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Short paper

Effects of High Intracellular Calcium Concentration by Ouabain on VTG Production in the Primary Hepatocyte Cultures of Rainbow Trout, *Oncorhynchus mykiss.*

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무지개송어(*Oncorhynchus mykiss*) 간세포배양에 있어서 Ouabain에 의한 세포내 高Calcium농도가 Vitellogenin 합성에 미치는 효과

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Effects of high concentration of intracellular calcium on estradiol-induced vitellogenin (VTG) induction were examined using ouabain in primary hepatocyte culture in the rainbow trout *Oncorhynchus mykiss*. Ouabain increases cytosolic free calcium as a result of inhibition of Na^+-Ca^{2+} exchanger. Ouabain markedly reduced VTG production to the control level, despite of calcium concentrations in the incubation medium. Therefore, ouabain would reduce VTG production not by increasing intracellular calcium but directly by inhibiting Na^+-K^+ ATPase.

Key words : Ouabain, EGTA, Intracellular calcium, Vitellogenin production, Rainbow trout (*Oncorhynchus mykiss*)

Vitellogenin (VTG), a calcium-binding and highly phosphorylated protein, is a precursor molecule of egg yolk in oviparous vertebrates. VTG is synthesized in the liver under the influence of estrogen (Wallace, 1985). VTG contains about 0.7% calcium in the rainbow trout (Fremont and Riazi, 1988) and about 2.0% calcium in the bass (Urist and Schjeide, 1961).

Calcium is required for protein synthesis in a variety of cells (Brostrom et al., 1983 ; Brostrom et al., 1986 ; Chin et al., 1987 ; Chin et al., 1988). Recently, Yeo and Mugiya (1997) found that VTG production is more susceptible to calcium than are other hepatocyte-derived proteins in the rainbow trout. Moreover, they suggested that trout hepatocytes have receptor-operated and verapamil-sensitive calcium channels that are functionally related to VTG production. It was also reported that the depletion of intracellular sequestrators by calcium agonists reduced the production of VTG (Yeo and Mugiya, 1998). Therefore, VTG production may be highly dependent on an intracellular calcium state, because VTG is a calcium-binding protein and highly phosphorylated nature.

In addition, Yeo and Mugiya (1998) found that A23187 reduced VTG production in a concentration-dependent way. A23187 rapidly increases cytosolic free calcium by

facilitating the entry of extracellular calcium and by releasing sequestered calcium (Albert and Tashjian, 1986). Brostrom et al. (1989) suggested that A23187 inhibits calcium-dependent translational inhibition of protein production through facilitating the mobilization of intracellular sequestered calcium in mammalian eukaryotic cells. However, intracellular mechanisms of inhibition are not well understood. Therefore, the effect of high concentration of intracellular calcium in VTG induction was examined using ouabain(Research Biochemicals Inc.).

Rainbow trout weighting 150-200 g were obtained from the Nanae Fish Culture Experimental Station, Hokkaido University, and maintained at about 14℃ in outdoor ponds at our laboratory. Hepatocytes were prepared following Hayashi and Ooshiro (1975) as described by Kwon et al.(1993). The culture medium was Leibovitz-15 medium(Ca 1.3 mM, Life Technol. Inc.) containing 0.2 μM bovine insulin(Sigma), streptomycin(100 μg/mℓ), and penicillin (70 μg /mℓ). Isolated hepatocytes were precultured for 2 days, and then estradiol-17β(E_2, 2×10^{-6}M in 3 $\mu\ell$ of 95% ethanol) and ouabain (2×10^{-6}M in 3 $\mu\ell$ of distilled water) were simultaneously added to the dishes. The culture medium contained 1.3 mM calcium, which was lowered to a level of 0.5 mM by adding 0.8 mM EGTA, according to the method described by Yeo and Mugiya(1997). The medium was changed every day throughout the preculture and experimental periods.

Synthesized proteins were analyzed by 5-20% gradient SDS-PAGE according to the method of Laemmli (1970). Identification of the VTG band was based on the results of a previous study (Kwon et al., 1993) in which isolated rainbow trout hepatocytes that were incubated with E_2 synthesized a protein of the same molecular weight (175 kDa) as in the present study. After SDS-PAGE, the integrated optical density (IOD) of the main VTG band was measured by a Bio Image (Millipore) and expressed as a percentage of the experimental to control (E_2 only). This type of expression has the benefit of excluding effects of variations in the number of cultured cells and in the amount of proteins applied to the lanes of electrophoresis (Mugiya and Thanahashi, 1998 ; Yeo and Mugiya, 1998).

Effects of ouabain on VTG production at different calcium concentration of 0.5 mM and 1.3 mM were examined on day 7 after E_2 addition. A low calcium concentrations of 0.5 mM reduced VTG production to about 35% of the control(Ca 1.3 mM)(Fig. 1). Ouabain markedly inhibited VTG production. The rate of decrease reached about 95% and did not differ from the background level without E_2. The addition of ouabain and EGTA to the incubation medium also reduced VTG production to about 92% of the control.

