perspiration at P7 (A), P9 (B), P11 (C), P13 (D), P15 (E), P17 (F), P21 (G) and adult (H). The presentative images of alpha-adrenergic agonist adrenaline hydrochloride-induced perspiration at the dose of 0.01 mg/kg body weight (I), 0.1 mg/kg body weight (J) and 0.2 mg /kg body weight (K). Pilocarpine-induced positive secretory response by starch-iodine test (L); Fine black dots represent functional sweat glands (arrows).
studied the sweat response of developing footpads to adrenergic and cholinergic agonists. The results showed that pilocarpine-induced secretory response was detected in both the developing and developed footpads, indicating cholinergic-induced actively sweating. There was no measurable adrenergic sweating when stimulated by alpha/beta-adrenergic agonist adrenaline hydrochloride, beta-adrenergic agonist isoprenaline hydrochloride and alpha-adrenergic agonist noradrenaline bitartrate, at all examined postnatal days, suggesting that adrenergic transmitters played no roles in sweating. The results in our study were consistent with the study by Weihe et al., but a slightly different from the study by Stevens & Landis [4, 16]. The study by Stevens & Landis showed that adult sweat glands were responsive to both cholinergic agonist acetylcholine and alpha- and beta-adrenergic agonists 6-fluoronorepinephrine and isoproterenol, although the response to adrenergic agonists was relatively infrequently and with reduced sweat volumes of sweat compared with cholinergic agonist [16]. In immature rats, neither the alpha-adrenergic agonists, clonidine and 6-fluoronorepinephrine, nor the beta-adrenergic agonist, isoproterenol, could elicit sweating, even the concentrations as high as 50 µM [16].

As for the disagreement between our current study and the study by Stevens & Landis’ study, the sudomotor function was detected by silicon impression molds, while in our study, iodine-starch test was used [16]. Starch-iodine sweat test and impression mold technique are two methods used to detect the direct sweat response, which had similar sensitivity and limitation [26]. Therefore, the two different sweating detection methods cannot account for the divergence.

The cholinergic secretion mainly depends on the activation of Ca²⁺−dependent K⁺ conductance and Cl⁻ conductance, whereas the beta-adrenergic secretion mainly involves the activation of cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ conductance, in summary, in the study we demonstrated that rat eccrine sweat glands to adrenergic agonists, it was difficult to explain. In Stevens & Landis’ study, the sudomotor function was detected by silicon impression molds, while in our study, iodine-starch test was used [16]. Starch-iodine sweat test and impression mold technique are two methods used to detect the direct sweat response, which had similar sensitivity and limitation [26]. Therefore, the two different sweating detection methods cannot account for the divergence.

Conflict of Interest

We declare we have no competing financial, personal or other relationships with other people or organizations.

Funding

This manuscript was supported in part by the National Natural Science Foundation of China (81772102, 81471882).

References

1. Saga K. Structure and function of human sweat glands studied with histochemistry and cytochemistry. Prog Histochem Cytochem. 2002; 37: 323–386. [Medline] [CrossRef]
2. Sato K, Kang WH, Saga K, Sato KT. Biology of sweat glands and their disorders. I. Normal sweat gland function. J Am Acad Dermatol. 1989; 20: 537–563. [Medline] [CrossRef]
3. Taniguchi Y, Sugeno N, Nishimura N, Iwase S, Matsumoto T, Shimizu Y, et al. Contribution of central versus sweat gland mechanisms to the seasonal change of sweating function in young sedentary males and females. Int J Biometeorol. 2011; 55: 203–212. [Medline] [CrossRef]
4. Weihe E, Schütz B, Hartschuh W, Anlauf M, Schäfer MK, Eidcn LE. Coexpression of cholinergic and noradrenergic phenotypes in human and nonhuman autonomic nervous system. J Comp Neurol. 2005; 492: 370–379. [Medline] [CrossRef]
5. Ouyang Z, Li HH, Zhang MJ, Xie ST, Cheng LH. Differential innervation of secretory coils and ducts in human eccrine
sweat glands. Chin Med J (Engl). 2018; 131: 1964–1968. [Medline] [CrossRef]

6. Behm JK, Hagiwara G, Lewistond NJ, Quinton PM, Wine JJ. Hyposecretion of beta-adrenergically induced sweating in cystic fibrosis heterozygotes. Pediatr Res. 1987; 22: 271–276. [Medline] [CrossRef]

7. Amano T, Shitara Y, Fujii N, Inoue Y, Kondo N. Evidence for beta-adrenergic modulation of sweating during incremental exercise in habitually trained males. J Appl Physiol 1985. 2017; 123: 182–189. [Medline]

8. Salinas DB, Kang L, Azen C, Quinton P. Low beta-adrenergic sweat responses in cystic fibrosis and cystic fibrosis transmembrane conductance regulator-related metabolic syndrome children. Pediatr Allergy Immunol Pulmonol. 2017; 30: 2–6. [Medline] [CrossRef]

