Effect of various proteinase inhibitors on ovulation of explanted hamster ovaries

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Summary. Hamster ovaries explanted at 21:00–24:00 h on the day of pro-oestrus were incubated with microbial proteinase inhibitors until 10:30 h on the next morning and the ovulatory blocking effect of these inhibitors was examined. Amongst 11 proteinase inhibitors examined, talopeptin, a specific inhibitor for metallo-proteinases, and α -MAPI, a specific inhibitor for serine and thiol proteinases, were the strongest blockers. These 2 inhibitors exhibited a chronological discrepancy in their blocking effect on ovulation. S-SI, plasminostreptin, elastatinal, antipain and chymostatin, which are inhibitors for serine proteinases, partly but significantly inhibited ovulation. The results suggest that, in addition to a metallo-proteinase reported previously, a proteinase which is sensitive to α -MAPI is essential for the ovulatory process, and that serine proteinase(s) also participate in ovulation of the hamster ovary.

Introduction

Since the initial report by Schochet in 1916, several studies have suggested that proteolytic enzymes are associated with the ovulatory process. The injection of proteolytic enzymes into the follicular antrum caused rupture of rabbit Graafian follicles (Espey & Lipner, 1965). The follicle wall becomes flaccid and distensible in the final stages of the ovulatory process (Espey, 1967a, b; Rondell, 1970), and proteinases, such as collagenase, elastase, trypsin, α -chymotrypsin and plasmin, weaken the tensile strength of the porcine follicle wall (Espey, 1970; Beers, 1975). Furthermore, several kinds of proteinases have been recognized in follicles (Espey, 1974; Fukumoto, Yajima, Okamura & Midorikawa, 1981). A specific inhibitor of metallo-proteinase, talopeptin, completely blocked ovulation in the hamster ovary explanted at 21:00 and 22:00 h on the day of pro-oestrus; the inhibition markedly decreased in ovaries excised at 23:00 h, whereas the integrity of the apex of the Graafian follicles of these ovaries was still maintained (Ichikawa, Ohta, Morioka & Murao, 1983). These observations suggest that some proteinases other than collagenase may also be involved in the mammalian ovulatory process before and after collagenolysis.

The present study was designed to confirm the participation of proteolytic enzymes other than metallo-proteinases in the ovulatory process by utilizing a series of microbial proteinase inhibitors which cover all proteinase groups, i.e. carboxyl-, metallo-, thiol- and serine proteinases. The temporal relationship between treatment and inhibition by the collagenase and other proteinase inhibitors was also studied.

Materials and Methods

The methods of animal handling and ovarian incubation have been described previously (Ichikawa *et al.*, 1983). Female hamsters were housed at $24 \pm 2^\circ\text{C}$ under a light schedule of 14 h light (05:00–19:00 h). Animals showing 4-day cycles and weighing 80–150 g were used. Ovaries were excised on the day of pro-oestrus at specified times ± 15 min and incubated in the medium until 10:30 h on the following day. The ovaries from each hamster were treated differently, so that ovaries in each treatment were taken from different animals. Ova in the oviducts and in the culture medium were counted under a dissecting microscope at the time of autopsy and at the end of incubation, respectively. Amongst the proteinase inhibitors used in this study, S-PI (*Streptomyces* pepsin inhibitor), talopeptin, thiolstatin, S-SI (*Streptomyces* subtilisin inhibitor) and α -MAPI (microbial alkaline proteinase inhibitor) were prepared by S. Murao; elastatinal, leupeptin, antipain and chymostatin were purchased from the Peptide Institute, Inc., Osaka, Japan; E-64 was supplied by Dr S. Ohmura of Taisho Pharmaceutical Co., Tokyo and plasminostreptin by Dr A. Kakinuma of Takeda Pharmaceutical Co., Osaka, Japan. The inhibitory spectra of these proteinase inhibitors are shown in Table 1.

