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—Research Note—

Changes of HCO_3^-/Cl^- Exchanger Activity During Meiotic Maturation in Balb/c Strain Mouse Oocytes and Zygotes

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Abstract. Intracellular pH-regulatory mechanisms are acquired by growing mouse oocytes with meiotic competence, and these mechanisms become fully active when the oocytes develop to the germinal vesicle (GV) stage as shown in CF1 and Balb/c strains mice. On the other hand, there is some evidence showing that intracellular pH-regulatory mechanisms are inhibited at the stages of Metaphase I (MI) and II (MII) oocytes in the CF1 strain mouse and hamster. Since it has been shown that the intracellular pH regulatory mechanism can be functionally different among mouse strains (e.g., CF1, Balb/c), the aim of this study was to investigate the activity of HCO_3^-/Cl^- exchanger (anion exchanger, AE), which protects cells against alkalosis during the meiotic maturation process, in the GV oocyte up to the pronuclear (PN) zygote derived from the Balb/c strain mouse. Intracellular pH (pH_i) was recorded using a microspectrofluorometric technique during meiotic maturation stages. KSOM-based solutions were used as culture and recording solutions. AE activity was determined using a Cl^- removal assay and was reported as the change in pH_i per minute. AE activity was high in GV stage oocytes but was significantly inhibited at the MI and MII stages. AE activity was higher in the PN zygote stage. This activity was significantly inhibited in all oocyte and zygote stages by 4,4′-Diisocyanatostilbene-2,2′-disulfonic acid disodium salt. After alkalosis induction, the pH_i of MI and MII stage oocytes did not completely recover; however, almost complete recovery occurred in the GV stage oocytes and PN zygotes. These results suggest that AE is inhibited during the meiotic maturation process in the Balb/c strain mouse.
Key words: Alkalosis, Anion exchanger, Meiotic maturation, Mouse, Oocyte

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Intracellular pH (pH_i) regulation is an important component of mammalian cell homeostasis. It is maintained within a narrow range with three major regulatory-mechanisms, the HCO_3^-/Cl^- exchanger (Anion Exchanger, AE), which alleviates alkalosis, and the Na^+/H^+ (NHE) and Na^+, HCO_3^-/Cl^- exchangers (NCBE), both of which alleviate acidosis. AE mediates the electroneutral exchange of extracellular Cl^- for intracellular HCO_3^-, and therefore pH_i decreases. NHE mediates the electroneutral exchange of extracellular Na^+ for intracellular H^+ [1–3]. The key feature of pH_i regulatory transporters is activation by changes in pH_i; NHE and NCBE are quiescent unless pH_i falls below a threshold, or "set-point," where transport is activated [3–5]. Conversely, the AE is activated when pH_i rises above the set-point [5–7].

The regulatory mechanisms of pH_i have been well documented in preimplantation stage embryos [8–12]. However, few studies have been conducted concerning the development and regulation of these mechanisms during the process of oogenesis. The pH_i of oocytes in primary follicles is regulated by the support of granulosa cells [13]. As oocytes grow, they become meiotically competent when they reach 80% of their full size. At this time, the activities of AE and NHE in oocytes increase, and thus the oocytes can regulate their own pH_i independently from the follicular structure [14]. Therefore, when oocytes are in the fully-grown germinal vesicle stage, the activities of AE and NHE reach high values [13, 14]. While the activity of NHE is high in GV stage oocytes, it has surprisingly been determined that this activity decreases during the MI stage in the Balb/c strain mouse [15]. During the MII stage, it has been shown that NHE activity remains at a low level in the hamster and mouse [10, 15]. The activity of NHE returns to a high level after fertilization, at the time of pronuclear formation [10, 15], and it stays at a high level during all preimplantation (PI) embryonic stages [10, 12, 16, 17]. Similar regulatory pattern have been reported for the AE activity of CF1 strain mice [18]. In that study, it was determined that AE activity was regulated via the MAP kinase pathway. In contrast to this, while the NCBE activity was negligible in the GV stage oocyte, the contribution of NCBE activity to recovery from acidosis was obvious in the MII oocytes and pronuclear stage zygotes of Balb/c strain mice [19]. Thus, it has been suggested that the activity of NCBE seems to develop a completely different pattern from those of AE and NHE activities during meiotic maturation. On the other hand, it has been reported that embryos derived from different strains of female mice differ in their control of mechanisms for pH_i regulation [12].

