博 士(理 学) エカテリーナ トゥーマ

学位論文題名

Collapsin Response Mediator Protein-2 cleavage and its impact on beading formation during neurite degeneration

(神経突起変性におけるコラプシン応答メディエータタンパク質-2の分解とそのビーズ形成への影響に関する研究)

学位論文内容の要旨

Neurite degeneration is no more considered a passive event following cell body death of a neuron. More and more evidence states that its mechanisms are not only totally different from the apoptotic program but also happen way before it. Although neurite degeneration, rather than apoptosis is now known to be the cause of main symptoms of many neurodegenerative diseases, its exact mechanisms are largely unknown. In vitro neuritic degeneration can be reproduced by different paradigms, including NGF (nerve growth factor) deprivation, Wallerian degeneration (cutting of the axon from the cell body) and treating the neurons with microtubule disturbing agents like vinblastine. Wallerian degeneration became the preferred method for many since the spontaneous discovery of WLDs (Wallerian degeneration slow) mutant type mice. In the neurons taken from these mice, Wallerian degeneration is markedly delayed. This delay was later to be proven to take place in other paradigms of neurite degeneration as well. To investigate further into the events underlying neurite degeneration in vitro we used NGF deprivation in SCG (superior cervical ganglia) cultures. Using WLDs mice as a control we ran SDS-PAGE for lysates of both NGF-deprived and treated neurites. As a result we found two proteins being markedly up-regulated, which upon MADLI-TOF-MS came out to be CRMP-2 and CRMP-4. Interestingly their C-terminal sequence was not seen by MS as well as their molecular weight was smaller. Taking into account that the cleaved forms were not identified in the lysates taken from the WLDs slow mutant mice made us speculate about the possibility of their cleavage in the course of the neurite degeneration. Knowing that alteration of protein levels of CRMP-2 was mentioned in other diseases as well such as Parkinson's, depression, schizophrenia, epilepsy, in some of which cleavage of CRMP-2 was involved, made us even more interested to explore more about its cleavage during neurite degeneration in our cell culture system.

Collapsin Response Mediator Protein 2 (CRMP-2) is known to play important role in neuronal growth cone guidance, polarization and regeneration. It binds many proteins, including tubulin dimers, actin filaments, Numb, Sra-1/WAVE1complex, such motors as kinesins and dyneins, calmodulin and voltage-gated calcium channels. It is mainly phosphorylated to modulate its binding and activity by different kinases, with the most highly phosphorylated form found bound to PHFs (paired helical filaments) during the course of Alzheimer's disease. Using different protease inhibitors we could identify calpain as the protease responsible for CRMP-2 cleavage during neurite degeneration. These results were further confirmed by the incubation of SCG lysates with purified calpain in the presence and absence of calcium. Wallerian degeneration as well as vinblastine (microtubule disturbing agent) treatment also showed same increase in the CRMP-2 cleaved form in the SCG lysates, suggesting a common mechanism involved in different paradigms of neurite degeneration. Calpain activation has been reported in the process of neurite degeneration, however cleavage of CRMP-2 by calpain was detected only in the

neuronal death following excitotoxicity. Therefore, our work was the first to identify that CRMP-2 is cleaved by calpain during the process of neurite degeneration. By studying the cleavage site specificity of calpain in CRMP-2 protein sequence we could identify possible cleavage sites, which would result in protein of the same size that we saw previously (58KDa). Furthermore, C-terminal end of the protein was found to be very proteolytically susceptible and exposed to the outside of the protein core, therefore even in the absence of the specific cleavage site, it is ready to be cleaved by proteases.

CRMP-2 cleavage site by calpain overlays with its kinesin binding site, therefore its binding to this motor protein would be definitely affected. Tubulin dimers as well as dynein binding regions lie much upstream to the cleavage site, however their transport which is directly bound to kinesin along microtubules will be halted. To investigate more into the possible role and localization of cleaved CRMP-2 we used both immunocytochemical and molecular techniques. Following the appearance of the cleaved form of CRMP-2 (58kDa) during the course of neurite degeneration, we could deduce that cleavage of CRMP-2 occured almost at the same time as beading formation in neurites. Therefore it was logically to assume that CRMP-2 cleaved form was localized to the beads of dying neurites. To confirm that further, we first stained degenerating neurites with C4G antibody and detected the fluorescence under the confocal microscope. Since at the time of beading formation all of CRMP-2 was converted into the CRMP-2 cleaved form, fluorescence that we saw in the beads was surely the cleaved form of CRMP-2. After that by using primers to overlay CRMP-2 truncated form (presumably at the calpain cleavage site) we have inserted it into the mammalian expression vector pEGFP-C1, by using specific restriction enzymes. This vector containing truncated CRMP-2 form was transfected into Neuro2a and PC-12 cells. In both cases fluorescence was evident in the beads following colchicine treatment or NGF deprivation. Next we used *E-coli* expression system in order to express high quantity of CRMP-2 protein to be used later in proteomic studies. The DNA sequence of C RMP-2 was sub-cloned into pGEX-KG plasmid expression vector containing *lac* promoter which could be induced upon the addition of IPTG (Isopropyl β-D-1-thiogalactopyranoside), in order for CRMP-2 to be expressed. However, working hard to get CRMP-2 protein expressed, we have encountered two main problems: protein came out cleaved and aggregated. Using different IPTG concentration as well as changing the expression conditions didn't help to eliminate the problems. Interestingly, the cleavage of CRMP-2 inside *E-coli* gave rise to almost the same size cleaved CRMP-2 as we saw in degenerating neurites upon calpain cleavage. This finding made us speculate: could cleavage of CRMP-2 actually lead to its aggregation, which would promote beading formation during neurite degeneration in vitro? Aggregate formation of different misfolded and cleaved proteins during the course of different neurodegenerative diseases is a known fact. Furthermore, highly phosphorylated form of CRMP-2 is known to be bound to PHFs in Alzheimer's disease. Moreover, CRMP-2 is protected from calpain cleavage by phosphorylation, which is impossible in *E-coli* expression system, making it even more vulnerable to cleavage. More experiments are needed to put light on this particular finding as well as on the ability to express full-length soluble CRMP-2 protein for precise determination of the calpain cleavage site.