Table 1. Microbial proteinase inhibitors and their inhibitory spectra

Proteinase inhibitor	Proteinase inhibited	Reference
Carboxyl-proteinase inhibitor S-PI (Ac-pepstatin)	Pepsin, cathepsin D, microbial acid proteinase	Murao & Sato (1971) Sato & Murao (1973)
Metallo-proteinase inhibitor Talopeptin (MK-I)	Thermolysin, collagenase, microbial neutral proteinase	Murao <i>et al.</i> (1980) Fukuhara <i>et al.</i> (1982)
Thiol proteinase inhibitor E-64	Papain, cathepsin B	Hanada <i>et al.</i> (1978)
Thiolstatin	Papain, cathepsin B	S. Murao (unpublished data)
Serine proteinase inhibitor S-SI	Microbial alkaline proteinase	Murao & Sato (1972) Sato & Murao (1974)
Plasminostreptin	Plasmin, trypsin, microbial alkaline proteinase	Kakinuma <i>et al.</i> (1978)
Elastatinal	Elastase	Umezawa <i>et al.</i> (1973)
Serine and thiol proteinase inhibitor α -MAPI	α -Chymotrypsin, papain, microbial alkaline proteinase	Murao & Watanabe (1977) Watanabe & Murao (1979)
Leupeptin	Trypsin, plasmin, papain	Aoyagi <i>et al.</i> (1969)
Antipain	Trypsin, plasmin (weakly), papain	Suda <i>et al.</i> (1972)
Chymostatin	Chymotrypsin, papain	Umezawa <i>et al.</i> (1970)

Antipain, chymostatin and α -MAPI were dissolved in 50% methyl alcohol–saline (9 g NaCl/l) solution and the remaining inhibitors were dissolved in saline (9 g NaCl/l), at a concentration of 0.5, 1.0, 2.0 and 4.0 mM. The basic medium was Medium 199 (Nissui, Co., Tokyo) supplemented with 3.0 mg glucose, 0.1 mg L-glutamine, 0.05 mg ascorbic acid, 0.035 mg insulin, 1.9 mg sodium bicarbonate, 100 units penicillin and 0.12 mg streptomycin/ml. The inhibitor solution (0.4 ml) was added to 10 ml of the basic medium. Control ovaries were incubated in basic medium to which 0.4 ml saline or 50% methyl alcohol–saline solution was added in place of the inhibitor solution. Since control values obtained in the two culture media were not statistically different, they were combined.

Differences in ovulation rate between inhibitor groups and non-inhibitor controls were assessed by Fisher's exact probability test and differences in mean number of ova by Student's *t* test.

Results

Effects of various proteinase inhibitors on ovulation

As shown in Table 2, the ovulatory inhibitory effects of talopeptin and α -MAPI were the strongest amongst those of the 11 inhibitors examined. Talopeptin at a concentration of 0.16 mM completely blocked ovulation of all ovaries explanted at 21:00 h on the day of pro-oestrus, and 17 ovaries treated with 0.08 and 0.16 mM- α -MAPI ovulated only 5 ova in all. A dose of 0.02 mM- α -MAPI effectively blocked ovulation. S-SI, plasminostreptin, antipain and chymostatin were less effective. The effect of antipain was not dose dependent. S-PI and elastatinal exhibited lesser but still significant effects.

Table 2. Effect of proteinase inhibitors on ovulation of hamster ovaries explanted at 21:00 h on the day of pro-oestrus

Inhibitor	Inhibitor conc. (mM)	No. of ovaries		Mean $\pm$ s.e.m. no. of ova/ovary
		Examined	Ovulating	
None (control)	—	20	20	4.3 $\pm$ 0.3
S-PI	0.08	8	7	3.0 $\pm$ 0.6
	0.16	8	6	2.0 $\pm$ 0.7*
Talopeptin	0.02	8	8	3.1 $\pm$ 0.4
	0.08	8	4†	0.8 $\pm$ 0.3*
	0.16	8	0†	0.0
E-64	0.08	4	4	4.3 $\pm$ 1.3
Thiolstatin	0.08	8	8	3.5 $\pm$ 0.6
S-SI	0.08	8	6	1.5 $\pm$ 0.5*
Plasminostreptin	0.04	4	4	3.0 $\pm$ 0.4
	0.08	8	7	2.1 $\pm$ 0.6*
Elastatinal	0.08	8	7	2.4 $\pm$ 0.6*
α -MAPI	0.02	6	5	1.2 $\pm$ 0.4*
	0.08	9	2†	0.3 $\pm$ 0.2*
	0.16	8	2†	0.3 $\pm$ 0.2*
Leupeptin	0.08	8	8	3.6 $\pm$ 0.4
	0.16	8	7	4.0 $\pm$ 0.7
Antipain	0.08	9	5†	0.9 $\pm$ 0.4*
	0.16	8	7	1.9 $\pm$ 0.4*
Chymostatin	0.08	8	7	1.8 $\pm$ 0.5*
	0.16	8	7	1.5 $\pm$ 0.4*

† $P < 0.01$, compared to the control value by Fisher's exact probability test.

