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専　　攻　　名	人間環境医工学専攻
学位論文題名	Pre-treatment with amitriptyline causes epigenetic up-regulation of neuroprotection-associated genes and has anti-apoptotic effects in mouse neuronal cells (抗精神病薬アミトリプチリンはエピジェネティクス機構を介して神経細胞死を抑制する)
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学位論文内容の要旨

(Background)

Antidepressants, such as imipramine (Imi) and fluoxetine, are known to alter gene expression patterns by inducing changes in the epigenetic status of neuronal cells. There are also some evidences for the anti-apoptotic effect of various groups of antidepressants; however, this effect is complicated and cell-type dependent. Further, it is unclear whether epigenetic effect is a general characteristic and is related to the anti-apoptotic effect of antidepressants. Tricyclic antidepressant (TCA) family, in particular, amitriptyline (Ami), is suggested to have advantages in treatment of neurodegenerative disorders.

(Objects)

We examined whether Ami has an anti-apoptotic effect via epigenetic mechanisms by the following objects:

1. Examine changes in gene's expression in mouse primary neocortical neuronal cultures (PNC) after treatment with TCAs, Ami and Imi,
2. Elucidate the epigenetic effects of Ami at genes related to neuroprotection,
3. Anti-apoptotic effect of Ami in cell models of Parkinson disease (PD) and Alzheimer disease (AD).

(Methods)

PNC were prepared from ICR mouse fetuses on embryonic day 15. Cells at *in vitro* day 3 were treated with either 5 μM Imi or Ami for 48 h. To examine genes expression, DNA microarrays were performed. Then, chosen genes was confirmed at mRNA and protein levels using quantitative polymerase chain reaction (qPCR) and Western blot (WB) with proper primers and antibodies, respectively.

For epigenetic experiments, chromatin immunoprecipitation assays (ChIP) follow by qPCR was performed with antibodies for histone H3 lysine 9 acetylation (H3K9ac), histone H3

lysine 27 acetylation (H3K27ac), or histone H3 lysine 4 trimethylation (H3K4me3).
To investigate the neuroprotective effect, after treatment with Ami, cells were treated with 1-methyl-4-phenylpyridinium ion (MPP^+) or beta amyloid 1-42 ($A\beta_{1-42}$) as models for PD and AD, respectively. Cell death ratio was determined using MTT reduction and LDH-release assays. We performed WB for cleavage caspase 3, extracellular signal regulated kinase 1 and 2 (ERK1/2), phosphorylated ERK1/2 (p-ERK1/2), cAMP response element binding protein (CREB) and p-CREB to elucidate the molecular pathway.

(Results)

Performing global gene expression analysis, we found that 17 up-regulated genes and 27 down-regulated genes responded to both Ami and Imi. Among genes that were up-regulated, we selected three neuroprotection-associated genes, namely Activating transcription factor 3 (*Atf3*), Heme oxygenase1 (*Hmox1*), and Growth/differentiation factor 15 (*Gdf15*) for biological validation with qPCR and WB. *Atf3* and *Hmox1* were up-regulated at both mRNA and protein levels by treatment with Ami, while Imi only up-regulated *Hmox1*. None of the two drugs significantly up-regulated *Gdf15* at protein level, thus we excluded *Gdf15* from our study.
ChIP-qPCR revealed that Ami increased enrichments of H3K4me3 and H3K9ac, which indicates an active epigenetic status, in the promoter regions of *Atf3* and *Hmox1*.
Ami pretreatment attenuated MPP^+- or $A\beta_{1-42}$- induced neuronal cell death and reduced cleaved caspase 3, a critical marker for neuronal apoptosis. Pretreatment with Ami did not affect the p-ERK1/2 in control cells but significantly inhibited the activation of ERK1/2 in MPP^+ or $A\beta_{1-42}$ treated cells. Moreover, CREB was not affected by Ami.
In addition, we found that *Atf3* and *Hmox1* were also up-regulated after $A\beta_{1-42}$ treatment, and were further increased when pre-treated with Ami. Interestingly, the highest up-regulation of *Atf3* and *Hmox1*, along with the loss of neuroprotective effect, were observed after co-treatment with $A\beta_{1-42}$ and Ami.

