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IN VITRO EFFECT OF SOME FREE ESTROGENS OR ESTROGEN PRECURSORS ON COLLAGENASE ACTIVITY OF UTERINE CERVIX

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Synopsis In order to determine whether or not collagenase activity of the uterine cervix can be influenced by estrone (E_1), estradiol (E_2), estriol (E_3), equilin, dehydroepiandrosterone sulfate (DHA-S) or dehydroepiandrosterone (DHA), the collagenase activity of extract of connective tissue of the uterine cervix treated with the drug was measured. A higher collagenase activity was obtained in pregnant cervix than that in nonpregnant ones. There was no difference in collagenase activity of the uterine cervix extract between control and drug (5×10^{-5} M of E_1, E_2, E_3, equilin, DHA-S or DHA) groups both in nonpregnant and pregnant conditions.

After preincubation of tissue with 1×10^{-5} M of the said drug at 37°C for 60 min, the enzyme activity of the supernatant was also investigated. In nonpregnant conditions, no increase in the enzyme activity was observed. On the contrary, significant increase of the enzyme activity was obtained in pregnant uterine cervix preincubated with equilin, E_3, DHA-S or DHA comparing to that of control group. However, these increase was not observed by E_1 or E_2.

Elastase activity was also investigated on cervical connective tissue both in nonpregnant and pregnant conditions. No increase in elastase activity was observed in the pregnant cervix comparing to that of nonpregnant group.

Key words: Collagenase • Uterine cervix • Estrogens • Equilin • DHA-S

Introduction

The stimulating effect of intraveneous administration of conjugated estrogens (CE) or DHA-S on the ripening of the pregnant uterine cervix have been reported by many investigators. The detailed mechanism of ripening by these drugs, however, have not been explained sufficiently. This paper describes the collagenase activity which is thought to be a key factor in ripening mechanism of the uterine cervix, in nonpregnant and pregnant conditions, and the effect of estrogens or estrogen precursors on the enzyme.

Materials and Methods

Reagents:

N-carbobenzoxy-glycyl-prolyl-glycyl-glycyl-prolyl-alanine (CBZ-GPGGPA), glycyl-prolyl-alanine (GPA) and bovine serum albumin, bovine pancreas trypsin (type III), estrone, β-estradiol, estriol, equilin, succinyl-(L-Alanine)$_3$-p-nitroanilide were purchased from Fluka AG, Chemische Fabrik, Switzerland and Sigma Chemical Companies U.S.A.: DHA-S and DHA (Organon, OSS, Holland), Ninhydrin (Nakarai Chemicals, LTD, Kyoto, Japan), N-metyl-2-pyrolidone (Wako Pure Chemical Industries, LTD, Osaka, Japan).

Specimens of uterine cervix or uterine body were obtained during simple hysterectomy or cesarean section from 13 nonpregnant and 8 pregnant women. A portion of the specimens (700 to 800 mg wet weight of the tissue) was washed in cold saline and homogenized immediately in 5 ml of 0.1 M acetate buffer (pH 5.0), containing 0.1% of Triton-X 100 using a glass homogenizer. The homogenate was centrifuged at 6,000×g for 10 min in refrigerated centrifuge, 0.05 ml of the supernatant was subjected to enzyme assay. The protein content of the supernatant was determined by the method of Lowry et al.[8] (1951) with crystalline bovine serum albumin as standard.

(1) Collagenase activity of the supernatant fluid (=extract) of uterine tissues

Collagenase activity of the supernatant was measured by the procedures of Espey and Rondell[4] (1967) with some modifications. A reac-

tion mixture of 0.1 ml CBZ-GPGGPA solution (5 mg per ml of distilled water containing 0.001 M $CaCl_2$), 0.05 ml of the supernatant and 0.05 ml of 0.1 M acetate buffer (pH 5.0) was incubated at 37°C for 3 hours. The reaction was stopped by 0.1 ml of 10% trichloroacetic acid and resulting precipitate was removed by centrifugation at 2,000×g for 10 min. A reaction mixture of 0.1 ml supernatant, 0.02 ml of 1.0 M NaOH, 0.2 ml of ninhydrin reagent was placed in a boiling water bath for 15 min. After dilution of the mixture with 5 ml of an even mixture of n-propanol and water, the optical density was measured with Hitachi Model 200-20 spectrophotometer at 570 mμ.

