

Dual role of 20-hydroxyecdysone in the apoptosis of the anterior silk gland of the silkworm, *Bombyx mori*

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| 学位授与の題目 | Dual role of 20-hydroxyecdysone in the apoptosis of the anterior silk gland of the silkworm, <i>Bombyx mori</i> (カイコ <i>Bombyx mori</i> の前部絹糸腺のアポトーシスにおける20-ヒドロキシエクジソンの二重支配) |
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学位論文要旨

Anterior silk gland of the silkworm, *Bombyx mori* is a larval specific tissue and its apoptosis is induced by 20-hydroxyecdysone (20E). Larval specific tissue is known to undergo degeneration shortly after pupation.

I investigated the molecular mechanisms underlying the action of 20E as an inducing factor of apoptosis in the anterior silk gland. The results indicate that 20E exerts its effects on the progression of apoptosis through two distinctive processes. One is mediated by nuclear hormone receptors that activate transcription of the genes required for the apoptosis, and the other is independent from *de novo* gene expression. In the latter, dibutyryl cAMP was capable of substituting for 20E, and 20E induced an increase in intracellular cAMP concentration. These results indicate that the latter effect of 20E is mediated by a membrane receptor.

At the pupal metamorphosis of the silkworm, *Bombyx mori*, most of larval specific tissues degenerate through a cellular event known as programmed cell death (PCD), or apoptosis. *Bombyx* silk gland is a larval specific tissue and consisted of three parts, anterior, mid and posterior silk gland. Silk proteins are produced in the middle gland and spun out as silk thread after passing through an anterior silk gland. After completion of spinning of cocoon, silk glands begin to degenerate.

An anterior silk gland is a mere duct surrounded by a single layer of about 300 large flattened cells and lined with a thick cuticular intima at the lumen side. The PCD

of anterior silk glands of *Bombyx* last instar larvae was studied in respect of 20-hydroxyecdysone (20E) requirements *in vivo* and *in vitro*. The glands *in vivo* began to exhibit signs of PCD 2 days after gut purge and completed the PCD by 48 hours after pupation. *In vitro*, 20E precociously induced the PCD, which took 120-144 hours (6 days) for its completion. An oligo-nucleosomal ladder pattern was observed in DNA extracted at the end of PCD, and chromatin condensation occurred as revealed by acridine orange staining. These results indicate that the PCD of anterior silk gland is apoptosis that is induced by a steroid hormone. In the present thesis, I put 7 scores (score 0-6) to distinguished morphological changes (stage 0-6) associated with progression of apoptosis. The progression of apoptosis in the anterior silk gland is slower than apoptosis of other tissues or glands that are induced by 20E. This appears to make it easy to analyze each step of apoptosis at molecular level.

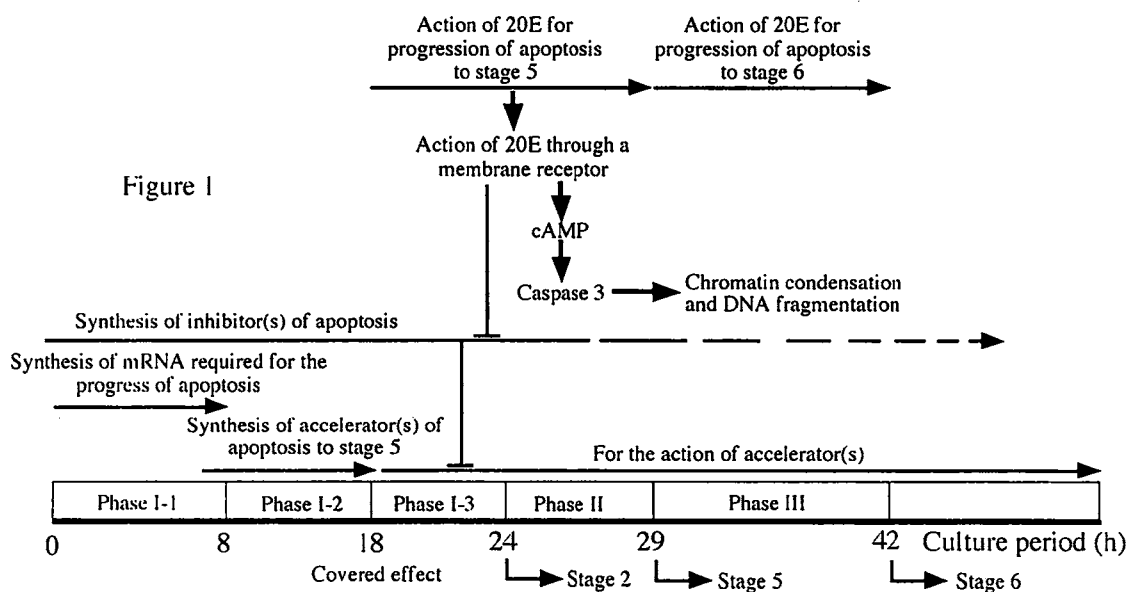
α -Amanitin, which is a mRNA synthesis inhibitor, and cycloheximide (CHX) and emetine, both of which are protein synthesis inhibitors, inhibited the apoptosis when added simultaneously with 20E. By contrast, α -amanitin, CHX and emetine did not inhibit the progressing of apoptosis when added to the culture 8, 18 and 18 h after 20E challenge, respectively. Accordingly, 20E-stimulated transcription and protein synthesis for apoptosis may be completed in 8 and 18 h, respectively. Nevertheless, withdrawal of 20E from the medium in the period of 24-42 h after 20E challenge suppressed the full apoptosis, showing that 20E must be present *in vitro* after completion of gene expression for the full apoptosis. This led an assumption that there are two distinct processes in the mode of action of 20E, one through nuclear hormone receptors and the other independent from *de novo* gene expression. Dibutyryl cAMP was capable of substituting for 20E. In the period after 18 h, 20E induced an increase in an intracellular cAMP concentration, and the increase was observed within one minute after 20E challenge. These results led a proposal that the later effect of 20E is mediated by a membrane receptor. Although the central dogma of a steroid signaling shows that ecdysteroids act through nuclear receptors that are hormone-regulated transcriptional factors, studies in the last decade argue that steroid hormones may also exert their effects without gene expression.

