厚生労働科学研究費補助金

創薬基盤推進研究事業

生体防御タンパク質に注目した、漢方薬の作用メカニズムの解明・有効成分の同定と新規治療薬の開発

平成 24 年度　総括研究報告書

研究代表者　水島　徹

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研究代表者　水島　徹　慶應義塾大学薬学部教授

研究要旨

平成24年度我々は、HSP70が紫外線によるシワを防ぐこと、及びその分子機構を解明し、***J. Invest. Dermatol.*** (皮膚科学の分野で最も評価の高い学術誌)に掲載した。これによりHSPを増やす生薬や天然物への注目が高まり、本研究の成果であるアルニカやヤバツイの様々な商品への応用が開始されている。

一方、HSP70誘導生薬（ヤバツイ）から新たに同定した、HSP70誘導物質、ユーパリノライドA, ユーパリノライドBに関しては、その成果を***Biochem. Pharmacol.***で発表するとともに、そのHSP誘導機構を解明した。

またサルビアから単離したSOD誘導物質に関しては、COPDや慢性腎不全のモデルでも高い有効性を示すことを見いだした。また、HSP47誘導生薬として単離したワイルドタイムに関しては、その外用剤を開発し、抗シワ効果を確認した。一方、HO-1誘導生薬として単離した、マジョラムに関しては、肺線維症、小腸潰瘍、喘息に関する有用性を示した。さらに、ワイルドタイムからHSP47誘導物質三種、マジョラムからHO-1誘導物質二種の同定に成功した（特許出願準備中）。

A. 研究目的

我々はこれまで、様々な疾患に対して HSP や SOD などの生体防御タンパク質が保護的に働くことを報告してきた。HSP は様々なストレスによって誘導され、細胞をストレスに耐性化する。また我々は、HSP が抗炎症作用やタンパク質の変性を抑制する作用を持つことを発見した。一方我々はテプレノン（胃薬）が HSP を誘導する（但し、誘導能はあまり高くない）ことを発見し、テプレノンはこの作用により胃潰瘍を抑制していることを証明した。さらに小腸潰瘍や炎症性腸疾患（炎症と細胞死が主な原因）、及びアルツハイマー病などの神経変性疾患（タンパク質の変性が原因）に対しても HSP が保護的に働くこと、及びテプレノンが有効であることを見出した（現在これらの疾患に対するテプレノンの臨床試験を行っている）。

活性酸素による組織傷害は間質性肺炎や炎症性腸疾患（いずれも難病）などの炎症性疾患の主要な原因である。そこで活性酸素を消去する SOD は古くから注目されてきたが、その血中安定性が低いために医薬品としての開発は成功しなかった。そこで我々は、SOD にリン脂質を結合させ安定化させた PC-SOD を開発し、間質性肺炎、及び炎症性腸疾患に対する第二相臨床試験でその有効性を示した。しかし生物製剤である PC-SOD は生産コスト、及び製剤としての安定性に問題があり、低分子の SOD 誘導薬が望まれている。

長年使われてきた漢方薬は、その安全性・有効性が確認されていることから、医薬品原料として注目されてきた。しかし現在まで、漢方薬由来の物質が新規医薬品として認可されたケースは少ない。我々はその原因として、そのような医薬品開発の多くが、受容体や酵素の阻害など西洋医薬品と同じ機構をターゲットとしており、漢方薬の特徴である緩やかな作用・副作用の少なさとマッチしていないこと、即ち漢方薬は西洋医薬品とは違う独自のターゲット（生体防御タンパク質の効果を高めるなど）を持っていることを考えている（研究計画で述べるように、この考えを支持する成果を最近あげた）。

そこで本研究で我々は、漢方薬（生薬）ライブラリーから HSP 誘導生薬、及び SOD 誘導生薬を検索し、誘導物質の同定、及び動物モデルでの評価を行い、疾患治療薬として開発する化合物を決定する。

ゲノム創薬などにより、21 世紀は新薬の開発ラッシュになると予想されていた。しかし現実には、発売される新薬の数は年々減少しており、製薬企業は医薬品開発戦略の変更を迫られている。この主な原因は臨床試験で発生する副作用であり、作用の強い医薬品より副作用の少ない医薬品を開発すべきであると考えられる。これまでの医薬品は受容体や酵素の阻害・活性化剤が主であり、生体内のバランスを大きく変えることにより副作用を導くと考えられる。そこで我々は、疾患というストレスに対して生体が自らを守るために誘導する生体防御タンパク質を増強させるタイプの医薬品が有用であると考えている。即ち、疾患に対する生体防御タンパク質の誘導が不十分であるために疾患が発症すると考え、医薬品によりその不足分を補うという考えである。生体が本来持っている反応を助けるだけであるので、副作用を起こしにくいと期待される。HSP 誘導薬や PC-SOD が間質性肺炎などの難病に有効であるという臨床結果は、このような医薬品は安全面で優れているだけでなく、従来型の医薬品では効果をあげられなかった疾患にも有効であることを示唆している。

本研究が成功すれば、種々の難病に対する治療薬が生まれるだけでなく、新しい医薬品開発戦略（生体防御タンパク質をターゲットとする医薬品を検索する材料として漢方薬を用いる）を製薬企業へ示すことになり、大きな波及効果が期待できる。

B. 研究方法

最近我々は、共同研究している中国企業（北京泰徳製薬）から得た漢方薬（生薬）ライブラリー（約 600 種）から HSP の誘導生薬をスクリーニングし、テプレノンよりも強力、かつ安全な数多くの HSP 誘導生薬を得た（特許出願済み）。我々はこの中からヤバツイを選択し、その HSP 誘導物質の同定に成功した（特許出願準備中）。この誘導物質

を小腸潰瘍、炎症性腸疾患、アルツハイマー病の動物モデルで評価したところ、テプレノンよりも強力な効果を示した。この結果は、漢方薬（生薬）ライブラリーからスクリーニングした HSP 誘導物質が医薬品として有用であることを示唆している。また最近我々は、HSP が間質性肺炎（有効な治療薬はなく、致死率は 80%を超える）、COPD（世界中で患者数が増大しており、有効な治療薬がない）、及び ALS やハンチントン舞踏症などの神経変性疾患の発症を抑制することを見出した。

そこで本研究で我々は、この漢方薬（生薬）ライブラリーをさらに充実させ、HSP 誘導生薬のスクリーニングを行い、有望な生薬を複数選択する。そして、誘導物質の同定、及び動物モデルでの評価を行い、種々の疾患治療薬として開発する HSP 誘導物質を決定する。

一方最近我々は、PC-SOD が間質性肺炎や炎症性腸疾患だけでなく、活性酸素による組織傷害がその主な原因となっている、腎炎、肝炎、膵炎、喘息、COPD、アトピー性皮膚炎の動物モデルにおいて有効性を示すことを見出した。そこで本研究で我々は、上述のライブラリーから SOD 誘導物質を検索・同定し、種々の疾患治療薬として開発する SOD 誘導物質を決定する。

（1） 漢方薬（生薬）ライブラリーの整備

上述の HSP 誘導生薬（ヤバツイ）は、化粧品として商品化が決定している。この成果を評価した北京泰徳製薬は中国政府から特別の許可を得て、2000 種以上の生薬を供与してくれることになった（最近では生薬を海外に出すことに中国政府は慎重になっており、このようなライブラリーを有する研究機関は国内にほとんどない）。そこでこの生薬の溶解法や投与法を確立し、スクリーニングの準備を行う。

（2） HSP、及び SOD 誘導生薬のスクリーニングと、誘導物質の同定

HSP、あるいは SOD 遺伝子プロモーターの下流にルシフェラーゼ遺伝子を挿入したプラスミドを導入した細胞を用いて一次スクリーニングを行い、イムノブロット法で二次スクリーニングを行う。毒性の少ない誘導薬を得たいので、三次スクリーニングではその生薬の細胞毒性を調べ、細胞毒性を示さない濃度で HSP、あるいは SOD を誘導するものを選択する。四次スクリーニングではその生薬をマウスに投与し、目的のタンパク質を誘導するかを検討する。

これらの結果から有望な生薬を選択し、その誘導物質の同定を行う。オープンカラムで粗分けした後、分取用 HPLC で分画し、誘導物質の構造を決定する。合成可能な物は合成し、難しいものは大量の生薬から精製する。

（3） HSP、及び SOD 誘導物質の疾患治療薬としての評価

それぞれの誘導物質の効果をまず試験管内で評価する。HSP 誘導物質に関しては、炎症抑制作用、細胞保護作用、及びタンパク質凝集抑制作用の程度を調べる。また SOD 誘導物質に関しては、活性酸素消去作用を調べる。次にその効果が HSP、あるいは SOD を介しているかを、siRNA を用いて検証する。