* $P < 0.01$, compared to the control value by Student's t test.

Table 3. Effect of 0.16 mM-talopeptin and - α -MAPI on ovulation of hamster ovaries explanted at various times on the day of pro-oestrus

Time of explantation (h)	No. of ovaries ovulating/ no. of ovaries examined			Mean $\pm$ s.e.m. no. of ova/ovary		
	Control	Talopeptin	α -MAPI	Control	Talopeptin	α -MAPI
21:00	8/8	0/8 ^{a**}	2/8 ^{ab**}	4.4 $\pm$ 0.4	0.0 $\pm$ 0.0	0.3 $\pm$ 0.2 ^{a**}
22:00	8/8	3/12 ^{a**}	0/8 ^{a**}	4.6 $\pm$ 0.5	0.4 $\pm$ 0.2 ^{a**}	0.0 $\pm$ 0.0
23:00	8/8	12/12 ^b	5/10 ^{b*}	4.5 $\pm$ 0.5	2.4 $\pm$ 0.4 ^{b**}	0.7 $\pm$ 0.3 ^{a**}
24:00	8/8	10/10 ^b	7/10 ^b	4.8 $\pm$ 0.6	3.1 $\pm$ 0.5 ^{b*}	2.4 $\pm$ 0.7 ^{b*}

Within each column figures without a common alphabetical superscript differ significantly, $P < 0.05$.

* $P < 0.05$; ** $P < 0.01$ compared to the corresponding controls.

Temporal relationships of the blocking effects of proteinase inhibitors on ovulation

Talopeptin and α -MAPI strongly inhibited ovulation of ovaries explanted at 21:00 and 22:00 h on the day of pro-oestrus (Table 3). The inhibitory effect of talopeptin decreased markedly in ovaries explanted at 23:00 h or later, but strong inhibition by α -MAPI was still observed in ovaries explanted at 23:00 h. Amongst the ovaries explanted at 24:00 h, 2 ovaries in the control group and 1 ovary in the talopeptin-treated group had already ovulated 1 or 2 ova when they were killed. These ova were excluded from the data.

The blocking effect of antipain, chymostatin and plasminostreptin on ovaries at 20:00 h was not different from the effect on ovaries at 21:00 h (Table 4).

Table 4. Effects of 0.08 mM-antipain, -chymostatin and -plasminostreptin on ovulation of hamster ovaries explanted at 20:00 and 21:00 h on the day of pro-oestrus

Inhibitor	No. of ovaries ovulating/ no. of ovaries examined		Mean $\pm$ s.e.m. no. of ova/ovary	
	20:00 h	21:00 h	20:00 h	21:00 h
None	14/16	20/20	3.6 $\pm$ 0.6	4.3 $\pm$ 0.3
Antipain	7/8	5/9**	1.5 $\pm$ 0.4*	0.9 $\pm$ 0.4**
Chymostatin	7/8	7/8	1.6 $\pm$ 0.5*	1.8 $\pm$ 0.5**
Plasminostreptin	6/8	7/8	1.1 $\pm$ 0.3*	2.1 $\pm$ 0.6**

Values at 21:00 h are cited from Table 2.

* $P < 0.05$; ** $P < 0.01$ compared to the corresponding controls.

Discussion

We previously reported that a talopeptin-sensitive metallo-proteinase, probably collagenase, was indispensable for ovulation in the hamster ovary (Ichikawa *et al.*, 1983). We now propose that another proteinase which is specifically inhibited by α -MAPI is also indispensable for the ovulatory process. This is based on the following observations in the present study: (1) α -MAPI, an inhibitor for serine- and thiol-proteinase, completely blocked ovulation of the explanted hamster ovary, as did talopeptin; (2) there was chronological discrepancy in the ovulatory blocking effect between talopeptin and α -MAPI, i.e. α -MAPI strongly inhibited ovulation of ovaries explanted at 23:00 h, whereas the inhibitory effect of talopeptin had decreased by this time. The proteolysis by the α -MAPI-sensitive proteinase seems to proceed in succession, rather than in parallel, to the collagenolysis by metallo-proteinase. It is not known which protein is attacked by the α -MAPI-sensitive proteinase in the follicular wall, but the proteinase may degrade a protein involved in the junctional complex between cells or an adhesive protein, such as cell surface fibronectin which binds cells to other cells (Yamada & Olden, 1978).

