称号及び氏名	博士（獣医学） Dwi Kesuma Sari
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論　文　名	「Effect of prolonged low level chemical exposures on the hypothalamo-pituitary-adrenal gland axis of adult female mice」 （低濃度化学物質の長期曝露が成熟雌マウス視床下部－下垂体－副腎軸に及ぼす影響）
論文審査委員	主査　小川　和重 副査　小谷　猛夫 副査　岡田　利也 副査　塚本　康浩

論文要旨

Introduction

Multiple chemical sensitivity (MCS) has been described as a chronic syndrome that is characterized by patterns of multiple somatic, cognitive and affective symptoms in response to low-level chemical exposures that are considered safe for the general population. Sick building syndrome (SBS), which is a form of MCS, is characterized by non-specific complaints such as mental fatigue, headache, nausea or dizziness. It occurs in humans following chemical exposure such as formaldehyde (FA) or toluene (TOL) inhalation. FA is a low molecular weight organic compound that is often found in materials used in occupational environments (textiles, paper, resins, wood composites) and domestic environments (insulating materials, chipboard and plywood, fabrics, and heating and cooking emissions). Many studies have looked at the relation between indoor FA concentration and the SBS. TOL is widely used in glues, lacquers and paint removers and induces MCS in humans as well as FA. The hypothalamo-pituitary-adrenal gland (HPA) axis responds to stress by increasing the secretion of corticotropin releasing hormone (CRH) in the hypothalamus, adrenocorticotropin hormone (ACTH) in the anterior pituitary gland and corticosteroids in the adrenal gland. Previous studies have found a relation between the inhalation of low-level FA and disorders of the respiratory organs in humans, rats and mice. To date,

however, there has been no report on the effect of low-level FA or TOL inhalation on the CRH neurons in the hypothalamus and ACTH cells in the anterior pituitary gland. Several reports have found that most SBS patients are woman with allergic disease. In the present study, I investigated the effect of prolonged low level FA or TOL inhalation on the HPA axis of non allergy (NAG) and allergy (AG) mice to make an animal model for MCS or SBS using immunocytochemical, morphometrical and RT-PCR methods.

Chapter 1: Effect of prolonged exposure to low concentration of FA on the HPA axis of adult female mice.

1.1. Animals and methods

Female C3H/He mice were obtained from Charles River Japan at 8 weeks old and used at 10 weeks old in this experiment. Eighty mice were divided into two groups (NAG model group and AG model group). NAG group comprised sham control mice. AG group was made allergic by injection of ovalbumin (OVA) and alum prior to exposure to FA. These animals were further exposed to aerosolized OVA as a booster four times during the exposure period. Female mice of two groups were divided into subgroups of 10 mice, designed 0, 80, 400 and 2000 subgroups since they were exposed to different concentrations (0 (clean air), 80, 400, 2000 ppb) of FA inhalation for 16 hrs/day, 5 days/week, for twelve weeks. The ministry of Health and Welfare of Japan proposed the guideline value (0.1 mg/m3=80 ppb) as an acceptable indoor concentration of FA. The hypothalamus (n=10), adrenal gland (n=10) and pituitary gland (n=5) were fixed and embedded in Tissue Prep. The hypothalamus and pituitary gland were cut coronally and sagitally in serial sections 10 μm thick, respectively. The adrenal gland was cut in serial sections of 5 μm thickness and the sections were stained with hematoxylin-eosin and observed under an optical microscope. The immunohisto-chemical method used for CRH-immunoreactive (ir) neurons and ACTH-ir cells was the avian-biotin-peroxidase complex method. Proliferating cell nuclear antigen and in situ apoptosis tests were performed to observe proliferation of neurons and nuclear DNA fragmentation in paraventricular nucleus (PVN) neurons. The other pituitary gland (n=5) was mixed and analyzed for ACTH-mRNA expression by RT-PCR method.

1.2. Results

A major finding in this study is that the numbers of CRH-ir neurons and ACTH-ir cells, and the ACTH-mRNA expression of NAG mice, are all up-regulated according to the dose of FA inhaled. Thus, FA inhalation may act as a chemical stressor on the HPA axis. The other main finding is that the data from AG mice differ significantly from the data for NAG mice. The number of CRH-ir neurons, ACTH-ir cells, and