最終的には、種々の疾患動物モデルを用いて評価する。治療効果が見られた場合、その効果が HSP、あるいは SOD を介しているかを、そのタンパク質を誘導出来ないマウスを用いて判断する。有用な薬理効果が見られた場合には、他の臓器の状態を精査し副作用が表れていないかを調べる。尚、HSP 誘導物質の場合は GGA と、SOD 誘導物質の場合は PC-SOD と治療効果を比較する。結果を総合的に判断し、それぞれの疾患治療薬として開発する誘導物質を決定する。

C. 研究結果

平成24年度我々は、HSP70が紫外線によるシワを防ぐこと、及びその分子機構を解明し、***J. Invest. Dermatol.*** (皮膚科学の分野で最も評価の高い学術誌)に掲載した。これによりHSPを増やす生薬や天然物への注目が高まり、本研究の成果であるアルニカやヤバツイの様々な商品への応用が開始されている。

一方、HSP70誘導生薬（ヤバツイ）から新たに同定した、HSP70誘導物質、ユーパリノライドA, ユーパリノライドBに関しては、その成果を***Biochem. Pharmacol.***で発表するとともに、そのHSP誘導機構を解明した。

またサルビアから単離した SOD 誘導物質に関しては、COPD や慢性腎不全のモデル

でも高い有効性を示すことを見いだした。また、HSP47 誘導生薬として単離したワイルドタイムに関しては、その外用剤を開発し、抗シワ効果を確認した。一方、HO-1 誘導生薬として単離した、マジョラムに関しては、肺線維症、小腸潰瘍、喘息に関する有用性を示した。さらに、ワイルドタイムから HSP47 誘導物質三種、マジョラムから HO-1 誘導物質二種の同定に成功した（特許出願準備中）。

D.考察

結果の欄に記載した

E.結論

これまでの我々の研究から、HSP70、HSP47、HO-1、SOD に関する誘導生薬を数多く同定した。一方我々は、HSP がシミやシワに有効であることを遺伝子改変マウスを用いた研究などにより明らかにした。これにより、HSP 誘導生薬の化粧品としての応用へ道を開き、実際本研究期間中に七種もの化粧品が発売された。今後は、HO-1 や SOD の有用性を証明し、その誘導生薬の商品化につなげる。

一方本研究で我々は、上述の HSP70、HSP47、HO-1、SOD に関する誘導生薬から誘導物質の単離に成功し、その一部のものに関しては、肺線維症、小腸潰瘍、喘息、COPD、アルツハイマー病、紫外線皮膚傷害に有効であることを示した。今後はこれらの誘導物質の医薬品としての開発を進めていく。

F.健康危険情報

該当なし

G.研究発表

1. 論文発表

1. Hoshino, T., Namba, T., Takehara, M., Murao, N., Sugimoto, Y., Narumiya, S., Matsushima, T., Suzuki, T. and Mizushima, T. Improvement of cognitive function in Alzheimer's disease model mice by genetic and pharmacological inhibition of the EP4 receptor. ***J. Neurochem.*** 120, 795-805. (2012)
2. Yamashita, Y., Ikeda, T., Matsuda, M., Maji, D., Hoshino, T. and Mizushima, T. purification and characterization of hsp-inducers from *eupatorium lindleyanum* ***Biochem. Pharmacol.*** 82, 909-922. (2012)
3. Asano, T., Tanaka, K. Suemasu, S., Ishihara, T., Tahara, K., Suzuki, T., Suzuki, H., Fukudo, S. and Mizushima, T. Effects of *b*-(1,3-1,6)-D-glucan on irritable bowel syndrome-related colonic hypersensitivity. ***Biochem. Biophys. Res. Commun.*** 420, 444-449. (2012)
4. Tanaka, K., Sato, K., Aoshiba, K., Azuma, A. and Mizushima, T. Superiority of PC-SOD to other anti-COPD drugs for elastase-induced emphysema and alteration in lung mechanics and respiratory function in mice. ***Am. J. Physiol. Lung Cell. Mol. Physiol.*** 302, L1250-L1261. (2012)
5. Yamakawa, N., Suemasu, S., Okamoto, Y., Tanaka, K., Ishihara, T., Asano, T., Miyata, k., Ohtsuka, M. and Mizushima,

T. Synthesis and biological evaluation of derivatives of 2-{2-fluoro-4-[(2-oxocyclopentyl)methyl]phenyl}propanoic acid: Non-steroidal anti-inflammatory drugs with low gastric ulcerogenic activity. ***J. Med. Chem.*** 55, 5143-5150. (2012)

6. Mizushima, T. Development of NSAIDs with lower gastric side effect. ***Frontier of Gastrointestinal Research*** 30, 71-78. (2012)

7. Tanaka, K., Azuma, A., Miyazaki, Y., Sato, K. and Mizushima, T. Effects of lecithinized superoxide dismutase and/or pirfenidone against bleomycin-induced pulmonary fibrosis. ***CHEST*** 142, 1011-1019. (2012)

8. Tanaka, K. Shirai, A., Ito, Y., Namba, T., Tahara, K., Yamakawa, N. and Mizushima, T. Expression of 150-kDa oxygen-regulated protein (ORP150) stimulates bleomycin-induced pulmonary fibrosis and dysfunction in mice. ***Biochem. Biophys. Res. Commun.*** 425, 818-824. (2012)

9. Suemasu, S., Yamakawa, N., Ishihara, T., Asano, T. Tahara, K., Tanaka, K. Matsui, M., Okamoto, Y., Otsuka, M., Takeuchi, K., Suzuki, S. and Mizushima, T. Identification of a unique NSAID, fluoro-loxoprofen with gastroprotective activity. ***Biochem. Pharmacol.*** 84, 1470-1481. (2012)

10. Matsuda, M., Hoshino, T., Yamakawa, N., Tahara, K., Adachi, H., Sobue, G., Maji, D., Ihn, H. and Mizushima, T. Suppression of UV-induced wrinkle formation by induction of HSP70 expression in mice. ***J. Invest. Dermatol.*** 133, 919-928. (2012)

2. 学会発表（招待講演のみ）

1 水島徹　既存薬を利用したアルツハイマー病治療薬の開発　日本薬学会シンポジウム招待講演 (2012)（札幌）

2 水島徹　NSAID 潰瘍発症機構とその対策　日本消化器病学会招待講演　(2012)（東京）

3 Tohru Mizushima PC-SOD, as a drug for IPF. Invited lecture in Asan Medical Center (2012) (Seoul)

4 水島徹　ドラッグリプロファイリング研究　武田薬品工業（株）研究所での招待講演　(2012)（藤沢）

5 Tohru Mizushima Protective and therapeutic effects of lecithinized superoxide dismutase (PC-SOD) against pulmonary emphysema. American Thoracic Society International Conference (2012) (San Francisco)

6 水島徹　ドラッグリプロファイリ

ング研究　南風病院での招待講演 (2012)（鹿児島）

7 Tohru Mizushima Development of new type of NSAID with lower gastric side effects. Invited lecture in CJ Pharma (2012) (Tokyo)

8 水島徹　ドラッグリプロファイリング研究　わかもと製薬（株）研究所での招待講演　(2012)（大井町）

9 水島徹　ドラッグリポジショニングによる創薬アプローチの現状と今後の展望　味の素製薬（株）研究所での招待講演　(2012)（川崎）

10 水島徹　消化器治療薬をベースにした新薬開発-その現状と可能性-日本消化器病学会四国支部会 (2012)（徳島）

11 Tohru Mizushima Identification of a unique NSAID, fluoro-loxoprofen with gastroprotective activity. Invited lecture in International Union of Basic and Clinical Pharmacology, GI Satellite Meeting. (2012) (Tokyo)

12 水島徹　PC-SOD 吸入製剤の開発　日本 DDS 学会シンポジウム (2012)（札幌）

13 水島徹　セルベックスの新しい可能性を求めて　消化器病態生理勉強会での招待講演　(2012)（東京）

14 水島徹　ドラッグリプロファイリング研究　医学部薬学部合同サマースクールでの招待講演　(2012)（東京）

15 水島徹　ドラッグリプロファイリング研究　バイオメディカル分析科学シンポジウムでの招待講演 (2012)（東京）

16 水島徹　ブレオマイシン依存の肺線維化、呼吸機能障害に対するPC-SOD、及びピルフェニドンの効果　第１５回間質性肺炎細胞分子病態研究会での招待講演　(2012)（東京）

17 Tohru Mizushima Protective role for HSP70 against various gastrointestinal diseases and other diseases. Invited lecture in 11th International Congress of Hyperthermic Therapy (2012) (Kyoto)

18 Tohru Mizushima Development of new type of NSAID with lower gastric side effects. Invited lecture in 7th International symposium on cell/tissue injury and Cytoprotection/Organoprotection: Focus on GI Tract (2012) (Honolulu)