In sum, this study has shown for the first time that the cleavage of CRMP-2 during neurite degeneration is caused by calpain 1, suggesting that the cleavage is a common step in the process of dying neurites initiated by diverse causes including axonal cutting and NGF deprivation.

学位論文審査の要旨

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（神経突起変性におけるコラプシン応答メディエータタンパク質-2の分解とそのビーズ形成への影響に関する研究）

神経系の発生においては、神経栄養因子による神経細胞（ニューロン）の軸索および樹状突起の伸張・変性の制御が神経回路網形成に重要な役割を演じることが知られている。近年では、種々の脳疾患と関連して、ニューロン軸索および樹状突起の変性に関する研究も盛んに行われるようになってきている。しかし、現象的は種々の要因によって惹起されることが判明している神経細胞の突起変性について、そのしくみ、特に分子レベルでの変性メカニズムについては、詳細な知見がいまだ得られておらず、今後の研究解析の発展が待たれている状況にある。本論文は、このような現況にある突起変性の分子レベルでのメカニズムについて、軸索輸送で重要な役割を果たすタンパク質 CRMP-2 に着目して、突起変性におけるその機能的意義に関して生化学的・分子生物学的に研究し、神経変性に関する基礎的理解および臨床応用上の有益な知見を得ることを目的として調査した結果をまとめたものである。

申請者は、神経細胞死とともに生じる神経突起変性が、細胞死とは独立のメカニズムに基づ

くという事実に着目し、神経成長因子欠乏によって惹起される変性過程におけるタンパク質の消長を、マウス交感神経上頸神経節から単離した培養細胞を用いて二次元電気泳動法により調べた。その結果、神経軸索の成長・再生などで重要な働きをするタンパク質 CRMP（Collapsin response mediator protein）-2 が変性の過程で分解されることが判明した。CRMP-2 に対するモノクローナル抗体を用いたウエスタンブロッティングでは、変性に伴うビーズ形成と並行して 64kDa の CRMP-2 が 58kDa の短縮形に分解されることが示された。軸索切断後の神経突起変性がゆっくりと進む突然変異をもつウォーラー変性遅延型マウスでは、神経成長因子を欠乏させても CRMP-2 の分解が起こらなかったことから、突起変性と CRMP-2 分解との密接な関連が証明された。

さらに申請者は、この分解が calpain 1 と呼ばれるタンパク質分解酵素によって行なわれることを阻害剤投与（calpain の選択的阻害剤である ZLLH、MDL-28170 の投与は 58kDa の短縮形形成を抑制したが他の阻害剤では CRMP-2 の分解を抑制しなかった）および siRNA による calpain 遺伝子発現の抑制実験（calpain 遺伝子選択性の siRNA は CRMP-2 分解を抑制したが非選択性 siRNA は抑制しなかった）により明らかにするとともに、小脳顆粒細胞においてその神経変性初期段階で calpain 活性が増大することを確認した。また、CRMP-2 の 58kDa 短縮形への分解には、calpain の存在のみならずカルシウムイオンの存在が必要であることを見出し、分解反応が calpain によって調節されることを確認した。なお、calpain による CRMP-2 の分解は、ビンブラスチン（微小管重合阻害剤）および in vitro での軸索切断などによる変性でも確認されたことから、突起変性における共通のステップであることが示唆された。また、分解された CRMP-2 は、変性過程で形成されるビーズ構造に局在することが判明した。CRMP-2 の分解部位については、まだ最終的結論は得られていないが、軸索輸送などに関わるモータータンパク質 kinesin-1 との結合部位である C 末端が分解部位である可能性があり、ここでの分解によって CRMP-2 が機能不全に陥り、ビーズ形成に伴う自食作用によって吸収される可能性が示唆される。CRMP-2 はチューブリン二量体およびアクチン繊維と結合する能力を持ち、kinesin-1 と協力してこれら細胞骨格タンパク質を微小管に沿って運搬する機能を果たすと考えられている。calpain による分解では、微小管との結合は阻害されないが、軸索における遠心性輸送は影響を受けるものと考えられる。

以上を要するに、申請者は神経突起変性において CRMP-2 タンパク質がタンパク質分解酵素 calpain によってその C 末端領域で切断されること、またこの切断が、異なる原因によって惹起する多様な突起変性の共通ステップであることを実験的に証明したものであり、脳疾患の根底に存する神経突起変性に関する知見を大きく進展させたという意味で、基礎的のみならず臨床的な神経科学に貢献するところ大なるものがある。よって、著者は、北海道大学博士（理学）の学位を授与される資格あるものと認める。