ACTH-mRNA expression were significantly higher in the 0 subgroup of AG mice than in 0 subgroup of NAG mice. The allergy reaction may act as a stress on the HPA axis. In the 80 subgroup of AG mice, the number of CRH-ir neurons and ACTH-ir cells, and ACTH-mRNA expression, were all significantly higher than in the 0, 400 and 2000 subgroups of AG mice. The HPA-axis of AG mice in the 80 subgroup may therefore counteract such both stresses (allergy and FA). The allergic reaction and exposure to low level FA may act in some synergistic manner. On the other hand, fewer CRH-ir neurons and ACTH-ir cells and reduced ACTH-mRNA expression were found in the 2000 subgroup of AG mice compared to the 80 subgroup of AG mice or the 2000 subgroup of NAG mice. Thus, CRH neurons in the 2000 subgroup of AG mice, may have impaired function (synthesis and secretion of CRH). This reduction in the number of CRH-ir neurons may inhibit the proliferation and functioning of ACTH cells in the pituitary gland. SBS may therefore be due to a depressed condition of the CRH-ir neurons and ACTH-ir cells, which is unable to react to the further or secondary stress (headache, mental fatigue, nausea etc) induced by FA.

Chapter 2: Effect of TOL exposure before prolonged exposure to low-level FA on the HPA axis in adult female mice

2.1. Animals and methods

MCS may develop in two stages: (1) loss of specific tolerance following acute or chronic exposure to various environmental agents, such as pesticide or solvents and (2) subsequent triggering of MCS by extremely small quantities of chemicals. The treshold value for work exposure of TOL is chosen as approximately 100 ppm in many countries. Several studies have found that 80-100 ppm of TOL is an acceptable concentration for TOL inhalation. So, in this experiments, adult female mice of four groups were first exposed intranasally to 500 ppm TOL, which is a relatively highly concentration compared with these data, per mouse for 6 hr/day, for 3 days. Then, mice of each group were exposed to different concentrations (0 (air), 80, 400 and 2000 ppb) of FA for 16 hr/day, 5 days/week for twelve weeks.

2.2. Results

The number of CRH-ir neurons was up-regulated according to the amount of FA as well as inhalation of FA alone. The proportion of ACTH-ir cells increased according to the FA concentration, though there was no significant difference between the 400 and 2000 groups. The number of ACTH-ir cells was higher in the 400 group than in the other groups (0, 80, and 2000). Expression of ACTH-mRNA was also up-regulated according to the quantity of FA. The sinusoid in the anterior pituitary showed more dilatation in the 400 and 2000 groups than in the 0 group, especially in the 2000 group.

This result showed that the exposure to acute TOL prior to inhalation of FA has no effect on the HPA axis, although greater sinusoid dilatation was found in the anterior pituitary gland at higher concentrations of FA.

Chapter 3: Effect of prolonged exposure to low concentration of TOL on the HPA axis of adult female mice

3.1. Animals and methods

Two groups (NAG group and AG group) of adult female mice were exposed to different concentrations (0 (clean air) and 50 ppm) of TOL for 6 hrs/day, 5 days/week, for 12 weeks. The CRH-ir neurons in the hypothalamus were examined, together with the ACTH-ir cells and ACTH mRNA in the pituitary.

3.2. Results

Prolonged inhalation of 50 ppm TOL as well as allergy increased the number of CRH-ir neurons and the proportion and number of ACTH-ir cells, and increased the expression of ACTH-mRNA. Prolonged TOL inhalation and allergy may therefore act as a stressor on the HP axis (ie, neurotoxicity). Furthermore, TOL and allergy may act synergetically on CRH neurons and ACTH cells, as do low concentrations of FA inhalation and allergy. This experiment suggests that low concentration of TOL and OVA sensitization act on the HPA axis as a stressor.

Chapter 4: Conclusions

1. In the experiment of prolonged exposure to low concentrations of FA, the following results were obtained.
   1.1. In NAG mice, the number of CRH-ir neurons and ACTH-ir cells and ACTH-mRNA expression are up-regulated according to the dose of FA inhaled. FA may act as a stressor on the HPA axis.
   1.2. The data from AG mice differ significantly from those for NAG mice. In 80 subgroup of AG mice, the allergic reaction and exposure to low level FA may act on the HPA axis in a synergistic manner. In 2000 subgroup of AG mice, the HPA axis may have impaired function.
2. The acute exposure to low concentration TOL prior to inhalation of FA has no effect on the HPA axis and as a trigger of MCS, although greater sinusoid dilatation was found in the anterior pituitary gland at higher concentrations of FA.
3. The prolonged low concentration of TOL and OVA sensitization acts as a stressor on the HPA axis.
4. This experimental system may be a suitable animal model for SBS and/or MCS.