19 水島徹　ドラッグリポジショニング（DR）の現状と今後の展望 日本バイオインダストリー協会での招待講演　(2012)（東京）

20 水島徹　ドラッグリポジショニング（DR）の現状と今後の展望 医薬基盤研究所での招待講演　(2012)（大阪）

21 水島徹　ドラッグリポジショニン

グ（DR）研究（既存薬を利用した新薬開発）慶応義塾大学研究推進本部主催シンポジウムでの招待講演 (2012)（東京）

22 水島徹 種々の疾患における熱ショックタンパク質(HSP)の効果と、HSP 誘導薬による治療 第 6 回 S-Target 学術講演会での招待講演 (2012)（大阪）

23 水島徹 2010 年問題（新薬が産まれない）の解決策 熊本発の創薬研究での招待講演 (2012)（東京）

24 水島徹 ドラッグリポジショニング（DR） 慶応義塾大学医学部呼吸器内科での招待講演 (2012)（東京）

25 水島徹 薬剤性肺線維症発症機構の解明とその治療法の確立 臨床ストレス応答学会大会招待講演 (2012)（東京）

26 水島徹 ドラッグリポジショニング（DR）の現状と今後の展望 第一三共（株）研究所での招待講演 (2012)（東京）

27 水島徹 ヒートショックプロテインのシワ抑制効果 明日の化粧品科学を創造する FJ セミナー (2012)（東京）

28 水島徹 レシチン化 SOD の開発 東京大学大学院新領域研究科での招待講演 (2013)（柏）

29 水島徹 ドラッグリポジショニングとは何か、新薬開発にどのような道が開けるのか レギュラトリーサイエンス エキスパート研修会での招待講演 (2013)（東京）

30 水島徹 ドラッグリポジショニング-既存薬を利用した新薬開発-第 55 回鹿児島消化器病研究会での特別講演 (2013)（鹿児島）

31 水島徹 PC-SOD 吸入製剤の開発 日本薬学会シンポジウムでの招待講演 (2013)（横浜）

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H.知的財産権の出願・登録状況

1.特許取得

該当なし

2.実用新案登録

該当なし

3.その他

該当なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hoshino, T., Namba, T., Takehara, M., Murao, N., Sugimoto, Y., Narumiya, S., Matsushima, T., Suzuki, T. and Mizushima, T.	Improvement of cognitive function in Alzheimer's disease model mice by genetic and pharmacological inhibition of the EP_4 receptor.	***J. Neurochem.***	120	795-805.	2012
Yamashita, Y., Ikeda, T., Matsuda, M., Maji, D., Hoshino, T. and Mizushima, T.	Purification and characterization of hsp-inducers from *eupatorium lindleyanum*	***Biochem. Pharmacol.***	82	909-922.	2012
Asano, T., Tanaka, K. Suemasu, S., Ishihara, T., Tahara, K., Suzuki, T., Suzuki, H., Fukudo, S. and Mizushima, T.	Effects of *b*-(1,3-1,6)-D-glucan on irritable bowel syndrome-related colonic hypersensitivity.	***Biochem. Biophys. Res. Commun.***	420	444-449.	2012
Ishihara, T., Tanaka, K., Tashiro, S., Yoshida, K. and Mizushima, T.	Protective effect of rebamipide against celecoxib-induced gastric mucosal cell apoptosis.	***Biochem. Pharmacol.***	79	1622-1633.	2012
Tanaka, K., Sato, K., Aoshiba, K., Azuma, A. and Mizushima, T. 302,	Superiority of PC-SOD to other anti-COPD drugs for elastase-induced emphysema and alteration in lung mechanics and respiratory function in mice.	***Am. J. Physiol. Lung Cell. Mol. Physiol.***	302	L1250-L1261	2012
Yamakawa, N., Suemasu, S., Okamoto, Y., Tanaka, K., Ishihara, T., Asano, T., Miyata, k., Ohtsuka, M. and Mizushima, T.	Synthesis and biological evaluation of derivatives of 2-{2-fluoro-4-[(2-oxocyclopentyl)methyl]phenyl}propanoic acid: Non-steroidal anti-inflammatory drugs with low gastric ulcerogenic activity.	***J. Med. Chem.***	55	5143-5150.	2012
Mizushima, T.	Development of NSAIDs with lower gastric side effect.	***Frontier of Gastrointestinal Research***	80	920-931.	2012
Tanaka, K., Azuma, A., Miyazaki, Y., Sato, K. and Mizushima, T.	Effects of lecithinized superoxide dismutase and/or pirfenidone against bleomycin-induced pulmonary fibrosis.	***CHEST***	142	1011-1019	2012
Tanaka, K. Shirai, A., Ito, Y., Namba, T., Tahara, K.,	Expression of 150-kDa oxygen-regulated protein	***Biochem. Biophys. Res.***	425	818-824	2012

Yamakawa, N. and Mizushima, T. 425,	(ORP150) stimulates bleomycin-induced pulmonary fibrosis and dysfunction in mice.	***Commun.***			
Suemasu, S., Yamakawa, N., Ishihara, T., Asano, T. Tahara, K., Tanaka, K. Matsui, M., Okamoto, Y., Otsuka, M., Takeuchi, K., Suzuki, S. and Mizushima, T.	Identification of a unique NSAID, fluoro-loxoprofen with gastroprotective activity.	***Biochem. Pharmacol.***	84	1470-1481	2012
Matsuda, M., Hoshino, T., Yamakawa, N., Tahara, K., Adachi, H., Sobue, G., Maji, D., Ihn, H. and Mizushima, T. 133,	Suppression of UV-induced wrinkle formation by induction of HSP70 expression in mice.	***J. Invest. Dermatol.***	133	919-928.	2012

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ORIGINAL ARTICLE

Improvement of cognitive function in Alzheimer's disease model mice by genetic and pharmacological inhibition of the EP_4 receptor

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Abstract

Amyloid-β peptide (Aβ), which is generated by the β- and γ-secretase-mediated proteolysis of β-amyloid precursor protein (APP), plays an important role in the pathogenesis of Alzheimer's disease (AD). We recently reported that prostaglandin E_2 (PGE_2) stimulates the production of Aβ through both EP_2 and EP_4 receptors and that activation of the EP_4 receptor stimulates Aβ production through endocytosis and activation of γ-secretase. We here found that transgenic mice expressing mutant APP (APP23) mice showed a greater or lesser apparent cognitive deficit when they were crossed with mice lacking EP_2 or EP_4 receptors, respectively. Mice lacking the EP_4 receptor also displayed lower levels of Aβ plaque deposition and less neuronal and synaptic loss than control mice. Oral administration of a specific EP_4 receptor antagonist, AE3-208 to APP23 mice, improved their cognitive performance, as well as decreasing brain levels of Aβ and suppressing endocytosis and activation of γ-secretase. Taken together, these results suggest that inhibition of the EP_4 receptor improves the cognitive function of APP23 mice by suppressing Aβ production and reducing neuronal and synaptic loss. We therefore propose that EP_4 receptor antagonists, such as AE3-208, could be therapeutically beneficial for the prevention and treatment of AD.

Keywords: Alzheimer disease, aging, infammation, memory, neurodegeneration.

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Alzheimer's disease (AD) is the most common neurodegenerative disorder of the central nervous system and the leading cause of adult onset dementia, affecting 5% of the population over the age of 65. Pathological characters of AD are accumulation of neurofibrillary tangles and senile plaques and senile plaques are composed of amyloid-β peptides (Aβ), such as Aβ40 and Aβ42 (Mattson 2004). In order to generate Aβ, β-amyloid precursor protein (APP) is first cleaved by β-secretase and then by γ-secretase (Sisodia and St George-Hyslop 2002). Monomeric Aβ easily self-assembles to form oligomers and protofibrils, which play an important role in the induction of the neuronal and synaptic loss that results in cognitive decline (Haass and Selkoe 2007). γ-Secretase is composed of four core components, including presenilin (PS)-1 and PS-2 (Haass 2004). Early onset familial AD is linked to three genes, *app*, *ps1* and *ps2* (Haass 2004), strongly suggesting that Aβ is a key factor in the pathogenesis of AD. Consequently, cellular factors that affect the production of Aβ represent good targets for drugs to prevent or treat AD.