Ouabain indirectly inhibits the outflux of intracellular calcium as a result of inhibition of Na^+-Ca^{2+} exchanger (Garvin et al., 1988). In the present study, ouabain markedly reduced VTG production to the control level (background level). Therefore, it is possible that this reduction is due to too high intracellular calcium concentrations as a result of an increase in sodium concentra-

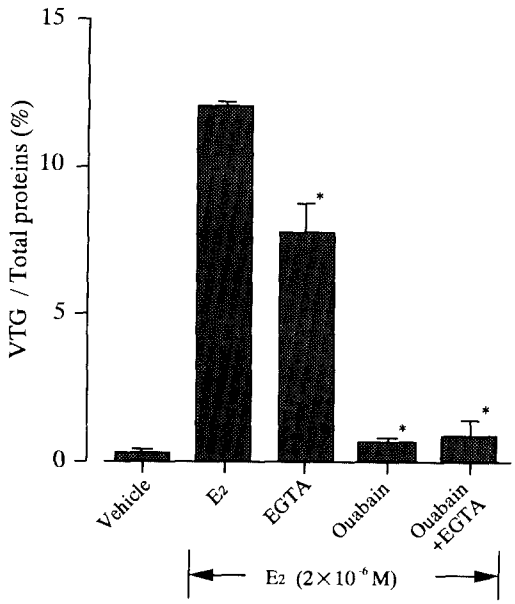

Fig. 1. Effects of ouabain on the E_2-induced production of VTG at calcium concentrations of 0.5 mM (EGTA) and 1.3 mM. Hepatocytes were cultured in the media containing E_2, ouabain(2×10^{-6} M), and/or EGTA for 7 days. Vertical bars represent the SE of mean for triplicate.
***P>0.01 for control(E_2 alone).**

tions in the cytoplasm. To make this point clear, a low extracellular calcium was tested by the addition of EGTA, which then would result in a low intracellular calcium concentration(Brostrom and Brostrom, 1990). However, a reduction in extracellular calcium concentration by EGTA did not recover the ouabain effect. Therefore, ouabain would reduce VTG production not by increasing intracellular calcium but directly by inhibiting Na^+-K^+ ATPase. Mechanisms in which Na^+-K^+ ATPase is involved in VTG production remain to be studied.

References

Albert, P. R. and A. H, Tashjian Jr., 1986. Ionomycin acts as an ionophore to release TRH-regulated Ca^{2+} stores from GH_4C_1 cells. Am. J. Physiol., 251 : C887-C891.

Brostrom, C. O., S. B. Boccking, and M. A. Brostrom, 1983. Identification of a Ca^{2+} requirement for protein synthesis in eukaryotic cells. J. Biol. Chem., 258 : 14390-14399.

Brostrom, C. O., S. B. Boccking, M. A. Brostrom, and E. M. Galuska, 1986. Regulation of protein synthesis in isolated hepatocytes by calcium-mobilizing hormones. Mol. Pharmacol., 29(3) : 104-111.

Brostrom, C. O., K.-V. Chin, W. L. Wong, C. Cade, and M. A. Brostrom, 1989. Inhibition of translational initiation in eukaryotic cells by calcium ionophore. J. Biol. Chem., 264 : 1644-1649.

Brostrom, C. O. and M. A. Brostrom, 1990. Calcium-dependent regulation of protein synthesis in intact mammalian cells. Annu. Rev. Physiol., 52 : 577-590.

Chin, K.-V., C. Cade, C. O. Brostrom, E. M. Galuska, and M. A. Brostrom, 1987. Calcium-dependent regulation of protein synthesis at translational initiation in eukaryotic cells. J. Biol. Chem., 262 : 16509-16514.

Chin, K.-V., C. Cade, M. A. Brostrom, and C. O. Brostrom, 1988. Regulation of protein synthesis in intact rat liver by calcium mobilizing agents. Int. J. Biochem., 20 : 1313-1319.

Fremont, L. and A. Riazi, 1988. Biochemical analysis of vitellogenin from rainbow trout (*Salmo gairdneri*) : Fatty acid composition of phospholipids. Reprod. Nutr. Develop., 28 : 939-952.

Garvin, J. L., K. R. Spring, and P. A. Santi, 1988. Secretion of endolymph by semicircular canals of the shark. Am. J. Physiol., 255 : F711-F719.

Hayashi, S. and Z. Ooshiro, 1975. Gluconeogenesis and glycolysis in isolated perfused liver of the eel. Bull. Jpn. Soc. Sci. Fish., 41 : 201-208.

Kwon, H. C., S. Hayashi, and Y. Mugiya, 1993. Vitellogenin induction by estradiol-17β in primary hepatocyte culture in the rainbow

trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol., 104B : 381−396.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature., 227 : 680−685.

Mugiya, Y. and A. Thanahashi, 1998. Inhibitory effects of aluminium on vitellogenin induction by estradiol-17β in primary culture of hepatocytes in the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol., 109 : 37−43.

Urist, M. R. and A. O. Schjeide, 1961. The partition of calcium and protein in the blood of oviparous vertebrates during estrus. J. Gen. Physiol., 44 : 743−756.

Wallace, R. A. 1985. Vitellogenin and oocyte growth in nonmammalian vertebrates. In "Developmental Biology"(Edited by L. Browder), Pergamon Press, New York, 127−177.

Yeo, I.-K. and Y. Mugiya, 1997. Effects of extracellular calcium concentrations and calcium antagonists on vitellogenin induction by estradiol-17β in primary hepatocyte culture in the rainbow trout *Oncorhynchus mykiss*. Gen. Comp. Endocrinol., 105 : 294−301.

Yeo, I.-K. and Y. Mugiya, 1998. Effects of calcium agonists on vitellogenin induction by estardiol-17β in primary hepatocyte culture in the rainbow trout *Oncorhynchus mykiss*. Fish. Sci., 64(3) : 443−447.