審査結果の要旨

本態性多種化学物質過敏状態(MCS)の研究の中で、低濃度ホルムアルデヒドとトルエンの長期曝露が及ぼす影響を調べる研究は、シックハウス症候群(SBS)の解明に結びつく研究である。視床下部―下垂体―副腎(HPA)軸はストレスに対応する軸である。本研究では、低濃度ホルムアルデヒドとトルエンの長期曝露が視床下部室旁核の副腎皮質刺激ホルモン放出ホルモン(CRH)神経細胞と下垂体の副腎皮質刺激ホルモン(ACTH)細胞にどのような影響を与えるかを免疫組織化学的方法、計量計測学的方法、半定量的 RT-PCR 法により解析・検討している。シックハウス症候群に罹患した患者の多くはアレルギーを発症している女性である事から、実験動物として卵白アルブミン(OVA)を前処置して作製したアレルギー(AG)雌マウスと前処置していない非アレルギー(NAG)雌マウスを使用し、低濃度ホルムアルデヒド長期曝露の影響を解析している(第 1 章)。また、前処置としてアレルギーを惹起しないトルエンを高濃度曝露したマウスを使用して、OVA 前処置で発症したアレルギーの意義を解析している(第 2 章)。さらに、低濃度トルエンを長期曝露しホルムアルデヒドとの相違を比較・解析している(第 3 章)。このように本研究は、MCS あるいは SBS 研究のモデル動物を作製することとその発症機構を解明することを目的としている。研究結果の概要を以下に示す。

第 1 章では、NAG 群、AG 群ともに、0ppb、80ppb、400ppb、2,000ppb 濃度のホルムアルデヒドを 12 週間曝露し HPA 軸に及ぼす影響を検討した。AG マウスは、曝露前に抗原として卵白アルブミン(OVA)10μg とミョウバン 2mg を腹腔に投与し、以降 3 週間ごとに OVA を腹腔に投与し作製した。NAG マウスの CRH-免疫陽性(ir)神経細胞数、下垂体の ACTH-ir 細胞出現率と数、下垂体の ACTH-mRNA の発現量はホルムアルデヒド曝露量依存的に増加した。ホルムアルデヒド非曝露群間を比較すると、CRH-ir 神経細胞数、下垂体の ACTH-ir 細胞出現率と数、下垂体の ACTH-mRNA の発現量はいずれも、AG マウスの方が NAG マウスに比べ有意に増加していた。一方 AG マウスでは、CRH-ir 神経細胞数、ACTH-ir 細胞の出現率と数、ACTH-mRNA の発現量は、80ppb ホルムアルデヒド曝露群が最高値を示し NAG マウスの 2,000ppb 曝露群の値まで増加したが、400ppb と 2,000ppb 曝露群ではこれらの値は減少した。

第 2 章では OVA 前処置によるアレルギー発症の意義を解析するために、ホルムアルデヒド曝露前に 500ppm の高濃度トルエンを 3 日間経気道曝露して、トルエンの前曝露が HPA 軸に及ぼす影響を検討した。本群の CRH-ir 神経細胞数、ACTH-ir 細胞の出現率と数、ACTH-mRNA の発現量は、ホルマリン曝露量依存的に増加し、第 1 章の NAG 群と類似の傾向を示した。下垂体前葉の静脈洞は、本群対照群より 400ppb と 2,000ppb 群で有意に拡大していた。

第 3 章では、NAG マウスと AG マウスに 0ppm か 50ppm のトルエンを 12 週間曝露し、HPA 軸に及ぼす低濃度トルエン長期曝露の影響を検討した。NAG マウスでは、非曝露群と比較してトルエン曝露群は CRH-ir 神経細胞数、ACTH-ir 細胞の出現率と数、ACTH-mRNA の発現量が増加していた。AG マウスでは、トルエン曝露群の CRH-ir 神経細胞数、ACTH-ir 細胞の出現率、ACTH-mRNA の発現量が、非曝露群に比べて増加していた。

本研究で、ホルムアルデヒドとトルエンがそれぞれストレッサーとして HPA 軸に作用していることが明示された。一方、AG 群の解析から、アレルギー状態下における高濃度(2,000ppm)ホルムアルデヒド曝露で HPA 軸は障害を受けることが明らかになった。従って、SBS とは、アレルギーとホルムアルデヒドの２つのストレスの相乗作用で HPA 軸が損傷を受け、更なるストレス(腹痛、頭痛など)を処理できない状態であることが推察され、これが、SBS 発現の機構であると思われる。アレルギー発症モデルである AG 群とトルエン前曝露群から得られた結果の比較・解析から、アレルギー性炎症はホルムアルデヒド曝露に対する HPA 軸の反応に悪影響を与えるが、アレルギー炎症を惹起しないトルエン前処置ではなんらの影響も与えていないことが示唆された。また、以上の結果を総合的に判断すると、本研究で作製したマウスは MCS あるいは SBS のモデルマウスとして有用であると考えられる。このように本研究の成果は，医学・獣医学分野における本態性多種化学物質過敏症の研究の発展に大きく貢献するものであり，最終試験の結果と併せて博士（獣医学）の学位を授与することを適当と認める。