It has been suggested that inflammation is important in the pathogenesis of AD; chronic inflammation has been observed

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Abbreviations used: Aβ, amyloid-β peptide; AD, Alzheimer's disease; APP, β-amyloid precursor protein; COX, cyclooxygenase; CTF, C-terminal fragment; LTP, long-term potentiation; NeuN, neuronal nuclei; NSAIDs, non-steroidal anti-inflammatory drugs; PGE_2, prostaglandin E_2; PKA, protein kinase A; PS, presenilin.

in the brains of AD patients, and trauma to the brain and ischemia, both of which can activate inflammation, are major risk factors for the disease (Ikonomovic *et al.* 2004; Wyss-Coray 2006). Cyclooxygenase (COX), which exists as two subtypes, COX-1 and COX-2, is essential for the synthesis of prostaglandin E_2 (PGE_2), a potent inducer of inflammation. COX-1 is expressed constitutively, whereas COX-2 expression is induced under inflammatory conditions and is responsible for the progression of inflammation (Srinivasan and Kulkarni 1989; Smith *et al.* 1998). It has been suggested that the COX-2-mediated production of PGE_2 plays an important role in the pathogenesis of AD. For example, elevated levels of PGE_2 and over-expression of COX-2 have been observed in AD patient brains (Kitamura *et al.* 1999; Montine *et al.* 1999); the extent of COX-2 expression correlates with the degree of progression of AD pathogenesis (Ho *et al.* 2001); transgenic mice constitutively over-expressing COX-2 show aging-dependent memory dysfunction (Andreasson *et al.* 2001); PGE_2 stimulates the production of reactive oxygen species in microglia and activates β-secretase (Liang *et al.* 2005); and prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs), inhibitors of COX, delays the onset and reduces the risk of AD (in t' Veld *et al.* 2001; Imbimbo *et al.* 2010). Thus, in order to identify molecular targets for the development of AD drugs, it is important to understand the molecular mechanism involved in the PGE_2-mediated progression of the disease.

We recently reported that PGE_2 stimulates the production of Aβ in cells stably expressing a form of APP with two mutations (K651N/M652L; APPsw) that elevates cellular and secreted levels of Aβ (Hoshino *et al.* 2007). Using agonists and antagonists specific for each of the four PGE_2 receptors (EP_1, EP_2, EP_3 and EP_4 receptors), we found that both EP_2 and EP_4 receptors are involved in the PGE_2-stimulated production of Aβ *in vitro* (Hoshino *et al.* 2007). With respect to the mechanism underpinning this stimulation, we also recently demonstrated that activation of the EP_2 receptor stimulates the production of Aβ through activation of adenylate cyclase, an increase in the cellular level of cAMP and activation of protein kinase A (PKA) (Hoshino *et al.* 2009). In contrast, EP_4 receptor activation causes its co-internalization with PS-1 (γ-secretase) into endosomes, a process that activates γ-secretase (Hoshino *et al.* 2009). Furthermore, we showed that deletion of the EP_2 or EP_4 receptor decreases brain levels of Aβ in transgenic mice expressing APPsw (APP23, a mouse model for AD), suggesting that EP_2 or EP_4 receptor activation stimulates the production of Aβ *in vivo* (Hoshino *et al.* 2007). These previous results suggest that EP_2 and/or EP_4 receptors could represent valuable molecular targets for the treatment of AD. However, the effect of deletion of these receptors on other AD-related phenotypes, such as neuronal and synaptic loss and cognitive deficits, has not been tested. The effect on cognitive performance is particularly important, because functional phenotypes (cognitive dysfunction) and pathological phenotypes (such as an increase in the brain level of Aβ) are not always directly linked (Roberson *et al.* 2007; Kanninen *et al.* 2009). In this study, we therefore examined the effect of EP_2 and EP_4 receptor inhibition on cognitive function in APP23 mice, revealing that genetic inhibition of the EP_4 receptor but not the EP_2 receptor not only suppresses neuronal and synaptic loss but also improves cognitive performance. Similarly, oral administration of AE3-208, an EP_4 receptor-specific antagonist, improved the cognitive function of the APP23 mice. These results suggest that the EP_4 receptor is a valuable molecular target for the development of drugs to prevent or treat AD.

Materials and methods

Materials and animals

See Appendix S1.

Morris water maze test

The Morris water maze test was conducted in a circular 90- or 150-cm diameter pool filled with water at a temperature of 22.0 + 1°C, as described previously (Kobayashi *et al.* 2000; Huang *et al.* 2006), with some minor modifications. Details are described in Appendix S1.

ELISA for Aβ and β- and γ-secretase-mediated peptide cleavage assay

Aβ40 and Aβ42 levels and β- and γ-secretase activity in the brain were determined as described previously (Hoshino *et al.* 2007). Details are described in Appendix S1.

Thioflavin-S staining and immunohistochemical and immunofluorescence analyses

Thioflavin-S staining and immunohistochemical and immunofluorescence analyses were performed as detailed in Appendix S1.

Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). One- or two-way ANOVA followed by the Tukey test was used to evaluate differences between more than two groups. The Student's *t*-test for unpaired results was used for the evaluation of differences between two groups. Differences were considered to be significant for values of $p < 0.05$.

Results

Effect of deletion of EP_2 or EP_4 receptor on cognitive function in APP23 mice

We first used a Morris water maze to compare the spatial learning and memory of 6-month-old APPsw/EP_2−/− and APPsw/EP_4−/− mice with that of APPsw/EP_2+/+ and APPsw/EP_4+/+ mice, respectively. Mice were trained for 7 days to learn the location of a hidden platform, and the time required to reach the platform (escape latency) was

measured. As shown Fig. 1(a), APPsw/EP_2−/− mice required a longer time than APPsw/EP_2+/+ animals to reach the platform, suggesting that EP_2 receptor deletion exacerbates the cognitive deficit in the APP23 mice. In contrast, the APPsw/EP_4−/− mice tended to take less time to reach the platform than the corresponding control animals (APPsw/EP_4+/+) (Fig. 1b), suggesting that deletion of the EP_4 receptor ameliorates the cognitive deficit. These differences did not reflect differences in swimming ability, because swimming speed and the ability to locate a visible platform were similar between the groups (data not shown).

Given that the above results suggest that the EP_4 receptor may represent the better potential molecular target for the development of AD drugs, we next compared AD-related phenotypes, such as the formation of plaques and neuronal and synaptic loss, between four strains of mice (WT/EP_4+/+, WT/EP_4−/−, APPsw/EP_4+/+ and APPsw/EP_4−/−). We first repeated the Morris water maze test using 6-month-old mice, under slightly different experimental conditions (such as the size of swimming pool and tracking period). As shown Fig. 2(a), APPsw/EP_4+/+ mice required a longer time than WT/EP_4+/+ mice to reach the platform and this result is consistent with previous reports (Van Dam *et al.* 2003). Again, this difference did not reflect reduced swimming ability, as the swimming speed and the ability to locate a visible platform were similar between the four strains (data not shown). APPsw/EP_4−/− mice required a shorter time to reach the platform than APPsw/EP_4+/+ mice (Fig. 2a). Furthermore, there was no significant difference in the escape latency between APPsw/EP_4−/− and WT/EP_4+/+

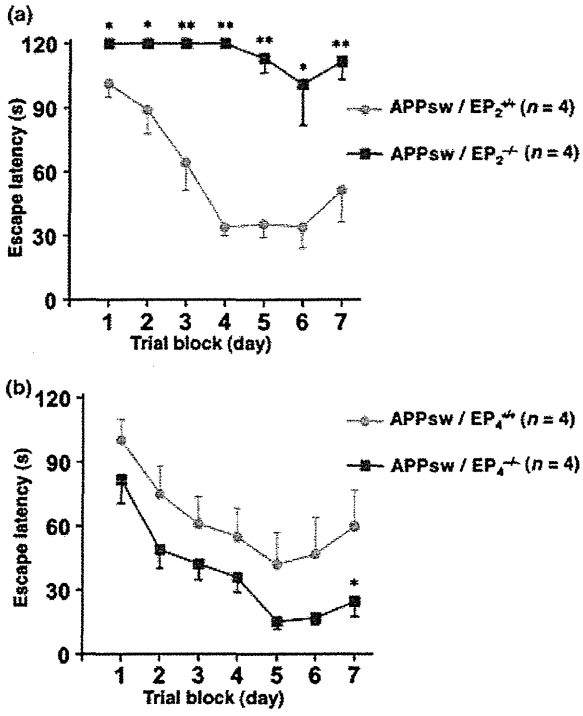

Fig. 1 Effects of deletion of the EP_2 or EP_4 receptor on spatial learning and memory in APP23 mice. Cognitive behavioral tests were carried out, using the Morris water maze, on 6-month-old APPsw/EP_2+/+ and APPsw/EP_2−/− (a) or APPsw/EP_4+/+ and APPsw/EP_4−/− (b) mice as described in the Materials and methods. Swimming paths in a circular 150-cm diameter pool were tracked for 120 s and the average (four tests) escape latency in each trial block was determined for 7 days. Values are given as mean ± SEM. Student's *t*-test: $^{**}p < 0.01$ and $^{*}p < 0.05$.

