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Inhibition of 20α-Hydroxysteroid Dehydrogenase Activity in Mouse Oocyte by Human 20α-HSD (AKR1C1) Inhibitor

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ABSTRACT

Inhibitors of the enzyme 20α-HSD (AKR1C18) are required to investigate AKR1C18 activity that occurs with meiotic resumption and progression in mouse oocytes. However, currently, no effective inhibitors of AKR1C18 are available. Therefore, we treated mouse oocytes with 3-bromo-5-phenylsalicylic acid (5-PBSA), an inhibitor of human 20α-HSD (AKR1C1), to ascertain whether it suppressed AKR1C18 activity. We also examined the effect of this inhibitor on oocyte maturation and viability.

In the germinal vesicle stage of mouse oocytes treated with 300 and 400 μM 5-PBSA, the number of oocytes showing AKR1C18 activity in the cytoplasm was 0 (0/34 and 0/35); the activity was significantly suppressed as compared with AKR1C18 activity in the control (25/29, 86.2%). In oocytes treated with >200 μM 5-PBSA, the number of degenerated oocytes was significantly increased compared with that in the control, and 17/37 (45.9%) of the oocytes treated with 300 μM were degenerated. In addition, in oocytes treated with 300 and 400 μM 5-PBSA, the number of oocytes that resumed meiosis was 2/20 (10%) and 0/16 (0%), respectively, and the resumption of meiosis was significantly suppressed compared with that in the controls (29/32, 90.6%).

These results revealed that 5-PBSA inhibits not only human AKR1C1 but also mouse AKR1C18 activity. In addition, suppression of meiotic resumption by treatment with 300 μM 5-PBSA suggests that AKR1C18 activity was involved in meiotic resumption. However, because 5-PBSA concentrations as high as 300 μM may also inhibit the activity of the AKR1C subfamily other than AKR1C18, the effects of 5-PBSA on these phylogenetically similar subfamilies must be investigated in the future.

Keywords: 20α-Hydroxysteroid dehydrogenase activity, Meiotic resumption, Mouse oocyte, 20α-Hydroxysteroid dehydrogenase inhibitor, Aldo-keto reductase 1C.

INTRODUCTION

Hydroxysteroid dehydrogenases (HSDs) are classified into AKR1Cs, which are a subfamily of the aldo-keto reductase superfamily. Two proteins corresponding to human and mouse 20α-HSD are AKR1C1 and AKR1C18 respectively [1,2]. These 20α-HSDs act to reversibly convert physiologically active progesterone into inactive 20α-hydroxyprogesterone [3]. Because mice deficient in 20α-HSD expression by gene disruption maintained high progesterone levels, indicating a delay in parturition, it was demonstrated that 20α-HSD is involved in pregnancy and parturition through progesterone metabolic regulation [4]. Furthermore, 20α-HSD is used as a tumor marker because AKR1C1 and its mRNA are actively expressed in various human tumors [5-8]. Moreover, 20α-HSD is used in the development of psychotropic drugs because its metabolite works as a neurosteroid [9]. Considering these facts, 20α-HSD has attracted attention in recent years. The specific inhibition of human 20α-HSD activity prevents the inactivation of neurosteroids such as progesterone and suppresses tumor growth. Because these are clinically beneficial, various inhibitors have been developed; to date, among them, 3-bromo-5-phenylsalicylic acid (5-PBSA), synthesized for use as an anti-cancer agent for humans, most strongly inhibits AKR1C1 [10].

Furthermore, 20α-HSD (AKR1C18) activity is presumed to be involved in meiosis in mouse oocytes. In mouse oocytes, 20α-HSD activity appears in the cytoplasm with the resumption of meiosis, and with its progress, the number of oocytes showing 20α-HSD activity increases [11]. However, the role of 20α-HSD activity associated with the resumption and progression of meiosis in oocytes remains unclear. In the *in vitro* culture of the oocyte, the completion of oocyte maturation is determined by the nucleus reaching metaphase II. Clarification of the role of AKR1C18 activity may lead to the adoption of this activity in the cytoplasm as a new indicator of oocyte maturation. In general, nuclear maturation progressed normally, in oocytes cultured *in vitro*, but their subsequent fertilization and developmental ability were inferior to that of oocytes matured *in vivo* [12,13]. This is presumed to be caused by insufficient cytoplasmic maturation even when the nuclear maturation is normal. Therefore, if AKR1C18 activity were an indicator of cytoplasmic maturation, this indicator would contribute to the development of breeding technology using cultured oocytes of domestic animals, and to *in vitro* oocyte culture techniques in human assisted reproduction technology, respectively. A deeper understanding of the role and mechanism of AKR1C18 activity may be important in improving these technologies. Unfortunately, the AKR1C18 inhibitor

necessary for investigating the role of 20α-HSD in mouse oocytes has not yet been developed. However, because 5-PBSA also weakly inhibits the activities of isoforms of AKR1C1—AKR1C2, AKR1C3, and AKR1C4— we anticipated that this inhibitor may also inhibit mouse AKR1C18 in a subfamily of AKR1C [1].

