Dihomo-\(\gamma\)-linolenic acid prevents the development of atopic dermatitis through prostaglandin \(D_1\) production in NC/Tnd mice

Yosuke Amagai \(^a\), Kumiko Oida \(^a\), Akira Matsuda \(^b\), Kyungsook Jung \(^c\), Saki Kakutani \(^d\), Takao Tanaka \(^d\), Kenshiro Matsuda \(^a\), Hyosun Jang \(^a\), Ginae Ahn \(^a\), Yan Xia \(^a\), Hiroshi Kawashima \(^d\), Hiroshi Shibata \(^d\), Hiroshi Matsuda \(^a,b,*\), Akane Tanaka \(^a,c,*\)

\(^a\) Cooperative Major in Advanced Health Science, Graduate School of Bio-Applications and System Engineering, Tokyo University of Agriculture and Technology, Tokyo, Japan
\(^b\) Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Institute of Agriculture, Tokyo, Japan
\(^c\) Laboratory of Comparative Animal Medicine, Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan
\(^d\) Institute for Health Care Science, Santory Wellness Ltd., Osaka, Japan

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A B S T R A C T

Background: Atopic dermatitis (AD) is a chronic and relapsing skin disorder with pruritic skin symptoms. We previously reported that dihomo-\(\gamma\)-linolenic acid (DGLA) prevented the development of AD in NC/Tnd mice, though the mechanism remained unclear.

Objective: We attempted to investigate the mechanism of preventive effect of DGLA on AD development in NC/Tnd mice.

Methods: The clinical outcomes of NC/Tnd mice that were given diets containing DGLA, arachidonic acid, or eicosapentaenoic acid were compared. Lipid mediator contents in the skin in each group were also quantified. In addition, release of lipid mediators from RBL-2H3 mast cells treated with either DGLA or prostaglandin \(D_1\) (PGD\(_1\)) was measured. Furthermore, effect of PGD\(_1\) on gene expression of thymic stromal lymphopoietin (TSLP) in PAM212 keratinocyte cells was determined.

Results: Only DGLA containing diet suppressed the development of dermatitis in vivo. By quantifying the 20-carbon fatty acid-derived eicosanoids in the skin, the application of DGLA was found to upregulate PGD\(_1\), which correlated with a better outcome in NC/Tnd mice. Moreover, we confirmed that mast cells produced PGD\(_1\) after DGLA exposure, thereby exerting a suppressive effect on immunoglobulin E-mediated degranulation. PGD\(_1\) also suppressed gene expression of TSLP in keratinocytes.

Conclusion: These results suggest that oral administration of DGLA causes preventive effects on AD development in NC/Tnd mice by regulating the PGD\(_1\) supply.

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1. Introduction

Atopic dermatitis (AD) is a common, chronic inflammatory skin disease associated with dryness and itching of the skin [1]. The etiology of the disease is multifactorial; genetic, environmental, immunological, psychological, and physical factors are considered to be involved in the onset and exaggeration of AD [1–4]. Among them, food intake is an important factor affecting AD symptoms. In particular, fatty acids have been considered to play a critical role in inflammatory responses because they are a source of various kinds of lipid mediators [5–7]. Therefore, many attempts to investigate the efficacy of a diet containing essential fatty acids in the...
treatment of AD have been made, though most of them were unsuccessful [8,9]. In addition, the efficacy of eicosanoids has been investigated using a spontaneous AD mouse model [10–12]. The group of Nakaie et al. [10–12] focused on the role of prostaglandin D2 (PGD2) and showed a negative correlation between PGD2 and AD progression. After mechanical scratching, PGD2 levels in the skin were increased in BALB/c mice but not in NC/Nga AD model mice [13]. These observations indicated that PGD2 might have suppressive effect on itch sensation and the subsequent development of AD in the mice. However, topical application of a PGD2 receptor agonist did not exert a potent inhibitory effect on AD symptoms [14].