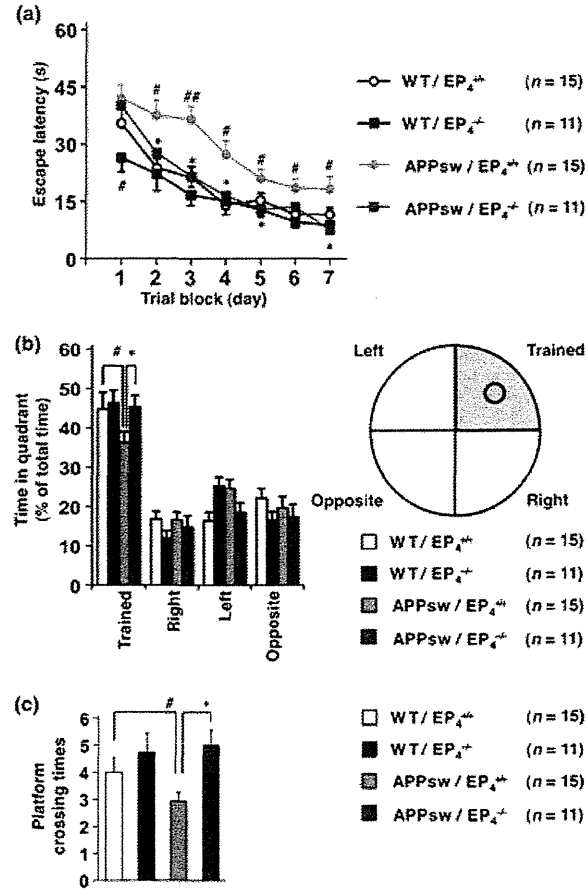

Fig. 2 Effects of deletion of the EP_4 receptor on spatial learning and memory in APP23 mice. Cognitive behavioral tests were carried out on 6-month-old WT/EP_4+/+, WT/EP_4−/−, APPsw/EP_4+/+ and APPsw/EP_4−/− mice. The swimming path in a circular 90-cm diameter pool was tracked for 60 s (a). Mice were subjected to a transfer test in which the platform was removed. The spatial memory for a platform location was estimated by per cent search time in each quadrant (the platform had been located in the 'trained' quadrant) (b) or platform crossing times (c). Values are given as mean ± SEM. One-way (b, c) or two-way (a) ANOVA followed by Tukey test: $^{*}p < 0.05$, versus APPsw/ EP_4+/+ mice; $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$, versus WT/ EP_4+/+ mice.

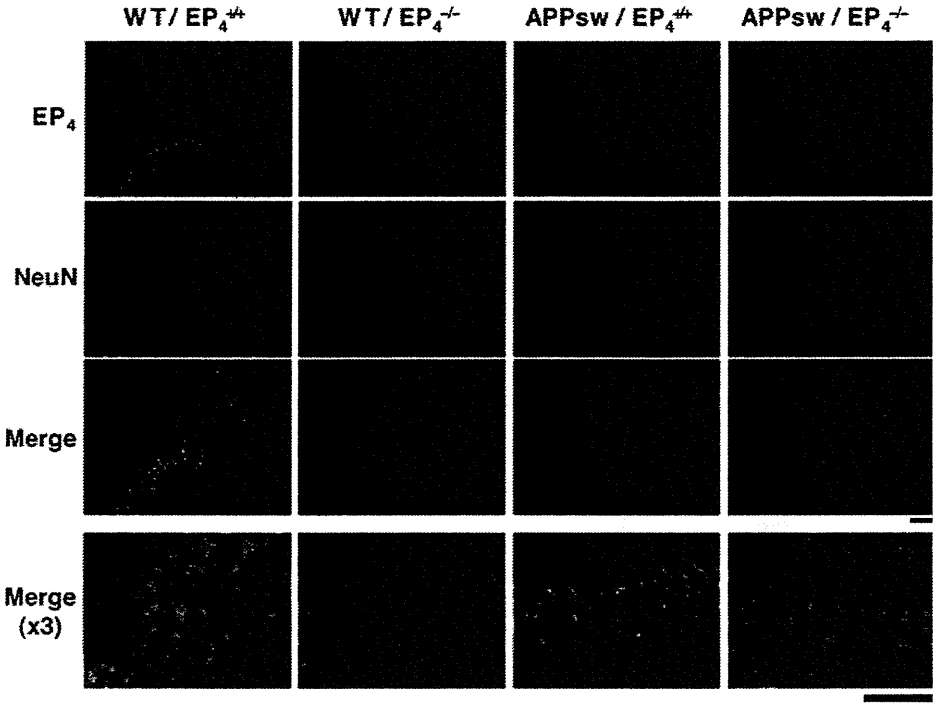

Fig. 3 EP_4 receptor expression in hippocampal neurons. Brain sections from 18-month-old WT/EP_4+/+, WT/EP_4−/−, APPsw/EP_4+/+ and APPsw/EP_4−/− mice were immunohistochemically labeled by immunofluorescence technique with antibodies against the EP_4 receptor and NeuN. The hippocampal CA3 region is shown (scale bar, 100 μm).

mice (Fig. 2a). These results suggest that the expression of APPsw disturbs spatial learning and memory and this effect can be ameliorated by deletion of the EP_4 receptor. WT/EP_4−/− mice took a significantly shorter time to reach the platform than WT/EP_4+/+ mice; however, the difference was observed only at day 0 (Fig. 2a).

We then did a transfer test to examine the spatial memory of platform location. After a 7-day training period as described above, each mouse was subjected to a Morris water maze test where the platform was removed and we measured the per cent search time for each quadrant. As shown in Fig. 2(b), the percentage of time spent in the trained quadrant was lower for the APPsw/EP_4+/+ group than for either the WT/EP_4+/+ or the APPsw/EP_4−/− mice. The crossing time of the area where the platform had been located was lower in the APPsw/EP_4+/+ group than in the WT/EP_4+/+ and APPsw/EP_4−/− cohorts (Fig. 2c). There was no significant difference between APPsw/EP_4−/− and WT/EP_4+/+ mice or between WT/EP_4−/− and WT/ EP_4+/+ mice for these indices (Fig. 2b and c). These results showed that deletion of the EP_4 receptor ameliorates the spatial memory deficits of APP23 mice.

We then examined EP_4 receptor expression in the brain of 18-month-old mice (hippocampal CA3 region) by immunofluorescence analysis. As shown in Fig. 3, expression of the receptor was clearly observed in the brains of WT/EP_4+/+ and APPsw/EP_4+/+ mice. Staining with antibody against neuronal nuclei (NeuN) confirmed expression of the EP_4 receptor in neurons (Fig. 3), consistent with previous results (Choi *et al.* 2006). However, co-staining with antibody against NeuN and that against glial fibrillary acidic protein (a maker for astrocytes) or F4/80 (a maker for microglia) was not so clear (Fig. S1).

Effect of deletion of EP_4 receptor on Aβ plaque deposition and neuronal and synaptic loss in APP23 mice

We have previously reported that the levels of Aβ40 and Aβ42 in soluble and insoluble brain fractions prepared from 6-month-old APPsw/EP_4−/− mice are lower than those from APPsw/EP_4+/+ mice (Hoshino *et al.* 2007), a finding that we confirmed here (Fig. 4a). We also examined Aβ plaque deposition by thioflavin-S staining using 18-month-old mice. As shown in Fig. 4(b) and (c), in both the hippocampus and the cerebral cortex, the level of Aβ plaque deposition was much lower in APPsw/EP_4−/− mice than in APPsw/EP_4+/+ animals.