In the present study, to confirm whether 5-PBSA, which blocks human AKR1C1, inhibits AKR1C18 in mouse oocytes, we treated mouse oocytes with various concentrations of 5-PBSA, and examined the inhibitor concentration effective in inhibiting AKR1C18 activity. Furthermore, we aimed to observe meiosis and the degeneration of the oocytes treated with various concentrations of 5-PBSA. We investigated the effect of 5-PBSA on oocyte meiosis and viability, and the possibility that 5-PBSA could elucidate the function of AKR1C18 in mouse oocytes.

MATERIALS AND METHODS

Animals

We used female mature mice of the ICR strain. We housed them in a metal cage in a room kept at 24℃, under lighting conditions of 14 h from 4:00 to 18:00.

Collection and culture of oocytes

We collected the oocytes by tearing the antral follicles at 48 h after intraperitoneally injection of PMSG (ASKA Pharmaceutical. Co. Ltd. Tokyo, Japan) (5 IU) to observe their maturation process *in vitro*. We cultured the collected oocytes in TYH medium containing 5% fetal bovine serum (FCS, Gibco Lab., MA, USA) and 10 IU/ml of PMSG and an AKR1C1 inhibitor (3-bromo-5-phenylsalicylic acid; 5-PBSA) (CAL Biochem, CA, USA) at 10, 20, 50, 100, 200, 300, or 400 μM, respectively [14]. We cultured the oocytes at 37 °C for 14 h in incubator with 5% CO_2 and 95% air. We dissolved the 5-PBSA in advance in DMSO (Wako Pure Chemical Industries, Ltd. Oosaka, Japan) diluted with culture medium at the concentrations mentioned above. As we had adjusted the DMSO concentration in the culture medium to 0.1%, we used the oocytes cultured in medium containing only 0.1% DMSO as a control.

Effect of 20α-HSD activity of AKR1C1 inhibitor-treated oocyte

We immersed the cultured oocytes in phosphate buffered saline (PBS, pH 7.4) containing 0.1% hyaluronidase (Sigma-Aldrich, MO, USA) [15]. We removed the cumulus cells surrounding the oocytes by capillary manipulation, and observed the denuded oocytes for morphology under a stereomicroscope, using normal, non-degenerated oocytes for subsequent treatments.

We employed the method of Dickmann and Dey to detect 20α-HSD activity. We prepared a substrate solution by dissolving 1.8 mg of 20α-hydroxyprogesterone (Sigma-Aldrich), 4.0 mg of NADP (Sigma-Aldrich), and 2.0 mg of nitro blue tetrazolium (Sigma-Aldrich) into 10 ml of 0.1 M phosphate buffer (pH 7.5) [16]. We immersed the denuded and non-degenerated oocytes treated with 5-PBSA and controls in the substrate solution at 37°C for 60-120 min. We used the oocytes immersed in a substrate-free solution containing the solvent (acetone) as a negative control. We used 20α-HSD (AKR1C18) activity in the oocyte for observation with a binocular stereomicroscope.

Effect of nuclear maturation and degeneration of AKR1C1 inhibitor-treated oocyte

We cultured the oocytes collected from antral follicles at 48 h after PMSG injection under the same conditions as described above. As we had adjusted the DMSO concentration in the culture medium to 0.1%, we used the oocytes cultured in medium containing only 0.1% DMSO as a control. After 14 h, we immersed the oocytes cultured for observation of nuclear maturation and degeneration in phosphate buffered saline (PBS, pH 7.4) [15] containing 0.1% hyaluronidase (Sigma-Aldrich), and removed the cumulus cells surrounding oocytes by capillary manipulation. At this point, we counted the number of degenerated oocytes. We fixed the oocytes for observation of nuclear maturation in acetic acid:ethanol (1:3) at 4 ℃ for 24 h. After fixation, we observed the nuclear maturation of oocytes stained with 1% acetolacmoid solution (Chroma Gesellschaft Schmidt & Co. Stuttgart, Germany) under an optical microscope. We classified the nuclear maturation of oocytes treated with the AKR1C1 inhibitor at 10 μM, 20 μM, 50 μM, 100 μM, 200 μM, 300 μM, and 400 μM, and controls (0 μM) into three stages: the germinal vesicle (GV) stage, from the diakinesis stage to the telophase of the first meiotic division, and the metaphase of the second meiotic division. All the procedures performed were in accordance with the ethical standards of the facility where the study was conducted.

Statistical analysis

We subjected the numerical values of the HSD activity to a multiple comparison test using Ryan's method if a significant difference was found by the Chi-square test [17]. We considered P values of less than 0.05 to be statistically significant.