We recently reported that oral administration of dihomo-γ-linolenic acid (DGLA) prevented the development of AD in NC/Tnd mice [15]. DGLA is metabolized to either the series 1 PGs (PGD1, PGE1 or PGF1α) as well as arachidonic acid (AA) which is a source of various kinds of leukotrienes and prostanooids such as PGD2 and PGE2 (Fig. 2A) [16]. Inhibitory effects of PGD1 on the PGD2/E1/E2-mediated increase in vascular permeability were reported [17]; however, there has been little information about the effects of the series 1 PGs on allergic inflammation, including AD. In this study, we compared the clinical outcomes of several diets containing different fatty acids in NC/Tnd mice to clarify the lipid mediators that mainly contribute to the better outcome of mice following DGLA supplementation. Our results revealed that PGD1 upregulation is involved in the prevention of AD by suppressing both mast cells and keratinocytes activations.

2. Materials and methods

2.1. Reagents

PGD1, PGE1, PGF1α, PGD2, PGE2, PGF2α, PGD3, PGE3, PGF3α, PGD2-d4, PGE2-d4, 8(S)-hydroxyeicosatrienoic (HETE), 15(S)-HETE, 5(S)-hydroxyeicosatetraenoic acid (HETE), 8(S)-HETE, 12(S)-HETE, 15(S)-HETE, 5(S)-hydroxyeicosapentaenoic acid (HEPE), 8(S)-HEPE, 12(S)-HEPE, 5(S)-HEPE-d8, and 15(S)-HEPE-d8 were obtained from Cayman Chemical (Ann Arbor, MI).

2.2. Mice

NC/Tnd mice were maintained in conventional circumstances, as described previously [18]. All experiments with animals complied with the standards specified in the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology, as well as with the guidelines for the use of laboratory animals provided by Science Council of Japan.

2.3. Diet

Mice were fed a defined diet containing several types of fatty acid (Table 1) from 8 to 13 weeks of age. The mice were given a pellet diet and water ad libitum. The diet was a modified AIN-76A containing 5% (w/w) lipids, which consisted of a mixture of corn oil, lard, olive oil, and either DGLA oil, AA oil, or eicosapentaenoic acid (EPA) ethyl ester (Table 1). DGLA oil was obtained as described previously [15]. AA oil and EPA ethyl ester was obtained from Suntrky Ltd. (Osaka, Japan) and Bizen Chemical (Okayama, Japan), respectively. The fatty acid compositions of the diets were adjusted to be similar to each other and for the amounts of total n-6 fatty acids (linoleic acid [LA] + γ-linolenic acid [GLA] + DGLA) to be approximately 40%.

2.4. Clinical severity score and scratching analysis

The clinical features of dermatitis were scored twice a week according to criteria described previously [18], which consisted of five major clinical signs and symptoms of AD: itching, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. The scratching behavior of the mice was analyzed using a SCALABA-Real™ system (Noveltec Inc., Kobe, Japan) [19]. Scratching behavior was quantified as the frequency and duration of scratching per 20 min in the double-blind evaluation.

2.5. Analysis of fatty acids of skin homogenates

The quantification of each fatty acid in the skin was conducted according to a previously described method [15].