Although the regulatory mechanisms of pH_i are fully active in the GV stage oocytes of CF1 and Balb/c strains mice, it is interesting that the activities of AE and NHE seem to be regulated throughout the meiotic maturation stages as shown in the CF1 and Balb/c strain mice, respectively. Therefore, since intracellular pH regulatory mechanism can be functionally different among strains, our aim was to investigate the AE activity, which is the sole defence mechanism against alkalosis, in oocytes and zygotes derived from the Balb/c strain mouse during meiotic maturation.

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Materials and Methods

Five- to eight-week-old Balb/c strain mice were used in this study. Stable light (12-h light/dark cycle), temperature (21 ± 1 C) and humidity (40–60%) conditions were maintained in the animal laboratory. All procedures were approved by the Ethics Committee of the Medical Sciences Research and Application Centre, University of Cukurova.

GV oocytes were obtained mechanically from the ovaries of mice that had received an intraperitoneal (IP) injection of equine chorionic gonadotropin (eCG). One hundred μM of db cAMP were added into all holding and recording solutions to maintain meiotic arrest. To obtain MI stage oocytes, 5 IU human chorionic gonadotropin (hCG) were injected IP 48 h after eCG injection. After 3 h, MI oocytes were isolated from ovaries. MII oocytes were collected from ampullae 18–20 h after hCG injection. Cumulus cells were removed by short exposure (approximately 2 min) to 75 μg/ml hyaluronidase. Female mice injected with hCG 48 h following eCG injection were kept with male mice overnight. Twenty-two hours after hCG injection, PN zygotes were collected from ampullae as described for retrieval of MII stage oocyte.

Solutions and chemicals

All chemicals were purchased from Sigma-Aldirch Chemicals (St. Louis, MO, USA). All media were based on KSOM mouse oocyte/embryo culture medium [20]. Hepes-buffered KSOM (KFHM) was used for collecting oocytes/zygotes from the oviducts and for oocyte/zygote handling. This medium was produced by replacing 21 mM (of 25 mM) $NaHCO_3$ with equimolar Hepes (pH adjusted to 7.40 with NaOH).

For all fluorophore-loading and pH_i measurement, 9 mM Na lactate was replaced with NaCl (total 104 mM NaCl and 1 mM Na lactate) and no BSA was included (defined as pHKFHM). The HCO_3^-/CO_2-buffered medium used for pH_i measurement was referred to as pHKSOM and equilibrated with 5% CO_2/air. Cl^--free media were produced by replacing Cl^- salts with corresponding gluconate salts. For ammonium-containing solutions, 35 mM NaCl was replaced with equimolar NH_4Cl.

Stock solutions were prepared in dimetyl sulfoxide [Carboxyseminapthorhodafluor-1-acetoxymethyl ester (SNARF-1-AM); valinomycin], in ethanol (nigericin), in water (db cAMP) and in 0.1 M $KHCO_3$ (4,4'-Diisocyanatostilbene-2,2'-disulfonic acid disodium salt: DIDS).

Measurement of pH_i

Measurement of pH_i was performed using the pH-sensitive carboxyseminaphthorhodafluor-1 (SNARF-1) loaded into oocytes/zygotes by incubation with 5.0 μM SNARF-1-AM at 37 C for 30 min in pHKFHM. After SNARF-1 was loaded, the oocytes/zygotes were washed several times with pHKFHM and placed in a chamber that was modified to allow solution changes. Each replicate consisted of a group of 7–10 same-stage oocytes/zygotes whose average pH_i was measured. All measurements were performed in a temperature- and atmosphere-controlled chamber (37 C, 5% CO_2/air).

Measurement of pH_i was performed using photomultiplier tubes (PTI-814) attached to an epifluorecense microscope (TE-2000U; Nikon, Tokyo, Japan). Oocytes/zygotes loaded with SNARF-1 were excited at wavelength of 535 nm, and two emission wavelengths (640 and 600 nm) were determined. The ratio of the two intensities (640/600), which is mainly dependent on pH_i, was calculated by dividing the intensities after background subtraction. This ratio was calibrated to pH_i using calibration solution containing 10 μg/ml nigericin and 5 μg/ml valinomycin [8]. The initial pH_i was determined in pHKSOM solution after a 15-min stabilization period.