We next determined the number of neurons in the hippocampal CA3 region by NeuN staining using 18-month-old mice. As shown in Fig. 4(d) and (e), the number of NeuN-positive cells (neurons) was significantly

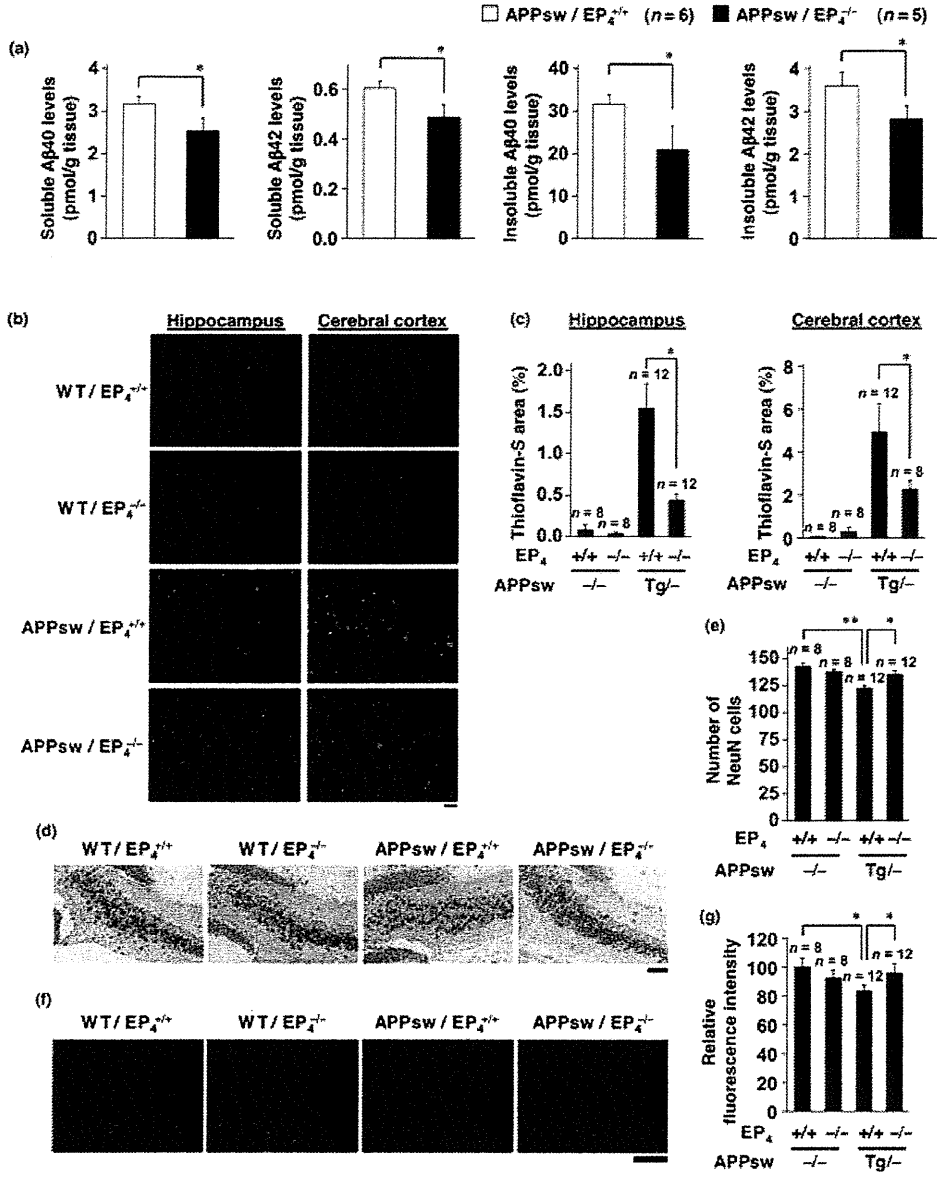

Fig. 4 Effects of EP_4 receptor deletion on Aβ levels, Aβ plaque deposition and neuronal and synaptic loss in APP23 mice. Soluble and insoluble fractions were prepared from the brains of 6-month-old APPsw/EP_4+/+ and APPsw/EP_4−/− mice. The amounts of Aβ40 and Aβ42 in each fraction were determined by ELISA as described in the Materials and methods (a). Values are given as mean ± SEM. Student's *t*-test: $^*p < 0.05$. Brain sections from 18-month-old APPsw/EP_4+/+, APPsw/EP_4−/−, WT/EP_4+/+ and WT/ EP_4−/− mice were applied to thioflavin-S staining (scale bar, 200 μm) (b) or immunohistochemical analysis with an antibody against NeuN (scale bar, 100 μm) (d) or immunofluorescence analysis with an antibody against synaptophysin (scale bar, 50 μm) (f). The relative area positive for thioflavin-S staining (c), the number of NeuN-positive cells in the hippocampal CA3 region (e) and the relative fluorescence intensity in the region (g) were determined (three sections per brain). Values are given as mean ± SEM. One-way ANOVA followed by Tukey test: $^{**}p < 0.01$; $^*p < 0.05$.

higher in the WT/EP_4+/+ and APPsw/EP_4−/− brain sections than in the APPsw/EP_4+/+ tissue, suggesting that the neuronal loss induced by Aβ was ameliorated by deletion of the EP_4 receptor. Similar results were observed for the hippocampal CA1 region (Fig. S2). We also estimated the number of synapses by synaptophysin staining using 18-month-old mice. The level of synaptophysin was higher in sections from both WT/EP_4+/+ and APPsw/EP_4−/− mice

than in those from APPsw/EP_4+/+ mice (Fig. 4f and g), indicating that Aβ-induced synaptic loss was suppressed by deletion of the EP_4 receptor. Taken together, these results suggest that deletion of the EP_4 receptor decreases the level of Aβ and Aβ plaque deposition in the brain and protects against Aβ-induced neurodegeneration. To confirm this further, stereological quantification of cell number that is more reliable should be performed in future studies.

Effect of oral administration of AE3-208 on AD-related phenotypes in APP23 mice

The results described above suggest that pharmacological inhibition of the EP_4 receptor ameliorates AD-related phenotypes in APP23 mice. In order to test this, we used an EP_4 receptor-specific antagonist, AE3-208. The K_i values of AE3-208 obtained by competition binding assay are 1.3, 30, 790 and 2400 nM for EP_4, EP_3, FP and TP, respectively, and more than 10 μM for the other prostanoid receptors (Kabashima *et al.* 2002). We have previously reported that AE3-208 suppresses the PGE_2-stimulated production of Aβ *in vitro* (Hoshino *et al.* 2007). APP23 and wild-type mice were fed either AE3-208-supplemented chow or a control diet between the ages of 3 and 6 months (the average dose of AE3-208 was calculated to be 17.8 mg/kg body weight/day). No significant differences were observed in the amount of chow consumed by the four groups of mice (APP23 or wild-type mice fed AE3-208-supplemented or control chow) during the experimental period. We then examined the spatial learning and memory of the 6-month-old animals in a Morris water maze test. Swimming speed and ability to locate a visible platform were indistinguishable between the four groups (data not shown). However, APP23 mice fed AE3-208-supplemented chow took significantly less time to find the hidden platform than the mice fed control chow (Fig. 5a). No significant difference in the escape latency was recorded between wild-type mice fed AE3-208-supplemented chow and wild-type mice fed control chow (Fig. 5a). These results suggest that the deficit in spatial learning and memory in the APP23 mice can be ameliorated by oral administration of AE3-208.

As shown in Fig. 5(b), the amount of time spent in the trained quadrant showed a tendency to be greater for the APP23 mice fed AE3-208-supplemented chow than for the mice fed control chow. Furthermore, the crossing time of the area where the platform had been located was significantly greater in the former case (Fig. 5c). However, the difference in Fig. 5(b) was not statistically significant and we have no clear explanation for the discrepancy between the 'time in quadrant' and 'platform crossings' outcomes.

In order to test whether pharmacological inhibition of the EP_4 receptor ameliorates AD-related pathological phenotypes in APP23 mice, we compared the amount of Aβ40 and Aβ42 in soluble and insoluble fractions prepared from the brains of APP23 mice fed either AE3-208-supplemented or

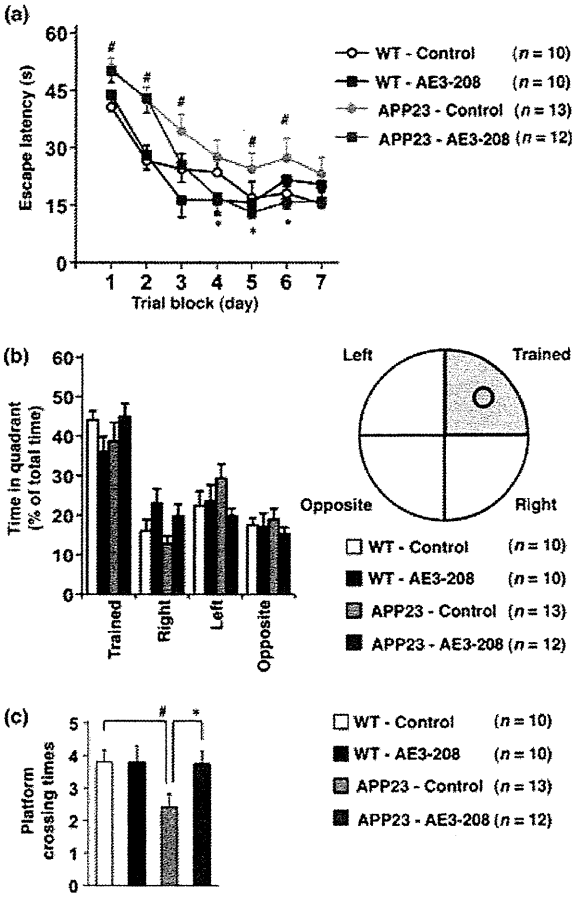

Fig. 5 Effects of oral administration of AE3-208 on spatial learning and memory in APP23 mice. Cognitive behavioral tests were carried out, using the Morris water maze, on 6-month-old wild-type mice (WT) and APP23 mice fed either AE3-208-supplemented chow (160 mg AE3-208/kg chow) or control chow between the ages of 3 and 6 months. Spatial learning and memory were tested as described in the legend of Fig. 2 (a–c). Values are given as mean ± SEM. One-way (b, c) or two-way (a) ANOVA followed by Tukey test: $^*p < 0.05$, versus APP23-control mice; $^\#p < 0.05$, versus WT-control mice.

control chow using 6-month-old mice. As shown in Fig. 6(a), the levels of Aβ40 and Aβ42 in the insoluble brain fractions from the former group were significantly lower. However, no significant difference was observed in the case of the soluble fractions (Fig. 6a).