We have no data at present on the enzymic properties of the α -MAPI-sensitive proteinase in the follicular wall, but it is unlikely that this enzyme is a chymotrypsin-like proteinase or thiol-proteinase, both of which are specifically inhibited by α -MAPI *in vitro*, because chymostatin, an inhibitor of chymotrypsin, was less effective than α -MAPI in blocking ovulation, and E-64 and thiolstatin which are inhibitors of thiol proteinases were ineffective blockers (Table 5). Furthermore the α -MAPI-sensitive proteinase is apparently different from serine proteinase, because serine proteinase inhibitors examined in this study were less effective than α -MAPI or unable to block ovulation from ovaries explanted at 21:00 h on the day of pro-oestrus (Table 5).

On the basis of various observations, Espey (1980) proposed that serine proteinases may participate in ovulation in mammals. In the present study S-SI, plasminostreptin, elastatinal, antipain and chymostatin, which are strong inhibitors against serine proteinases, significantly

Table 5. Inhibition of proteinases and ovulation by proteinase inhibitors

Proteinase inhibitor	Proteinase								Mean no. of ova/ ovary, at 0.08 mm inhibitor
	Acid Pepsin Cathepsin D	Metallo Collagenase Thermolysin	Thiol Papain Cathepsin B Ca ²⁺ -activated proteinase	Serine					
Acid S-PI	+ +	- -	- - -	-	-	-	-	-	3.0
Metallo Talopeptin	- -	+ +	- - -	-	-	-	-	-	0.8*
Thiol E-64	- -	- -	+ + +	-	-	-	-	-	4.3
Thiolstatin	- -	- -	+ + +	-	-	-	-	-	3.5
Serine S-SI	- -	- -	- - -	-	-	-	-	+	1.5*
Plasminostreptin	- -	- -	- - -	+	-	-	+	+	2.1*
Elastatinal	- -	- -	- - -	-	-	+	-	-	2.4*
Serine and thiol α -MAPI	- -	- -	+ + +	-	+	-	-	+	0.3*
Leupeptin	- -	- -	+ + +	+	-	-	+	+	3.6
Antipain	- -	- -	+ + +	+	-	-	±		0.9*
Chymostatin	- -	- -	+ + +	-	+	-	-		1.8*

The values in the last column are cited from Table 2. The asterisk indicates significant difference ($P < 0.01$) when compared to the control value of 4.3 in Table 2.

inhibited ovulation of the hamster ovary (Table 5). These observations support Espey's (1980) hypothesis. However, the ovulatory blockage by these 5 inhibitors in the ovaries explanted at 20:00 and 21:00 h was partial and not complete. Furthermore, leupeptin, a strong inhibitor of trypsin, was ineffective on ovaries explanted at 21:00 h. According to Werb & Aggeler (1978), plasmin and other serine proteinases activate procollagenase to collagenase in inflamed tissues, and trypsin does so in the rat Graafian follicle (Morales, Woessner, Howell, Marsh & LeMaire, 1978). It is therefore probable that the action of serine proteinase in the ovulatory process might have been essentially completed by the time of explantation of the ovaries at 20:00 h. If the inhibitors had been applied to ovaries explanted at 19:00 h or earlier they might have inhibited ovulation more strongly. However, when the hamster ovary was explanted at 19:00 h or earlier on the day of pro-oestrus and cultured by the method used in this study, the number of ova ovulated was too small to evaluate the ovulatory blocking effect of the inhibitor (Morioka & Ichikawa, 1982).

In conclusion, the present results suggest that at least three kinds of proteinases, i.e. serine proteinase(s), metallo-proteinase and an α -MAPI-sensitive proteinase, are involved in the ovulatory process of the hamster ovary, and that serine proteinases may act on the ovulatory process by 20:00 h on the day of pro-oestrus, metallo-proteinase by 23:00 h and the α -MAPI-sensitive proteinase by 24:00 h. While metallo-proteinase presumably degrades the collagen matrix in the follicular wall by 23:00 h, α -MAPI-sensitive proteinase may act on other components of the follicular wall before rupture.

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