Cl^- removal assay for AE activity

AE activity was quantified by a Cl^- removal method [14, 18, 21]. In brief, AE will reverse when cells are exposed to Cl^--free solution, resulting in intracellular alkalinization due to HCO_3^- influx coupled with Cl^- efflux [22]. Increased pH_i upon Cl^- removal thus indicates AE activity, and the initial rate of alkalinization provides a quantitative measure of activity [18, 22]. The initial rate of intracellular alkalinization upon Cl^- removal was determined using linear regression (Sigma Plot 8.0), and exchanger activity is reported as the change in pH_i per minute (pHU/min). The activity was confirmed by showing that the rise was abolished by DIDS (an inhibitor of AE, 500 μM) [7, 23].

Recovery from induced alkalosis

To determine whether oocytes/zygotes were able to recover from an increase in pH_i, intracellular alkalosis was induced using the permenant weak base NH_4Cl [8]. Following steady-state pH_i measurements, the solution was changed to NH_4Cl pHKSOM for 20 min. This pulse produces an immediate alkalinization due to the rapid equilibration of NH_3 across the membrane. For some of the recovery recordings, DIDS was added in NH_4Cl-pHKSOM solution to verify recovery resulting from AE activity. Following recovery, pH_i (pH_{if}) was determined by fitting the recovery to a single exponential by non-linear regression (Sigma Plot 8.0).

Statistics

Comparisons were made using one way ANOVA followed by the Tukey multiple comparisons test. The statistical analysis of differences of initial and final pH_i values for same stages oocytes/zygotes was performed using the paired *t*-test. For overall analysis, SPSS 13.0 was utilized. The data are presented as means ± SEM, and significance was accepted as $P<0.05$.

Results and Discussion

The initial (resting) pH_i values of oocytes and zygotes were stable before the alkalosis induction and Cl^- removal procedures. There was no significant difference between the initial pH_i values of the same stage oocytes/zygotes in the different treatment groups (as expected, since the media were identical during the period before alkalinization was induced).

HCO_3^-/Cl^- exchange was detected by replacing the medium with Cl^--free medium and by determining if there was an increase in pH_i [13, 14, 16, 18]. Particularly at the stages of GV oocyte and PN zygote, switching to Cl^--free medium resulted in fast development

of intracellular alkalinization; pH_i then stabilized at a new higher level. Therefore, the data presented here indicates HCO_3^-/Cl^- exchange activity as shown by a pH_i increase upon removal of Cl^- from external medium, which caused Cl^- efflux from the cell and hence HCO_3^- influx through any functional AE (Table 1). This pH_i rise was confirmed to be due to HCO_3^-/Cl^- exchange by abolition of the increase by an inhibitor of AE, DIDS (500 μM; Table 1) [7, 14]. Moreover, our findings indicate that recovery from induced-alkalosis was significantly inhibited by DIDS (Table 2), further indicating the presence of functional HCO_3^-/Cl^- exchange, particularly in the GV stage and PN zygote (Table 1).

The AE activity of the GV oocyte was 0.056 pHU/min, and it decreased in the MI and MII stages. AE activity, however, increased to a higher value in the PN stage zygote (Table 1). Our results and others have shown that the activities of AE and NHE are at a high level in GV stage oocytes [13, 14, 18]. Thus, the GV oocyte has defence mechanisms against alkalosis and acidosis as shown here with alkalosis. Interestingly, it has been reported that high NHE activity decreases to a low level during the MI stage in Balb/c strain mice [15] and remains at a low level as long as the oocytes are in the MII stage in this strain and in the hamster [10, 15]. However, this low NHE activity returns to its initial high level at the time of PN formation [10, 15]. Therefore, the changes in NHE activity during the meiotic maturation process seem to be similar to our AE activity findings. In line with our findings, Phillips *et al*. also reported that AE is quiescent in MII oocytes and only becomes activated hours after fertilization in CF1 strain mice [18]. In this study, it has been determined that AE activity was regulated via MAP kinase pathway [18]. Therefore, the major pH-regulatory mechanisms (exchanges of Na^+/H^+ and HCO_3^-/Cl^-) seemed to have a similar changing pattern during the meiotic maturation process independent from the mouse strain. On the other hand, it has been reported that embryos derived from different strains of female mice differ in their control of mechanisms for pH_i regulation [12]. Moreover, data obtained from the Balb/c strain mouse shows that while NCBE (the other pH_i defence mechanism that exchanges $Na^+,HCO_3^-/Cl^-$ against acidosis) activity is negligible in GV and MI stage oocytes, the contribution of NCBE activity to recovery from acidosis became evident in the MII stage and in the PN zygote [19]. Thus, the activity of NCBE seems to develop a completely different pattern than those of the AE and NHE activities during meiotic maturation [19].