We have previously reported that EP_4 receptor activation increases Aβ levels through its co-internalization into endosomes with PS-1 (γ-secretase), with resulting activation of γ-secretase *in vitro* (Hoshino *et al.* 2009). This finding was supported by our previous *in vivo* demonstration that brain γ-secretase activity is lower in APPsw/EP_4−/− mice than in APPsw/EP_4+/+ animals, and that the co-localization of PS-1 with Rab7 (a marker of late endosomes and

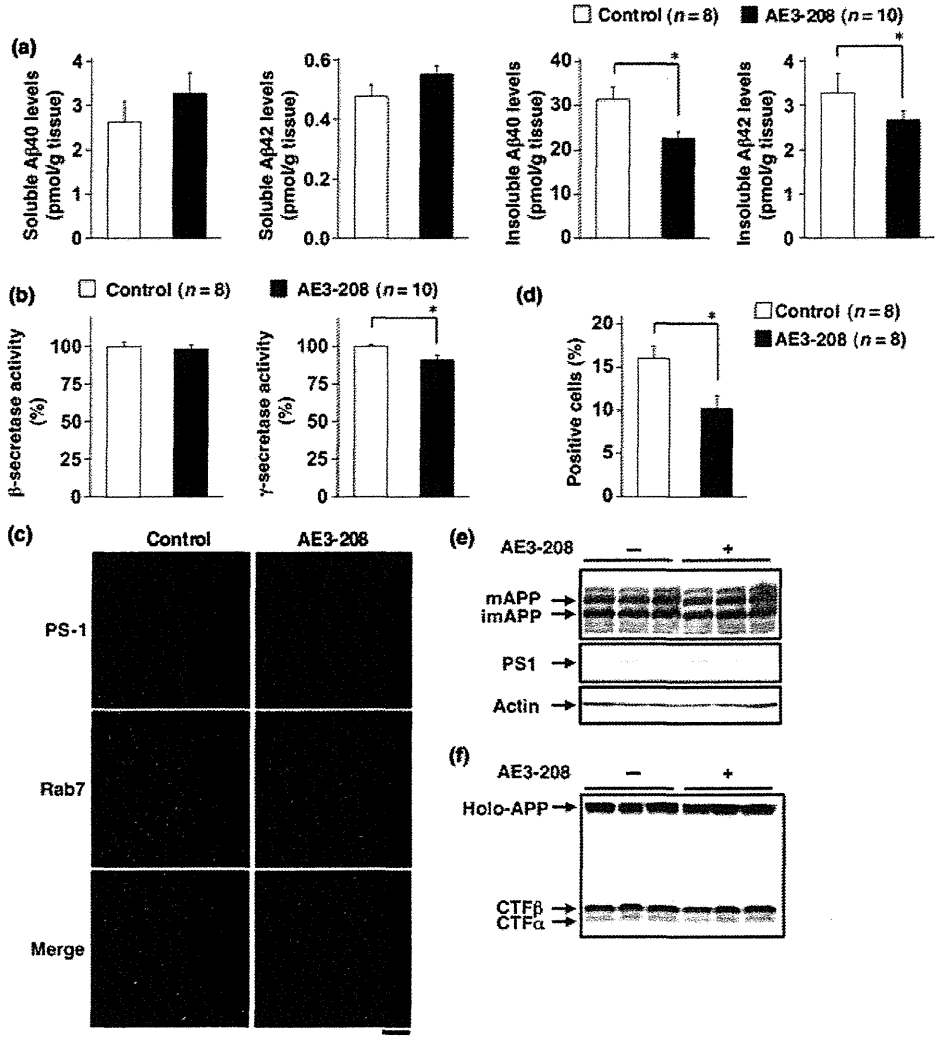

Fig. 6 Effects of oral administration of AE3-208 on Aβ levels, secretase activity, localization of γ-secretase and APP modulation in the APP23 mouse brain. APP23 mice were treated with AE3-208, as described in the legend of Fig. 5 (a–c). (6-month-old) The amounts of Aβ40 and Aβ42 were determined as described in the legend of Fig. 4 (a). Membrane fractions were prepared and subjected to a β- or γ- secretase-mediated peptide cleavage assay as described in the Materials and methods (b). Brain sections were immunostained with antibodies against PS-1 and Rab7 (scale bar, 200 μm) (c). Cells positive for both PS-1 and Rab7 staining were counted (d). Whole-cell extracts were subjected to immunoblotting with an antibody to APP (e, f), PS-1 (e) or actin (e). Values are given as mean ± SEM. Student's *t*-test: $^*p < 0.05$.

lysosomes) is not as apparent in the former group (Hoshino *et al.* 2009). In the present study, we examined the effect of oral administration of AE3-208 on the activity and localization of γ-secretase using 6-month-old mice. As shown in Fig. 6(b), the activity of γ-secretase, but not that of β-secretase, was lower in the brains of APP23 mice fed AE3-208-supplemented chow than in those of mice fed control chow. Furthermore, we found that the co-localization of PS-1 with Rab7 was not as apparent in the former group (Fig. 6c and d). We quantitatively examined the effect of AE3-208 on the expression of PS-1 staining and found that the effect was not statistically significant (data not shown).

We have previously reported that deletion of the EP_4 receptor in APP23 mice does not affect the modification of APP or α- and β-secretase activity (Hoshino *et al.* 2009), both of which are important for Aβ production. Here, we examined the effect of oral administration of AE3-208 on these processes using 6-month-old mice. We could separate by sodium dodecyl sulfate–polyacrylamide gel electrophoresis between the mature (*N*- and *O*-glycosylated) and

immature (*N*-glycosylated alone) forms of APP (mAPP and imAPP, respectively) (Tomita *et al.* 1998). As shown in Fig. 6(e), the total amount of APP and the ratio of mAPP and imAPP were similar between APP23 mice fed AE3-208-supplemented chow and those fed control chow, suggesting that the administration of AE3-208 does not affect APP modulation. We also found that the administration of AE3-208 did not affect the level of PS-1 (Fig. 6e). We then examined α- and β-secretase activity by comparing the level of secreted C-terminal fragment (CTF), representing an indirect index of secretase activity. We could not detect a CTFγ band under our experimental conditions. However, as shown in Fig. 6(f), CTFα and CTFβ were detected in the APP23 mice and the amounts of CTFα and CTFβ were indistinguishable between APP23 mice fed AE3-208-supplemented chow and those fed control chow, thereby suggesting that the administration of AE3-208 does not affect α- or β-secretase activity.

Taken together, these results suggest that the improvement in the cognitive function of the APP23 mice orally administered AE3-208 is mediated by a decrease in the brain levels of Aβ through suppression of co-internalization of the EP_4 receptor with γ-secretase into endosomes, thereby inhibiting the activation of γ-secretase.

Discussion

We have previously suggested that EP_2 and EP_4 receptors represent valuable molecular targets for the development of drugs to prevent or treat AD by showing that the amount of Aβ in the brains of APPsw/EP_2−/− and APPsw/EP_4−/− mice is lower than that in the respective control mice (Hoshino *et al.* 2007). However, among the antagonists specific for either the EP_2 or EP_4 receptor, or both, which type offers the most therapeutic potential? In order to address this issue, we herein compared the cognitive performance of APPsw/EP_2−/− or APPsw/EP_4−/− mice with that of their respective wild-type counterparts. This approach was adopted because, although AD is characterized by cognitive impairment, the functional (cognitive) phenotypes and pathological phenotypes (such as an increase in Aβ levels and Aβ plaque deposition) of the disease are not always directly linked. For example, some conditions ameliorate cognitive dysfunction in AD model mice without affecting the pathological phenotypes (Roberson *et al.* 2007; Kanninen *et al.* 2009). Our results suggested that APPsw/EP_4−/− mice but not APPsw/EP_2−/− mice display a higher level of cognitive function (spatial learning and memory) than their respective wild-type controls, suggesting that inhibition of the EP_4 receptor might prove the better therapeutic option.