9. Sato K, Kang WH, Saga K, Sato KT. Biology of sweat glands and their disorders. II. Disorders of sweat gland function. J Am Acad Dermatol. 1989; 20: 713–726. [Medline] [CrossRef]

10. Chen SF, Chang YT, Lu Ch, Huang CR, Tsai nW, Chang CC, et al. Sweat output measurement of the post-ganglion sudomotor response by Q-Sweat test: a normative database of Chinese individuals. BMC Neurosci. 2012; 13: 62. [Medline] [CrossRef]

11. Lu CP, Polak L, Keyes BE, Fuchs E. Spatiotemporal antagonism in mesenchymal-epithelial signaling in sweat versus hair fate decision. Science. 2016; 354: aah6102. [Medline] [CrossRef]

12. Cui CY, Yin M, Sima J, Childress V, Michel M, Piao Y, et al. Involvement of Wnt, Eda and Shh at defined stages of sweat gland development. Development. 2014; 141: 3752–3760. [Medline] [CrossRef]

13. Cui CY, Schlessinger D. Eccrine sweat gland differentiation and sweat secretion. Exp Dermatol. 2015; 24: 644–650. [Medline] [CrossRef]

14. Sato K, Sato F. Pharmacologic responsiveness of isolated single eccrine sweat glands. Am J Physiol. 1981; 240: R44–R51. [Medline] [CrossRef]

15. Sato K, Sato F. Cyclic AMP accumulation in the beta adrenergic mechanism of eccrine sweat secretion. Pfugers Arch. 1981; 390: 49–53. [Medline] [CrossRef]

16. Stevens LM, Landis SC. Development and properties of the secretory response in rat sweat glands: relationship to the induction of cholinergic function in sweat gland innervation. Dev Biol. 1987; 123: 179–190. [Medline] [CrossRef]

17. Nejsun LN, Kwon TH, Jensen UB, Fumagalli O, Frokiaer J, Krane CM, et al. Functional requirement of aquaporin-5 in plasma membranes of sweat glands. Proc Natl Acad Sci USA. 2002; 99: 511–516. [Medline] [CrossRef]

18. Li H, Chen L, Zhang M, Zhang B. Foxa1 gene and protein in developing rat eccrine sweat glands. J Mol Histol. 2017; 48: 1–7. [Medline] [CrossRef]

19. Landis SC. Development of cholinergic sympathetic neurons: evidence for transmitter plasticity in vivo. Fed Proc. 1983; 42: 1633–1638. [Medline]

20. Cowen T, Thrasivoulou C, Shaw SA, Abdel-Rahman TA. Transplanted sweat glands from mature and aged donors determine cholinergic phenotype and altered density of host sympathetic nerves. J Auton Nerv Syst. 1996; 58: 153–162. [Medline] [CrossRef]

21. Landis SC, Fredieu JR. Coexistence of calcitonin gene-related peptide and vasoactive intestinal peptide in cholinergic sympathetic innervation of rat sweat glands. Brain Res. 1986; 377: 177–181. [Medline] [CrossRef]

22. Schütz B, von Engelhardt J, Gördes M, Schäfer MK, Eiden LE, Monyer H, et al. Sweat gland innervation is pioneered by sympathetic neurons expressing a cholinergic/noradrenergic co-phenotype in the mouse. Neuroscience. 2008; 156: 310–318. [Medline] [CrossRef]

23. Schütz B, Schäfer MK, Gördes M, Eiden LE, Weihe E. Satb2-independent acquisition of the cholinergic sudomotor phenotype in rodents. Cell Mol Neurobiol. 2015; 35: 205–216. [Medline] [CrossRef]

24. Guidry G, Willison BD, Blakely RD, Landis SC, Habecker BA. Developmental expression of the high affinity choline transporter in cholinergic sympathetic neurons. Auton Neurosci. 2005; 123: 54–61. [Medline] [CrossRef]

25. Stanke M, Duong CV, Pape M, Geissen M, Burbach G, Deller T, et al. Target-dependent specification of the neurotransmitter phenotype: cholinergic differentiation of sympathetic neurons is mediated in vivo by gp 130 signaling. Development. 2006; 133: 141–150. [Medline] [CrossRef]

26. Buchmann SJ, Penzlin AI, Kubasch ML, Illigens BM, Siepmann T. Assessment of sudomotor function. Clin Auton Res. 2019; 29: 41–53. [Medline] [CrossRef]

27. Reddy MM, Bell CL. Distinct cellular mechanisms of cholinergic and beta-adrenergic sweat secretion. Am J Physiol. 1996; 271: C486–C494. [Medline] [CrossRef]

28. Reddy MM, Bell CL, Quinton PM. Cystic fibrosis affects specific cell type in sweat gland secretory coil. Am J Physiol. 1997; 273: C426–C433. [Medline] [CrossRef]

29. Bijman J, Quinton PM. Predominantly beta-adrenergic control of equine sweating. Am J Physiol. 1984; 246: R349–R353. [Medline]