Oocytes and zygotes were alkalinized by exposure to NH_4Cl to assess whether they could recover from intracellular alkalosis. Although there was no significant difference in the initial pH_i values of all stages of oocytes and PN zygotes, after recovery from alkalosis, the pH_{if} values of MI and MII stage oocytes stayed at higher values than those of GV stage oocytes and PN zygotes (Table 2). Therefore, although the pH_i of GV oocytes and PN zygotes could return to nearly physiological values after recovery, those of MI and MII stage oocytes could not recover effectively (Table 2). In particular, it was surprising that the MII stage oocyte, which is ready for fertilization, was defenseless against alkalosis, since the pH_i of MII oocyte could shift to an alkaline value due to the pH of oviductal fluid being alkaline (about 7.50–7.70) [17]. Therefore, the pH_i of fertilized MII oocytes (zygote) can remain alkaline until the time of reactivation of AE. It has been reported that alkaline pH_i is permissive for metabolic activation and development [24, 25]. Although the clear physiological reasons for this specific meiotic inactivation remain unclear, Baltz pointed out that it may be related to the need to conserve energy or maintain transmembrane ionic gradients in the ovulated egg until fertilization and

Table 1. The mean AE activity of GV, MI and MII stage oocytes and PN zygotes in pHKSOM and in pHKSOM containing DIDS (values were given as pHU/min)

	pHKSOM	pHKSOM + DIDS
GV	0.056 ± 0.008	0.001 ± 0.000**
	(N=8, n=52)	(N=7, n=51)
MI	0.024 ± 0.006*	0.002 ± 0.001**
	(N=8, n=61)	(N=7, n=61)
MII	0.030 ± 0.004*	0.003 ± 0.001**
	(N=7, n=44)	(N=6, n=57)
PN	0.040 ± 0.004	0.002 ± 0.001**
	(N=7, n=35)	(N=6, n=47)

* Significant compared to the value of GV stage oocytes in pHKSOM (P<0.05). ** Significant compared to the values of same stage oocytes/zygotes in pHKSOM (P<0.05). N: Mice used. n: Number of recording oocytes/zygotes.

Table 2. The mean values of initial pH_i and final pH_i (pH_{if}) determined after a 20-min recovery period in NH_4Cl pHKSOM and in NH_4Cl pHKSOM containing DIDS

	NH_4Cl pHKSOM		NH_4Cl pHKSOM + DIDS	
	pH_i	pH_{if}	pH_i	pH_{if}
GV	7.20 ± 0.017	7.27 ± 0.029*	7.19 ± 0.006	7.43 ± 0.044**
	(N=6, n=106)		(N=6, n=89)	
MI	7.18 ± 0.008	7.45 ± 0.041*	7.18 ± 0.012	7.66 ± 0.053**
	(N=7, n=51)		(N=5, n=33)	
MII	7.22 ± 0.002	7.43 ± 0.040*	7.19 ± 0.013	7.54 ± 0.056
	(N=6, n=38)		(N=5, n=53)	
PN	7.16 ± 0.015	7.29 ± 0.035*	7.20 ± 0.015	7.52 ± 0.032**
	(N=7, n=50)		(N=5, n=53)	

* Significant compared to the pH_i values of same stage oocytes/zygotes in NH_4Cl pHKSOM (P<0.05). ** Significant compared to the pH_{if} values of same stage oocytes/zygotes in NH_4Cl pHKSOM (P<0.05). N: Mice used. n: Number of recording oocytes/zygotes.

metabolic activation [16].

In summary, AE activity is inhibited during meiotic maturation in the Balb/c strain mouse, as in the other major intracellular pH regulator, NHE. Further investigations are needed to clarify the physiological reasons for this inactivation.

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