We have previously reported that PGE_2-stimulated production of Aβ *in vitro* is partially mediated by EP_2 receptor-dependent activation of the cAMP–PKA pathway (Hoshino *et al.* 2009), and that the amount of Aβ in the brains of APPsw/EP_2−/− mice is lower than that in control mice (Hoshino *et al.* 2007). Another group has also shown that deletion of the EP_2 receptor in AD model mice reduces Aβ plaque deposition (Liang *et al.* 2005). Thus, it is surprising that deletion of this receptor exacerbates cognitive dysfunction in APP23 mice, suggesting that deletion of the EP_2 receptor impaired cognitive performance through an Aβ-independent mechanism. It has previously been reported that Aβ inhibits long-term potentiation (LTP) through inhibition of the cAMP–PKA pathway (Vitolo *et al.* 2002), and that inhibition of the EP_2 receptor also suppresses LTP via a similar mechanism (Akaneya and Tsumoto 2006). Thus, deletion of the EP_2 receptor may exacerbate cognitive dysfunction in APP23 mice through inhibition of LTP, a process known to be important for memory formation. It was recently reported that deletion of the gene encoding EP_2 receptor in mice without the expression of APPsw have behavioral deficits (Savonenko *et al.* 2009), thus it is unclear whether the observed effects of EP_2 receptor deletion in this study are specific to the AD model. However, it was previously reported that siRNA for EP_4 did not affect LTP (Akaneya and Tsumoto 2006).

We have previously reported that EP_4 receptor activation stimulates the production of Aβ through its co-internalization with γ-secretase into endosomes, leading to the activation of γ-secretase (Hoshino *et al.* 2009). We also showed that there are lower levels of Aβ and less endosomal localization of γ-secretase in the brains of APPsw/EP_4−/− mice than in those of APPsw/EP_4+/+ animals (Hoshino *et al.* 2007, 2009). Furthermore, in the present study, we have demonstrated that APPsw/EP_4−/− mice display lower levels of Aβ plaque formation and neuronal and synaptic loss than APPsw/EP_4+/+ mice. These results suggest that deletion of the EP_4 receptor ameliorates cognitive dysfunction in APP23 mice by decreasing brain levels of Aβ and suppressing neurodegeneration.

The findings of the present study also demonstrate that oral administration of the EP_4 receptor-specific antagonist, AE3-208, ameliorates the spatial learning and memory deficits of APP23 mice. AE3-208 has been shown to have some therapeutically beneficial effects, including suppression of tumor growth (Terada *et al.* 2010) and suppression of autoimmune encephalomyelitis (Yao *et al.* 2009). However, it has been reported that AE3-208 exacerbates dextran sodium sulfate-induced colitis, an animal model for ulcerative colitis (Kabashima *et al.* 2002), and that a specific agonist for the EP_4 receptor stimulates bone formation and prevents bone loss (Yoshida *et al.* 2002), suggesting that EP_4 receptor antagonists, including AE3-208, have adverse effects on colitis and osteoporosis, possibilities that must be considered if these agents are to be developed for the clinical treatment of AD. Although the transitional character of orally administered AE3-208 to the brain has not yet been examined, the results of the present study suggest that it can

pass the blood–brain barrier. AD is a chronic disease that requires long-term drug treatment in order to produce therapeutic effects. Thus, this property of AE3-208 would be of great advantage for its clinical use. As for the mechanism underpinning the amelioration of cognitive dysfunction in the APP23 mice following the administration of AE3-208, we believe that this is mediated by a similar mechanism to EP_4 receptor deletion, given that oral administration of AE3-208 decreases levels of Aβ and γ-secretase activity and inhibits the localization of γ-secretase in endosomes. The soluble Aβ level was reduced in APPsw/EP_4−/− mice but not in mice administered with AE3-208. This difference would be because of the difference in extent of the inhibition; deletion of the gene encoding EP_4 receptor completely inhibits the function of this protein, whereas administration of the drug may cause partial inhibition. As for the difference between soluble and insoluble Aβ for the modulation by administration of AE3-208, we have no clear explanation at present. One possible explanation is that the temporal alteration in synthesis of Aβ may affect more drastically soluble Aβ level than insoluble one.

Although this study focused on how inflammation affects the pathogenesis of AD through PGE_2 but not on how inflammation is induced in association with AD progression, we examined the effect of inhibition of EP_4 receptor on the activation of astrocytes. As shown in Fig. S3, the expression of glial fibrillary acidic protein (a maker for the activity of astrocytes) was higher in 18-month-old APPsw/EP_4+/+ mice than in WT/EP_4+/+ and APPsw/EP_4−/− mice. As for AE3-208, because we used 6-month-old mice, the activation of astrocytes by the expression of APPsw was not so clear; however, the activity was a little lower in drug-treated mice than in control mice (Fig. S3). These results suggest that the inhibition of EP_4 receptor suppresses APPsw-mediated activation of astrocytes (inflammation). Based on previously reported results, the activation of EP_4 receptor seems to affect immune systems both positively and negatively. For example, EP_4 receptor-stimulated differentiation of T_H1 cells and production of IL-23 in dendritic cells and resulting inflammation in experimental autoimmune encephalomyelitis were reported (Yao *et al.* 2009). However, in microglia, the activation of EP_4 receptor was reported to suppress the LPS-stimulated production of pro-inflammatory cytokines (Shi *et al.* 2010).

As described in the introduction, NSAIDs have attracted considerable attention as a new class of drugs for the treatment and prevention of AD, although it should also be noted that some clinical studies have recorded negative results (Imbimbo *et al.* 2010). NSAIDs can be classified into two groups: newly developed COX-2-specific NSAIDs (such as celecoxib) and classical NSAIDs without COX-2 specificity (such as indomethacin). The clinical use of classical NSAIDs is associated with gastrointestinal side effects (Hawkey 2000), as a result of the strong protective effect of prostaglandins on the gastrointestinal mucosa (Vane and Botting 1996). Given that it is mainly COX-1, which is expressed in this mucosa, COX-2-specific NSAIDs cause less of an effect on prostaglandin levels in this region, and therefore produce fewer gastrointestinal side effects than classical NSAIDs. However, it has recently been shown that clinical use of COX-2-specific NSAIDs is associated with cardiovascular thrombotic side effects (Ray *et al.* 2004; Singh 2004). These side effects of NSAIDs are likely to prove problematic if the drugs are used long term for the prevention or treatment of AD.

Compared with NSAIDs, we consider that EP_4 receptor-specific antagonists have advantages in relation to both safety and efficacy, based on the following lines of evidence. EP_1 and EP_3 receptors have been reported to be involved in PGE_2-mediated protection of the gastrointestinal mucosa by stimulating the production of bicarbonate and gastric mucosal blood flow, respectively (Takeuchi *et al.* 1997; Araki *et al.* 2000). Therefore, antagonists specific for the EP_4 receptor would be gastrointestinally safer than NSAIDs. However, it is now believed that inflammation has both positive and negative effects in relation to the progression of AD; for example, inflammation activates the phagocytosis of Aβ by microglia (Shaftel *et al.* 2007; Chakrabarty *et al.* 2010). However, NSAIDs that inhibit overall inflammation inactivate microglial phagocytosis (Yan *et al.* 2003). Therefore, compared with general anti-inflammatory agents, inhibitors that specifically act on the inflammation-mediated progression of AD may be more effective. NSAIDs suppress inflammation through both COX-dependent and COX-independent mechanisms, such as activation of the peroxisome proliferators activated receptor-γ and inhibition of nuclear factor-kB (Tegeder *et al.* 2001), with COX-mediated inhibition and the resulting decrease in PGE_2 levels seen to play a major role in the anti-AD activity of NSAIDs (Qin *et al.* 2003; Heneka *et al.* 2005). Furthermore, it was recently reported that the ability of NSAIDs to decrease PGE_2 levels is important in NSAID-dependent protection of hippocampal LTP against Aβ toxicity and restoration of Aβ-mediated suppression of synaptic plasticity and memory function (Kotilinek *et al.* 2008). Based on the findings of the present study, we consider that PGE_2 impairs cognitive performance at least partly through activation of the EP_4 receptor. Thus, we propose that EP_4 receptor-specific antagonists, such as AE3-208, will prove therapeutically more effective than NSAIDs as a result of their greater safety and efficacy. However, although we previously suggested that EP_1 and EP_3 receptors are not involved in PGE_2-stimulated production of Aβ *in vitro*, it is not clear whether activation of EP_1 and EP_3 receptors affect cognitive performance. Furthermore, modulation of COX-2 expression by activation of EP_4 receptor was also suggested (Shi *et al.* 2010). Therefore, the mechanism by which PGE_2 modulates cognitive performance is unclear at present and understanding of such mechanism is important for the identification of other targets of AD drugs.