After 18 h of 20E challenge, CHX could not inhibit progression of apoptosis. Rather, addition of CHX to the culture of the glands induced abrupt progression of apoptosis. When added to culture medium at the early stages of apoptosis, it was nuclear condensation and DNA fragmentation after 24-48 h of addition of CHX. Same

phenomenon could be observed in the case of addition of emetine. Normally, the apoptosis progresses step by step *in vitro*. But, the progression skipped over the stage 2, 3 and 4 in the case of addition of CHX. Abrupt progression of apoptosis could be explained based on a hypothesis that there was an apoptosis inhibitor (s), and the abrupt progression was caused by inhibition of inhibitor(s) synthesis. On the other hand, addition of α -amanitin after 8 h of 20E challenge did not induce the abrupt progression. If this is the case, 20E may be involved in the regulation of such inhibitor(s).

Progression of apoptosis in the anterior silk gland is summarized as in Figure 1. The progression is divided three distinctive phases (phase I-III) according to 20E requirement to attain the morphologies of the glands, what are stage 2, 5 and 6. 20E-activation of gene expression is necessary during phase I-1 and phase I-2 but not sufficient for completion of apoptosis. Stimulation of 20E is needed after completion of gene expression (phase I-3 and thereafter). The stimulation of 20E may transmitted through membrane receptor to activation of adenylyl cyclase, and induce an increase in intracellular cAMP concentration. Finally, cAMP signal may be activate caspase cascade, which may induce chromatin condensation and DNA fragmentation. In addition, there is a possibility that the stimulation of 20E suppresses translation or action of inhibitor protein (s).

In this thesis, I argue that 20E-membrane receptor may exist and mediates activation of caspase cascade that is typical of signal pathway in apoptosis. 20E is a steroid hormone. The present thesis is thus the first report that describes dual roles of a same steroid hormone in the progression of apoptosis.



学位論文審査結果の要旨

動物の発生は、形態形成と細胞死の同時進行により進んでいく。近年予定細胞死、あるいはアポトーシスの研究は、その実行段階での分子機構に関しては格段の進歩を見せている。研究は、紫外線などの遺伝子外傷、あるいは内分泌因子による膜受容体を介したアポトーシスのシグナル伝達とその介在タンパク質に関するものであり、死の実行の最終段階における分子レベルでの理解が急速に進みつつある。一方、グルココルチコイドをはじめとするステロイドホルモンが予定細胞死の引き金になることは古くから知られてきた。しかし、その詳細はまったく不明であった。昆虫の発生において、幼虫特異的組織は蛹化とともに予定細胞死により崩壊・除去される。本論文提出者は、昆虫ステロイドホルモンである20-ヒドロキシエクジソン(20E)による絹糸腺の予定細胞死を研究対象とし、細胞死の初期段階から最終段階までのステロイドホルモン要求性に関する検討から研究をはじめた。幼虫特異的組織である全部絹糸腺20Eとともに培養すると、5-6日後に細胞死が完了する。まず、細胞死は典型的なアポトーシスであることを示した。アポトーシスに必要な遺伝子の転写は20E刺激後8時間で完了し、18時間以内にその翻訳が完了する。しかし、アポトーシスの完了には20Eによる刺激がその後も42時間まで必要であった。これはステロイドホルモンによる刺激に遺伝子発現を伴わない、すなわち核受容体を介さない作用発現があることを示唆した。ステロイドホルモンがcAMPレベルを1分以内に急激に増加させることを明確に示す実験系を構築することにより、ここではcAMPがセカンドメッセンジャーとして関与していることを示した。また、20E刺激12時間以降に翻訳阻害物質であるシクロヘキシミド(CHX)を与えると細胞死が急激に進行することを見出し、これは細胞死抑制因子の翻訳が阻害される結果であるとの仮説を立てるに至った。これらもろもろの結果をもとに、申請者は20Eによるアポトーシスの実行を、ホルモン要求性から3段階に区別し、これまであいまいであったステロイドホルモンによるアポトーシスの進行を整理した。すなわち、第一段階は核受容体を介するもの、第二段階は膜受容体を介するもの、第三段階は抑制因子との相克によりアポトーシスが完了する、というものである。これは、同一のホルモンが同一の細胞現象の引き金を引く上で、2種類の作用機構を介して作用し、それは経時的に整然とスイッチオーバーする、という説明である。この作業仮説は今後のステロイドホルモン作用の分子レベルでの研究に一石を投じるものであり、審査員一同は、本論文は学位(学術)を授与するに足るものと判断した。