The activity was determined by subtracting the OD in the absence of a substrate (blank test) from the total OD in test tubes containing both a substrate and the extract. The collagenase activities are expressed as ΔE_{570} per 3 hour per mg protein in the supernatant fluid.

(2) Drug influence on collagenase activity of the uterine cervix extract

After incubation of the initial reaction mixture with E_1, E_2, E_3, eqilin, DHA-S or DHA at final concentration of 5×10^{-5} M, the collagenase activities were measured by the same procedures described above.

(3) Collagenase activity of the uterine cervix slice preincubated with drug

Slice was made from another portion of tissue (40 to 50 mg wet weight) and was preincubated in 4.95 ml of Krebs-Ringer bicarbonate buffer (pH 7.3) containing E_1, E_2, E_3, equilin, DHA-S or DHA at final concentration of 1×10^{-5} M with or without 0.05 ml of trypsin, Type III, 12.700 BAEE units per mg protein, (1 mg per ml of saline) for 30 min at 37°C under 100% oxygen. The slice was washed in cold saline and homogenized in 2 ml of 0.1 M acetate buffer (pH 5.0) containing 0.1% of Triton-X 100 using a glass homogenizer. The homogenate was centrifuged at 6,000×g for 10 min in refrigerated centrifuge, 0.05 ml of the supernatant was subjected to enzyme assay by the method described above.

(4) Elastase activity of the uterine cervix

Elastase activity of the uterine cervix was also measured by the method of Bieth (1974). Supernatant for elastase assay was made from the cervical tissue homogenate using saline instead of 0.1 M acetate buffer (pH 5.0). The reaction mixture of 40 μl of succinyl-(L-Alanine)$_3$-p-nitroanilide solution, 4.8 ml of 0.05 M Tris-HCl buffer (pH 9.0) and 0.2 ml of the supernatant was incubated at 37°C for 20 min. The elastase activity is expressed as ΔE_{410} per 20 min per mg protein in the supernatant fluid. Trypsin effect of elastase activity was also investigated by the same way of that on collagenase except extension of the incubation time from 30 min to 60 min.

Results

(1) Collagenase activity of the supernatant fluid of uterine cervix

The collagenase activity of the supernatant fluid of connective tissue of the uterine cervix was measured and compared with those of uterine bodies in both nonpregnant and pregnant conditions. The following results (ΔE_{570} per 3 hours per mg protein) were obtained; connective tissue of nonpregnant cervix 41.8 ± 17.8 (n=13), connective tissue of pregnant cervix 61.8 ± 19.2 (n=8), nonpregnant myometrium 26.2 ± 8.1 (n=4), pregnant myometrium of placenta-implanting site 58.2 ± 6.6 (n=3), pregnant myometrium of placenta-non-implanting site 83.9 ± 28.3 (n=3) and connective tissue of nonpregnant portio vaginalis 67.7 ± 25.9 (n=3).

A significant increase of collagenase activity was obtained in connective tissue of pregnant uterine cervix comparing to that of nonpregnant ones.

There was no difference in the activities of this enzyme between connective tissue of uterine cervix and myometrium both in nonpregnant and pregnant conditions. Trypsin effect on collagenase activity was also investigated and no influence was observed (Table 1).

(2) Drug influence on collagenase activity of the uterine cervix extract

Drug influence on collagenase activity of the

Table 1. Comparison of collagenase activities at various portion of the uterus

	Number of Cases	Collagenase activity ΔE_{570}/3 hr/mg protein mean ± SE	Significant differences from 1) (*P*)
In nonpregnant condition:			
1) Connective tissue of cervix	13	41.8±17.8	
2) Myometrium	4	26.2± 8.1	0.5>P>0.1
3) Connective tissue of portio vaginalis	3	67.7±25.9	0.1>P>0.05
In pregnant condition:			
4) Connective tissue of the cervix	8	61.8±19.2	0.05>P>0.02
5) Myometrium			
Placenta implanting site	3	58.2± 6.6	0.2>P>0.1
Placenta nonimplanting site	3	83.9±28.3	0.01>P>0.001