RESULTS

20α-HSD activity in AKR1C1 inhibitor-treated oocytes

In **Table 1** we show the activity of 20α-HSD in oocytes treated with the 5-PBSA at 10 μM, 20 μM, 50 μM, 100 μM, 200 μM, 300 μM, 400 μM, and controls (0 μM) for 14 h. The 20α-HSD activity-positive oocytes had diformazan granules deposited throughout the cytoplasm. The number of oocytes showing 20α-HSD activity cultured in medium containing 10 μM, 20 μM, 50 μM, 100 μM and 200 μM of the inhibitor were 24/31 (77.4%), 33/34 (97.1%), 34/34 (100.0%), 34/35 (97.1%), and 22/34 (64.7%) of the observed oocytes, respectively. These numbers were not significantly different from that of the control oocytes (25/29, 86.2%). The number of oocytes showing 20α-HSD activity was significantly reduced when cultured at 300 and 400 μM of the inhibitor, these oocytes showing no 20α-HSD activity.

Nuclear maturation and degeneration in AKR1C1 inhibitor-treated oocytes

In **Table 2** we show nuclear maturation and degeneration in

Table 1: The 20α-HSD activity in AKR1C1 inhibitor-treated oocytes.

Inhibitor concentration (μM)	Number of observed oocytes	20α-HSD activity	
		+	-
0	29	25 (86.2) [i)] [abc]	4 (13.8)
10	31	24 (77.4)[bc]	7 (22.6)
20	34	33 (97.1)[ab]	1 (2.9)
50	34	34 (100.0)[a]	0 (0.0)
100	35	34 (97.1)[ab]	1 (2.9)
200	34	22 (64.7)[c]	12 (35.3)
300	34	0 (0.0)[d]	34 (100)
400	35	0 (0.0)[d]	35 (100)

Note: We used oocytes cultured for 14 h for observation. [i)] The value in parentheses is percent of the total number of observed oocytes. There are significant differences between numbers with different superscripts in the same lines ($p<0.05$).

Table 2: Nuclear maturation and degeneration in AKR1C1 inhibitor-treated oocytes.

Inhibitor concentration (μM)	Number of observed oocytes	Nuclear maturation stages[i)]				Degeneration[ii)]
		Germinal vesicle	≤ Diakinesis	Diakinesis ~ Telophase Ⅰ	Metaphase Ⅱ	
0	32	3 (9.4)[b]	29 (90.6)[a]	4 (12.5)	25 (78.1)	0 (0.0)[b]
10	36	2 (5.7)[b]	33 (94.3)[a]	5 (14.3)	28 (80.0)	1 (2.7)[b]
20	36	4 (11.1)[b]	32 (88.9)[a]	5 (13.9)	27 (75.0)	0 (0.0)[b]
50	34	2 (5.9)[b]	32 (94.1)[a]	3 (8.8)	29 (85.3)	0 (0.0)[b]
100	36	5 (13.9)[b]	31 (86.1)[a]	6 (16.7)	25 (69.4)	0 (0.0)[b]
200	34	9 (37.5)[ab]	15 (62.5)[ab]	5 (20.8)	10 (41.7)	10 (29.4)[a]
300	37	18 (90.0)[a]	2 (10.0)[b]	2 (10.0)	0 (0.0)	17 (45.9)[a]
400	36	16 (100.0)[a]	0 (0.0)[b]	0 (0.0)	0 (0.0)	20 (55.6)[a]

Note: We used oocytes cultured for 14 h for observation. [i)] The value in parentheses is percent of the total number of observed oocytes, excluding degenerated oocytes. [ii)] The value in parentheses is percent of the total number of observed oocytes. There are significant differences between numbers with different superscripts in the same lines ($p<0.05$).

oocytes treated with 5-PBSA at 10 μM, 20 μM, 50 μM, 100 μM, 200 μM, 300 μM, 400 μM, and controls (0 μM) for 14 h. The number of oocytes that resumed meiosis was not significantly different from the control at 10-200 μM inhibitor. At 5-PBSA concentrations of 300 μM and 400 μM, compared with the controls, 18/20 (90%) and 16/16 (100%) oocytes significantly suppressed meiotic resumption, respectively. The number of oocytes showing degeneration was significantly increased in cultures over 200 μM of the inhibitor. The degeneration number of oocytes cultured with 200 μM, 300 μM, and 400 μM inhibitors tended to increase to approximately 10/34 (29.4%), 17/37 (45.9%), and 20/36 (55.6%), respectively. There was, however, no significant difference between these degeneration numbers.