(Discussion)

In this study, we found neuroprotection genes are commonly up-regulated in neurons after treatment with Ami and Imi, supporting the reported neuroprotective effect of these drugs. We discovered that Ami induced enrichments of active histone marks, which caused up-regulation of Atf3 and Hmox1. To our knowledge, this is the first report on histone alterations by Ami. Lastly, we showed that Ami has anti-apoptotic effect in neuronal cells, which is related to MAPK/ERK1/2 pathway. It is known that activation of ERK1/2 may lead to pro-apoptosis (caspase 3) or anti-apoptosis (CREB). In our study, we showed that Ami only inhibited the ERK1/2/Caspase 3. Further, the neuroprotection can only be achieved by pretreatment with Ami, which induced changes in genes expression without harming cell viability. This observation was in line with the notion of preconditioning effect. Therefore, we suggested a preconditioning effect of Ami, which warrants further investigation.
Our study has some limitations: (1) we only examined drugs at one time point and one concentration, (2) Effects were observed on neurons at *in vitro* day 3, and (3) roles of *Atf3* and *Hmox1* in the effects of Ami are not fully understood.

(Conclusion)

Our results suggest that epigenetic up-regulation of *Atf3* and *Hmox1* genes may underlie the neuroprotective effect of Ami. The neuroprotection of Ami may be accomplished by a preconditioning effect that related to *Atf3* and *Hmox1*. However, further studies on *Atf3* and *Hmox1* knockout, as well as over expression, are needed to clarify the roles of these genes in the pathway of preconditioning and neuroprotective effects of Ami.

論文審査結果の要旨

抗鬱薬は神経細胞のエピジェネティックな変化を介した遺伝子発現パターンを変化させることが知られており、一部の抗鬱薬では抗アポトーシス的な効果があることが示されてきた。しかしながら、これらの作用機序は複雑であり詳細は明らかにされていない。本論文では、三環系抗うつ薬の一つであるアミトリプチリンについて、まず、マウスの新皮質由来の初代培養神経細胞の遺伝子発現の変化に及ぼす影響について解析し、つづいて、神経保護作用と関連する遺伝子発現のエピジェネティックな変化について検討し、さらに、パーキンソン病やアルツハイマー病の細胞モデルにおいて抗アポトーシス的な効果の有無について検討した。

まず、DNA マイクロアレイを用いて、アミトリプチリンまたはイミプラミンによってマウスの新皮質由来の初代培養神経細胞において発現が変化する遺伝子を検出し、さらに定量的 PCR ならびにウエスタンブロットを用いて、mRNA レベルおよびタンパクレベルが変化する遺伝子を確定した。その結果、アミトリプチリンによっては Atf3 および Hmox1 の発現が増加するが、イミプラミンでは Hmox1 の発現のみが増加した。つづいて、ChIP-qPCR による解析の結果、アミトリプチリンは Atf3 および Hmox1 のプロモータ領域のヒストンを修飾していることが明らかになった、さらに神経毒性のある MPP^+ および $A\beta_{1-42}$ の作用はアミトリプチリンによる前処理によって減弱することが示された。この減弱は ERK1/2 の活性化の抑制の結果、神経細胞のアポトーシスのマーカーである cleaved caspase 3 の減少によるものであることが示された。なお、Atf3 および Hmox1 の発現は $A\beta_{1-42}$ によっても増加し、さらに、$A\beta_{1-42}$ とアミトリプチリンの同時投与によりさらに増加した。

以上の実験結果から、アミトリプチリンがヒストンの修飾を介して Atf3 および Hmox1 の発現を増強することが明らかになったが、アミトリプチリンによるヒストンの修飾についてこれまでに報告はない。また、アミトリプチリンが ERK1/2 の活性化による cleaved caspase 3 の増加を抑制することによって抗アポトーシス的な効果を発揮することが明らかにされた。さらに、アミトリプチリンの神経保護作用は事前投与によってのみ認められることが明らかにされた。

本研究では、アミトリプチリンの効果発現における Atf3 および Hmox1 の作用は解明できていないものの、アミトリプチリンによるによるエピジェネティックな変化をはじめて明らかにした点、また、アミトリプチリンが ERK1/2/cleaved caspase 3 の増加抑制によって抗アポトーシス的な効果を発現することを初めて明らかにした点から、博士論文として妥当なものであると判断された。