Table 2. Drug influences on collagenase activity of the uterine cervix extract

	Collagenase activity ΔE_{570}/3 hr/mg protein mean ± SE	Significant differences from control (*P*)
Nonpregnant cases (n=4)		
control	29.8±14.7	P>0.5
estribe (5×10⁻⁵ M)	31.4±16.2	
estradiol (〃)	37.7±21.7	
estriol (〃)	26.2±11.1	
equilin (〃)	29.4±10.9	
DHA (〃)	28.0±13.5	
DHA-S (〃)	27.1±11.2	
Pregnant cases (n=6)		
control	42.7±13.2	P>0.5
estrone (5×10⁻⁵ M)	43.7±12.1	
estradiol (〃)	41.1±13.5	
estriol (〃)	41.1± 9.2	
equilin (〃)	41.2± 9.2	
DHA (〃)	42.3±12.6	
DHA-S (〃)	40.2±11.6	

uterine cervix extract was investigated by incubation of initial reaction mixture with E_1, E_2, E_3, equilin, DHA-S or DHA at final concentration of 5×10^{-5}M for 3 hours. The following results were obtained in nonpregnant materials (n=4); control 29.8 ± 14.7, E_1 31.4 ± 16.2, E_2 37.7 ± 21.7, E_3 26.2 ± 11.1, equilin 29.4 ± 10.9, DHA 28.0 ± 13.5 and DHA-S 27.1 ± 11.2, in pregnant materials (n=6); control 42.7 ± 13.2, E_1 43.7 ± 12.1, E_2 41.1 ± 13.5, E_3 41.1 ± 9.2, equilin 41.2 ± 9.2, DHA 42.3 ± 12.6, and DHA-S 40.2 ± 11.6.

There was no difference in collagenase activity between the supernatant of uterine cervix (control) and those added said drug both in nonpregnant and pregnant conditions (Table 2).

(3) Collagenase activity of the uterine cervix slice preincubated with drug

After preincubation with said drug at the final concentration of 1×10^{-5}M for 1 hour, the collagenase activity of the supernatant of the slice of the uterine connective tissue was measured both in nonpregnant and pregnant conditions. The following results were obtained in nonpregnant slices (n=6); control 42.0 ± 7.8, E_1 45.9 ± 10.3, E_2 48.4 ± 6.9, E_3 46.5 ± 8.4, equilin 46.6 ± 9.1, DHA 43.8 ± 10.0 and DHA-S 46.5 ± 9.0, in pregnant slices (n=6); control 42.4 ± 6.6, E_1 51.7 ± 11.5, E_2 49.7 ± 13.4, E_3 60.9 ± 13.2, equilin 68.8 ± 18.4, DHA 53.5 ± 6.7 and DHA-S 54.5 ± 8.7.

There was no increase of collagenase activity in the slices of nonpregnant cervix preincubated with said drugs. On the contrary, a marked (0.02>P) or significant (0.05>P>0.02) increase of the collagenase activity was obtained in the slices of pregnant uterine cervix preincubated with equilin, E_3, DHA-S or DHA comparing to that of control group. However, these increase was not observed by E_1 or E_2 (Table 3).

There was no difference on collagenase activity of the slices treated with the said drugs between control and trypsin treated materials both in nonpregnant and pregnant conditions.

(4) Elastase activity of the uterine cervix

Elastase activity was also calculated on cervical tissue both in nonpregnant or pregnant (39-42 weeks gestation) conditions. Elastase activities (ΔE_{410} per 20 min per mg protein of

Table 3. Collagenase activities of the uterine cervical slices preincubated with drug

	Collagenase activity ΔE_{570}/3 hr/mg protein mean ± SE	Significant differences from control (P)
Nonpregnant cases (n=6):		
control	42.0± 7.8	
estrone (1×10⁻⁵ M)	45.9±10.3	P>0.5
estradiol (〃)	48.4± 6.9	0.5>P>0.1
estriol (〃)	46.5± 8.4	0.5>P>0.1
equilin (〃)	46.6± 9.1	0.5>P>0.1
DHA (〃)	43.8±10.0	P>0.5
DHA-S (〃)	46.5± 9.0	0.5>P>0.1
Pregnant cases (n=6):		
control	42.4± 6.6	
estrone (1×10⁻⁵ M)	51.7±11.5	0.5>P>0.1
estradiol (〃)	49.7±13.4	0.5>P>0.1
estriol (〃)	60.9±13.2	0.02>P>0.01
equilin (〃)	68.8±18.4	0.02>P>0.01
DHA (〃)	53.5± 6.7	0.05>P>0.02
DHA-S (〃)	54.5± 8.7	0.05>P>0.02

supernatant fluid) were obtained 2.1 ± 0.7 (n=3) and 2.5 ± 1.4 (n=3) in nonpregnant and pregnant materials, respectively. There was no difference in elastase activity of the uterine cervix between nonpregnant and pregnant conditions. Trypsin effect on this enzyme was also investigated and no influence was observed.