DISCUSSION

In this experiment, we examined whether 20α-HSD (AKR1C18) activity of mouse oocytes was inhibited by using human 20α-HSD (AKR1C1) inhibitor (5-PBSA). We confirmed for the first time that 300 μM 5-PBSA inhibits AKR1C18 activity in mouse oocytes (**Table 1**). The AKR1C18 activity was also implicated as being involved in meiotic resumption and progression (**Table 2**). However, in humans, the inhibitor blocked 20α-HSD activity at approximately 10 μM [10]. The reason why a high concentration of 5-PBSA was required for inhibition of AKR1C18 activity is presumed to be the difference in the active site structure from AKR1C1. Acting as a competitive inhibitor, 5-PBSA is designed based on the active site structure of AKR1C1. The inhibitor-binding site of 5-PBSA in AKR1C1 comprises eight amino acid residues—Leu54, Tyr55, His117, His222, Glu224, Leu306, Leu308, and Phe311 [1]. In 5-PBSA, the inhibition constant (K_i value), which is an indicator of the inhibitory potency, was 4 nM [1]. In addition, 5-PBSA also inhibits isoforms of AKR1C—AKR1C2, AKR1C3, and AKR1C4. In the inhibitor-binding site, AKR1C1 and AKR1C2 differ in only one amino acid residue—that is, Leu54 in AKR1C1 and Val54 in AKR1C2. The K_i value of 5-PBSA in AKR1C2 was 87 nM, and the inhibitory potency was reduced to about 1/21 compared with that of AKR1C1 [1]. Similarly, His222, which is a part of the inhibitor-binding site of AKR1C1, is Gln222 in AKR1C3 and AKR1C4; the K_i value of 5-PBSA is 4,200 nM and 18,200 nM for AKR1C3 and AKR1C4, respectively. The inhibitory potency of 5-PBSA in AKR1C3 and AKR1C4 is greatly reduced compared with that of AKR1C1 [1]. According to the BLAST® program published on the (NCBI) website [18], the amino acid residues of His222, Glu224, Leu306, and Leu308 at the AKR1C1 (Sequence ID: NP_001344.2) inhibitor-binding site are replaced by Gln196, Tyr198, Phe281, and Ala283 in AKR1C18 (Sequence ID: AAH34259), respectively [1,18]. This difference in inhibitor-binding sites between AKR1C subfamilies reduces the competitive inhibitory potency of 5-PBSA. Therefore, we concluded that a high concentration of 5-PBSA may have been required to inhibit the AKR1C18 activity in this study.

As the concentration of 5-PBSA inhibiting AKR1C18 was greatly increased to 300 μM in this study, we realized the need to confirm effects other than AKR1C18 activity in oocytes with the inhibitor. Thus, we examined the nuclear maturation and the degeneration in oocytes cultured under the same conditions as in the previous experiment. Degenerated oocytes increased significantly with 5-PBSA at over 200 μM, and 46% of oocytes degenerated at 300 μM, inhibiting AKR1C18 activity. In addition, most oocytes remained in the GV stage due to suppression of meiotic resumption after treatment with more than 300 μM of 5-PBSA (**Table 2**). We visually confirmed all the oocytes used for observation of AKR1C18 activity as being non-degenerated oocytes. However, oocytes treated with high concentrations of 5-PBSA may have had abnormalities other than degeneration that could not be distinguished visually. In this case, we cannot clearly determine whether the loss of AKR1C18 activity was due to 5-PBSA or abnormalities other than degeneration; in future studies we will investigate the normality of oocytes cultured with over 200 μM inhibitor.

As described above, 5-PBSA inhibits AKR1C1 isoforms (AKR1C2, AKR1C3, and AKR1C4), although the inhibitory effect is weaker than that of AKR1C1 [1]. In this study, because the concentration of 5-PBSA inhibiting AKR1C18 activity was as high as 300 μM, the activity of aldo-keto reductases such as AKR1C14, AKR1C21, AKR1C6, and AKR1C20, which are subfamily of AKR1C and maintain a close phylogenetic relationship, may have been inhibited. As inhibition of AKR1C subfamilies other than AKR1C18 may have suppressed meiotic resumption and increased degenerated oocytes, there is a need to investigate the effects of inhibitors on subfamilies similar in structure to AKR1C18. In addition, we also hope that new inhibitors specific to AKR1C18 will be developed to more accurately examine how AKR1C18 activity is involved in meiotic resumption and progression.

CONCLUSION

The treatment of mouse oocytes with 300 μM AKR1C inhibitor, which inhibited human 20α-HSD activity, blocked resumption of meiosis along with 20 α-HSD activity. Therefore, we have concluded that 20α-HSD activity may induce meiotic resumption and it may have been an indicator of mouse oocyte maturation.

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AUTHORS CONTRIBUTION

Shin-ya Kawakami contributed to the analysis of the data throughout the study and the writing of the manuscript. Chihiro Nansai is contributed to the methods and experiments section. Prof. Sueo Niimura designed the research strategy and provided suggestions to other authors.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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