2.6. Analysis of lipid mediators of skin homogenates and RBL-2H3 cells

The extraction and analysis of lipid mediators were conducted based on previous methods with some modification [20,21]. Briefly, skin tissue frozen in liquid nitrogen was ground using a Multi-Beads Shocker MB701 (Yasui Kikai, Osaka, Japan) and homogenized with ice-cold ethanol. Fixed amounts of PGD2-d4, PGE2-d4, 5(S)-HETE-d8, and 15(S)-HETE-d8 were added as an internal standard. After centrifugation, each supernatant was dried by centrifugal evaporation, and the residues were dissolved in methanol and diluted with water/acetic acid (1000/5, v/v) for clean-up with solid phase extraction (SPE) cartridges (Empore disk cartridge C18-SD; 3 M, St. Paul, MN). An Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a Cadenza CD-C18 column (3 μm, 2 mm i.d. × 150 mm; Imtakt, Kyoto, Japan) and a quadrupole linear ion trap hybrid mass spectrometer, 4000 Q TRAP, with an electrospray interface (Applied Biosystems/MDS SCIEX, Concord, Canada) was used for quantification. The flow rate was 0.2 mL/min and the column temperature was set at 30°C. Solvent A was acetonitrile/water (50/50, v/v) and solvent B was methanol/formic acid (10,000/1, v/v). The analytes were separated using the following gradient: 0–2.5 min, 0–100% solvent B; 2.5–5 min, 100% solvent B; 5–5.75 min, 100–0% solvent B; and 5.75–14.8 min, 0% solvent B. The mass spectrometer was operated in negative ion mode with selected reaction monitoring (SRM) as summarized in Table 2. Lipid mediators in the culture medium of RBL-2H3 cells [22] were analyzed using the same method as the above through grinding and homogenization steps were skipped.

2.7. Cell culture and determination of cell activation

RBL-2H3 mast cells [22] were maintained in α-minimum essential medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; PAtlas, Brooklyn, Australia) and antibiotics. A β-hexosaminidase (β-HEX) assay was performed according to the method described by Ortega et al. [23]. For immunoglobulin E (IgE) cross-linkage, cells were first sensitized with 5 μg/ml monoclonal anti-dinitrophenyl (DNP) IgE (clone SPE7; Sigma Aldrich, Taufkirchen, Germany) for 4 h and stimulated by DNP-bovine serum albumin (DNP-BSA; Sigma Aldrich) for 3 h. The absorbance was measured with an Immuno- Mini NJ-2300 (Nalge Nunc International K.K., Tokyo, Japan).

Table 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Proportion of fatty acid (%)</th>
<th>DGLA</th>
<th>AA</th>
<th>EPA</th>
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<tr>
<td>Control</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>0.9</td>
<td>0</td>
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<tr>
<td>20:4 AA</td>
<td></td>
<td>0</td>
<td>12.1</td>
<td>0</td>
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<tr>
<td>20:5 EPA</td>
<td></td>
<td>0</td>
<td>0</td>
<td>12.8</td>
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</table>
Table 2

SRM transitions and collision energy conditions.

<table>
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<th>Analyte</th>
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<th>Q3 (m/z)</th>
<th>Collision energy (V)</th>
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<td>317</td>
<td>-20</td>
</tr>
<tr>
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<td>351</td>
<td>271</td>
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<td>307</td>
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<tr>
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</table>

2.8. Growth activity of RBL-2H3 cells

A 5-bromo-2′-deoxy-uridine (BrdU) incorporation assay was conducted according to the method described previously [24].

2.9. Determination of thymic stromal lymphopoietin (TSLP) mRNA expression

PAM212 keratinocyte cells [25] were maintained in Dulbecco’s modified Eagle medium (Life Technologies) supplemented with 10% FBS and antibiotics. Cells were incubated with DGLA and PGD<sub>1</sub> for 48 h, and then stimulated with 50 nM phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) or diethyl sulfoxide for 6 h. The real-time reverse transcription and polymerase chain reaction (RT-PCR) was performed using an ABI 7000 system (Life Technologies) according to the manufacturer’s instructions. The following primers were used: a TSLP-specific forward primer (5′-cga aat cca gga ctt gta g-3′), a TSLP-specific reverse primer (5′-ctt cgg gag tta ctt gtt acg-3′), an ACTB-specific forward primer (5′-cat ccc taa aga cta tgc cca c-3′) and an ACTB-specific reverse primer (5′-atg gag cca ccc atc cac a-3′).