Discussion

In the ripe uterine cervix, three to four folds increase of collagenolytic enzyme activity than those in the nonpregnant cervix was reported by Hirakawa[5] (1978). Our investigation was in agreement with his observation except for the increasing rate of activity of the enzyme. We think this difference may be depend upon the difference of the synthetic substrate used in each investigation. On the other hand, a slight increase of prolyl hydroxylase activity in the uterine cervix immediately after delivery was reported by Yamamoto et al.[17] (1980). Since, the increasing rate of the collagenolytic enzyme activity was more markedly than that of prolyl hydroxylase, key enzyme of collagen biosynthesis, in the ripe cervix, it was suggested that the mechanism of so-called collagen-dissociation involves mainly decomposition or degradation of collagen fibers and in addition to the collagen new-formation.

The stimulating effects of free estrogens, CE or DHA-S on the ripening of the uterine cervix or animals have been reported by many investigators (e.g. Puck and Hübner[11], 1956; Puck et al.[12], 1957; Overbeek and Visser[10], 1958; Boglin[2], 1959; Kushima et al.[7], 1961; Saito et al.[13], 1979; Saito et al.[14][15], 1981; Sekiba et al.[16], 1972; Chimura et al.[3], 1978; Kobayashi et al.[6], 1977). The detailed mechanism of ripening by these drugs, however, have not been explained sufficiently.

We have reported that DHA-S or CE (which contains E_1 and equilin) exhibited more prompt effects in increasing the wet weight of the cervix than did free estrogens in castrated rats. Judging from the close resemblance between the electronmicroscopic figures of the DHA-S or CE group and those of the free estrogens group, we postulated that the effect of DHA-S or CE might be due mainly to the action of free type estrogens converted from these drugs, and additionally, due to some unknown factors involved in the drugs in the rat.

Since the concentration of 17β-estradiol and total collagenase activity in the uterine cervical tissue of pregnant women were elevated after a single shot intravenous injection of 200 mg DHA-S, Mochizuki[9] (1977) concluded that DHA-S converted to 17β-estradiol in the placenta was considered to have acceleration effect on the collagenase activity of the cervix.

In order to determine whether or not the direct increasing of collagenase activity of the tissue can be brought about by these drugs in vitro, the investigation were carried out on the supernatant fluid of the human uterine cervical slice preincubated with the drug in nonpregnant and pregnant conditions.

There was no increase of the enzyme activity in the slice of nonpregnant cervix preincubated with E_1, E_2, E_3, equilin, DHA-S or DHA.

On the contrary, a marked or significant increase of the enzyme activity was observed in the slices of pregnant cervix preincubated with equilin, E_3, DHA-S or DHA comparing to that of control group, however, E_1 or E_2 was no effect.

The results strongly suggest that the mecha-

nism of cervical ripening induced by intravenous administration of CE or DHA-S might involve not only the action of free estrogens converted from these drugs but also the direct local action of these drugs to the collagenase activity.

The mechanism of increasing activity of the enzyme after preincubation with equilin, E_3 or DHA-S remains obscure.

The encrease in production or secretion of the enzyme will be considered.