2.10. Topical application of PGD<sub>1</sub> or PGD<sub>2</sub>

Conventional NC/Tnd mice aged 14–17 weeks were topically applied PGD<sub>1</sub> or PGD<sub>2</sub>, which are dissolved in ethanol at 0.1 mg/mL, respectively, habituated in a cage for 30 min, and the number of scratching on either the nape or rostral part of the back was measured for 20 min every hour. As a sham control, ethanol alone was applied. In order to compare alterations in the number of scratching behaviors in the same mice, they were measured in each mouse following application of either PGD<sub>1</sub> or PGD<sub>2</sub> a week after the same measurement following ethanol treatment.

2.11. Statistical analyses

Dunnett’s test, Tukey’s test, Steel’s test and Spearman’s rank correlation test were performed for statistical analysis, and a p value of <0.05 was considered statistically significant. The Spearman’s coefficients are denoted by r.<sup>s</sup>

3. Results

To investigate whether the preventive effect of DGLA on AD was obtained directly from DGLA-derived PGs or mediated through AA, diets containing either AA or DGLA (Table 1) were fed to NC/Tnd mice and clinical skin scores, as well as the scratching frequency of the mice, were compared. As controls, a diet containing another 20-carbon fatty acid, EPA, and a normal diet that contained neither of these fatty acids were administered (Table 1). As shown in Fig. 1A, DGLA abrogated the exaggeration of AD development, while both AA and EPA did not suppress the increase of clinical scores in the mice. In addition, compared to the control, only DGLA decreased the number and duration of scratching behaviors (Fig. 1B and C).

![Fig. 1](image-url)
Fig. 2. Analysis of lipid mediators produced in NC/Tnd mice that were fed diets containing different fatty acids. (A) Outline of the conversion pathway of eicosanoids from PUFAs. (B) Absorption of each fatty acid in NC/Tnd mice which were fed each fatty acid-containing diet. The mice were fed the each diet for 5 weeks, and the amount of DGLA was quantified. Data represent the mean and SE of the results from 7 mice in each group. ** p < 0.01 compared to the control by using Tukey’s test. (C) Lipid mediators in the skin of NC/Tnd mice. Mice were fed each diet for 5 weeks, and the content of each mediator in the skin was quantified. Data represent the mean and SE of the results from 7 mice in each group. *, ** p < 0.05, 0.01 compared to the control by using Tukey’s test, respectively. (D) Relationships between the number of scratching behaviors and the amount of lipid mediators in the skin. The data are based on the results obtained in mice after receiving each diet for 5 weeks. The amount of lipid mediators is indicated as the ratio to total PGs in the skin. The lines were calculated using the method of least squares. * and ** denote p < 0.05 and p < 0.01, respectively, for Spearman’s rank correlation. (E) Relationships between the clinical scores and the amount of lipid mediators in the skin. The data are based on the results obtained in mice after receiving each diet for 5 weeks. The amount of lipid mediators is indicated as the ratio to total PGs in the skin. The lines were calculated using the method of least squares.
Polyunsaturated fatty acids (PUFAs) are converted to various kinds of eicosanoids as shown in Fig. 2A. EPA, which is originated from α-linolenic acid (ALA; n-3 PUFA), is converted to the series 3 PGs. On the other hand, LA (n-6 PUFA) is converted to DGLA resulting in direct conversion to the series 1 PGs as well as in indirect conversion to the series 2 PGs through AA. Because skin absorption of fatty acids derived from each diet was confirmed (Fig. 2B for DGLA, and data not shown for other fatty acids), we assumed that amounts of each eicosanoid produced in NC/Tnd mice were different and some of them may be highly correlated with the exaggeration of AD symptoms. Therefore, we measured the amount of eicosanoids in the skin of NC/Tnd mice fed each diet for 5 weeks. PGs, especially the series 1 PGs, were significantly upregulated only in the DGLA diet-treated group, while there were no significant differences in most mediators that are converted by lipoxygenase (Fig. 2C). The series 3 PGs, produced from EPA, were detected only in the EPA diet-treated group. On the other hand, PGD2 and PGE2, which are converted from AA, were significantly increased following both DGLA and AA treatment, although there were no significant differences between those two groups (Fig. 2C).

To further explore the correlation between the production of lipid mediators and AD-related symptoms, the amount of lipid mediators and duration of scratching behavior in the mice fed each diet for 5 weeks were compared (Fig. 2D). Corresponding to

![Fig. 3](image)

**Fig. 3.** Involvement of mast cells in PG production and activation. (A–C) Production of PG mediators in RBL-2H3 cells. Cells were treated with DGLA, AA or EPA for 48 h, and the eicosanoids produced within the cells were quantified. Data represent the mean and SE of 3 independent experiments. (D) Degranulation responses of RBL-2H3 cells. Cells were treated with anti-DNP IgE and DNP-BSA after the treatment of DGLA, AA, or EPA for 48 h. Data represent the mean and SE of 3 independent experiments. *p < 0.05 compared to the control by using Dunnett’s test. (E) Effects of each fatty acid on the growth activity of RBL-2H3 cells. Cells were incubated with each fatty acid for 48 h in the presence of BrdU, and the incorporation of BrdU was quantified. Data represent the mean and SE of 3 independent experiments. (F) Degranulation responses of RBL-2H3 cells. Cells were treated with anti-DNP IgE and DNP-BSA after the treatment of PGD1 for 48 h. Data represent the mean and SE of 3 independent experiments. *p < 0.05 compared to the control by using Dunnett’s test.
the results shown in Fig. 2C, a significant negative correlation was observed between AD symptoms and PGD1 \((r_2 = -0.52)\) and a positive correlation was observed with PGE2 \((r_2 = 0.44)\). Regarding the correlation between clinical scores and lipid mediators, weak negative correlation with PGD1 \((r_2 = -0.21)\) and weak positive correlation with PGE2 \((r_2 = 0.26)\) was observed, though it was not statistically significant (Fig. 2E).

These results indicate that series 1 PGs, especially PGD1 play an important role in the prevention of AD development in NC/Tnd mice. However, few studies have determined the role and source of these PGDs. Because mast cells are a primary source of PGD2 in the skin [26,27], we hypothesized that PGD1 was also produced from mast cells. To test this possibility, DGLA, AA or EPA was supplemented to the culture medium of an RBL-2H3 mast cell line [22]. As shown in Fig. 3A, DGLA supplementation significantly upregulated PGD1 production in RBL-2H3 cells in a dose-dependent manner. In contrast, other series 1 PGs as well as series 2/3 PGs were not increased by DGLA treatment in these cells (Fig. 3B and C). Moreover, neither AA nor EPA increased the production of any PGs in the mast cells (Fig. 3A–C). We next evaluated the effect of each mediator on mast cell function. As shown in Fig. 3D, DGLA, but neither AA nor EPA, inhibited mast cell degranulation in a dose-dependent manner. In contrast, no mediators affected the growth of the cells (Fig. 3E). To further explore whether the inhibitory effect of DGLA on mast degranulation was mediated by PGD1, B-HEX assay was carried out with RBL-2H3 cells incubated with PGD1. Corresponding to the results obtained in DGLA-treated cells, PGD1 suppressed the degranulation of the cells (Fig. 3F).

TSLP is a cytokine released from epithelial cells and well-known to trigger allergic inflammation at the onset of AD [28,29]. Next we examined effects of DGLA on TSLP expression in keratinocyte. PAM212 keratinocytes were treated with either DGLA or PGD1, activated with PMA and real-time RT-PCR was carried out to quantify the expression of TSLP mRNA. As shown in Fig. 4, PGD1 suppressed the TSLP mRNA expression in PAM212 cells. Although DGLA also tended to suppress, there was no statistical difference (Fig. 4).

Finally, PGD1 was administered percutaneously to the established AD in NC/Tnd mice. As shown in Fig. 5, topical application of PGD1 at 0.1 mg/ml significantly decreased the number of scratching behaviors in NC/Tnd mice especially for the first 12 h, while PGD2 showed limited effect. However, 0.01 mg/ml or less concentration of PGD1 application did not show any inhibitory effect (data not shown). We also applied PGD1 daily for 8 days to evaluate effects on clinical severity, though little inhibitory effect was identified (data not shown).