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References

1. *Bieth, J., Spiess, B. and Wermuth, C.G.:* The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochem. Med., 11: 350, 1974.
2. *Boglin, N.E.:* Effect of estriol on the female genital tract. Acta Obstet. Gynec. Scand., 38: 157, 1959.
3. *Chimura, T., Kanasugi, H., Oda, T., Kawagoshi, S. and Hiroi, M.:* Augmentation of cervical ripeness and uterine activity by the administration of conjugated estrogen. The World of Obstetrics and Gynecology (Jap), 30: 377, 1978.
4. *Espey, L.L. and Rondell, P.:* Estimation of mammalian collagenolytic activity with a synthetic substrate. J. Appl. Physiol., 23: 757, 1967.
5. *Hirakawa, S.:* The mechanism of cervical ripening on pregnancy and labor in human and rabbit; biochemical changes in collagen, collagenolytic enzyme activity. Glycosaminoglycans (hyaluronic acid) and noncollagenous protein. Acta Obst. Gynaec. Jpn., 30: 885 and 1525, 1978.
6. *Kobayashi, T., Nakayama, T. and Tojo, S.:* Dehydroepiandrosterone sulfate and the ripening of pregnant uterine cervix. The World of Obstetrics and Gynecology (Jap), 29: 467, 1977.
7. *Kushima, K., Ichijo, M., Hirano, M. and Ohtomo, Y.:* Clinical and fundamental studies on estriol, (1) Effect on dilatation of the uterine cervix during labor. The World of Obstetrics and Gynecology (Jap), 13: 515, 1961.
8. *Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall R.J.:* Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265, 1951.
9. *Mochizuki, M., Honda, T. and Tojo, S.:* Collagenolytic activity and serum or myometrial steroid levels following a intravenous dose of dehydroepiandrosterone sulfate in human term pregnancy. Acta Obst. Gynaec. Jpn., 29: 995, 1977.
10. *Overbeek, G.A. and de Visser, J.:* A comparison of oestriol and oestradiol in female rat. Acta Endocrinol., 27: 73, 1958.
11. *Puck, A. und Hübner, K.A.:* Die wirkungen des oestriols auf uterus und vagina des kaninchens und meerschweinchens und auf die symphyse des meerschweinchens. Acta Endocrinol., 22: 191, 1956.
12. *Puck, A., Korte, W. und Hübner, K.A.:* Die wirkung des oestriol auf corpus uteri, Cervix uteri und vagina des frau. Dtsch. med. Wschr., 44: 1864, 1957.
13. *Saito, Y. and Takahashi, S.:* Effects of dehydroepiandrosterone sulfate (DHA-S) on ripening of the uterine cervix in rat. Acta Obst. Gynaec. Jpn. (Jap), 31: 1329, 1979.
14. *Saito, Y., Takahashi, S. and Maki, M.:* Effects of some drugs on ripening of uterine cervix in nonpregnant castrated and pregnant rat. Tohoku J. exp. Med., 133: 41, 1981.
15. *Saito, Y., Takahashi, S. and Maki, M.:* Effects of conjugated estrogens on ripening of uterine cervix in nonpregnant castrated and pregnant rats. Acta Obst. Gynaec. Jpn., 33: 14, 1981.
16. *Sekiba, K. and Kamada, S.:* The effects of conjugated estrogen (premarin) on ripening of the rigid cervix of late pregnant women. Toyoyakujiho (Jap), 13: 13, 1972.
17. *Yamamoto, S., Seida, A. and Okuyama, T.:* Collagen metabolism in the human cervix during pregnancy, delivery and puerperium. Acta Obst. Gynaec. Jpn., 32: 909, 1980.

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概要 E_3, Conjugated estrogens (CE), DHA-S などによる妊婦子宮頚熟化促進機序の一端に，これら薬剤による子宮頚結合織への局所作用が関与しているか否かを明らかにする目的で子宮頚結合織内 collagenase 活性におよぼすこれら薬剤の影響を in vitro で検討し以下の成績を得た.

1) 子宮頚結合織 homogenate 上清画分の collagenase 活性 (ΔE_{570}/3hr/mg protein) は非妊例に比べ妊末例で高値 ($p<0.05$) であつた. 本酵素活性測定に際し上清一合成基質 (CBZ-GPGGPA)・反応系に E_1, E_2, E_3, equilin, DHA あるいは DHA-S を 5×10^{-5}M (終末濃度) 添加しても得られた collagenase 活性値は対照 (無添加) 群と差を示さなかつた.

2) 上記薬剤を 1×10^{-5}M (終末濃度) 添加し子宮頚結合織 slice を Krebs Ringer 緩衝液 (pH 7.3)内で37℃, 60分振盪培養後, slice の collagenase 活性を対照 (薬剤無添加) 例と比較検討した. 非妊例では対照群と薬剤添加群とで本酵素活性に差はなかつた. 妊末例 ($n=6$) では対照群 (42.4±13.4) に比べ E_3 群 (60.9±13.2), equilin 群 (68.8±18.4), DHA 群 (53.6±6.6) および DHA-S 群 (54.5±8.7) で有意 ($p<0.05$～$p<0.02$) の collagenase 活性高値が得られた. E_1 と E_2 ではこの高値は得られなかつた.

なお, 子宮頚結合織内 elastase 活性もあわせて検討したが本酵素活性は非妊例と妊末例で有意差がなかつた.

以上の成績から E_3, CE, DHA,S 投与時の妊婦子宮頚熟化促進にはこれら薬剤による子宮頚結合織内 collagenase 活性上昇作用 (局所作用) が関与しているものと推定される.