4. Discussion

In this study, we revealed that DGLA prevents AD via PGD1 production for the first time. DGLA solely prevented AD development in NC/Tnd mice despite the fact that AA is converted from DGLA, indicating the significance of series 1 PGs in the inhibition of dermatitis progression (Fig. 6). The notion is also supported by the results that PGD1 suppressed TSLP mRNA expression and mast cell activation in vitro as well as the number of scratching behavior in vivo. In addition, systemic alteration of the composition of lipid mediators may be beneficial in the prevention of AD, considering our result that the serum levels of PGD1 were negatively correlated with the reduction in the frequency of scratching behaviors in NC/Tnd mice. Compared to healthy donors, patients with AD have been reported to have lower DGLA levels as well as normal LA levels in the serum [7], suggesting that AD patients may possess abnormalities in lipid metabolism, especially in the process related to the conversion of LA to DGLA. In fact, lower transcriptional levels of both Δ6-desaturase and elongase 5, which are the enzymes necessary for the conversion of LA to DGLA, have been noted in pediatric patients with AD [30]. Thus, supplements or agents that modify the enzymatic activity of those proteins or other molecules that target the metabolism of these lipids may help to prevent or ameliorate AD symptoms.

PGD2 receptor 1 (DP1) and DP2 are well-known receptors for PGD2 and PGE2 [31]. DP1 activation shows suppressive effects on allergic reactions, while DP2 activation promotes allergic inflammation [32–34]. Limited effects of PGD1 on AD symptoms in this study as well as the ones reported by Nakaie et al. [14] was probably due to DP2 activation mediated by PGD2 and its metabolites [31,35]. Though there is little information regarding the PGD1 and its receptors, several cellular responses to PGD1 are
similar to those induced by PGD₂. For example, both PGD₁ and PGD₂ activate peroxisome proliferator-activated receptors [36] and inhibit growth of glioma cells [37]. It suggests that DP1 and DP2 may also be provided as receptors for PGD₁. Since PGD₁ suppressed activation of keratinocyte and mast cell in vitro partially, there is a possibility that the binding affinity of PGD₁ to DP1 was stronger than that to DP2 on those cells. Different outcomes of DP1 and DP2 activation are characterized by the opposing effects on cyclic AMP (cAMP) production; while DP1 activates cAMP and downstream protein kinase A (PKA), DP2 downregulates both of them [38,39]. Interestingly, IgE-mediated mast cell degranulation requires cAMP downregulation [40], supporting the premise that PGD₁ activated DP1 signaling and resulted in functional suppression of mast cells via cAMP upregulation. Because PMA strongly increases intracellular cAMP and following TSLP expression [41], inhibitory effects of PGD₁ in vitro may be a consequence of cAMP consumption by DGLA pretreatment. It raises the possibility that DGLA supplementation can alter the cellular responsiveness to external stimuli and prevent excessive inflammatory reactions. Though PGD₁ may be a novel candidate for controlling AD symptoms, topical application of PGD₁ on the skin of NC/Tnd mice exerted little beneficial effect on their clinical symptoms, probably due to the instability and short half-life of PGD₁ [42]. Constant supplementation of PGD₁ through oral administration of DGLA may be superior to direct PGD₁ application. In addition, our in vivo experiments showed that DGLA uptake enhanced the production of several lipid mediators other than PGD₁ including PGE₁ and 15-HETE, which may also be involved in the control of AD symptoms directly or indirectly and providing the benefit of DGLA supplementation.

In conclusion, our results reveal that production of PGD₁ is one of the mechanisms of the preventive effects of DGLA on AD progression. Therefore, supplementation of DGLA or the modification of these metabolic pathways may provide a novel therapeutic strategy for AD.

References


