Curcumin promotes progression of AApooAII amyloidosis and peroxisome proliferation in mice by activating the PPARα signaling pathway

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Abstract:

Curcumin is a polyphenol compound that exhibits multiple physiological activities. To elucidate the mechanisms by which curcumin affects systemic amyloidosis, we investigated amyloid deposition and molecular changes in a mouse model of amyloid apolipoprotein A-II (AApoAII) amyloidosis, in which mice were fed a curcumin-supplemented diet. Curcumin supplementation for 12 weeks significantly increased AApooAII amyloid deposition relative to controls, especially in the liver and spleen. Liver weights and plasma ApoA-II and high-density lipoprotein concentrations were significantly elevated in curcumin-supplemented groups. RNA-sequence analysis revealed that curcumin intake affected hepatic lipid metabolism via the peroxisome proliferator-activated receptor (PPAR) pathway, especially PPARα activation, resulting in increased Apoa2 mRNA expression. The increase in liver weights was due to activation of PPARα and peroxisome proliferation. Taken together, these results demonstrate that curcumin is a PPARα activator and may affect expression levels of proteins involved in amyloid deposition to influence amyloidosis and metabolism in a complex manner.

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Introduction:

Amyloidosis is a group of diseases characterized by abnormal aggregation of proteins to form amyloid fibrils, and subsequent deposition in various tissues and organs, which can lead to severe functional failures. More than 30 amyloid proteins have been identified; some result in localized tissue deposits, such as Aβ in Alzheimer's disease and α-synuclein (αSyn) in Parkinson's disease that deposit in the brain, while others result in systemic amyloidosis and are widely deposited in various tissues and organs, such as ALλ and ALκ in immunoglobulin light chain amyloidosis and ATTR in transthyretin amyloidosis [1]. In general, when amyloid proteins are exposed to certain conditions that affect protein homeostasis (e.g., overexpression, gene mutation, enzyme cleavage), they may undergo structural changes into stable structures that are rich in β-sheets, and which promote subsequent aggregation to form oligomers, protofibrils and amyloid fibrils [2, 3]. Because the formation of amyloid fibrils is nearly irreversible, maintaining proteostasis and inhibiting amyloid aggregation presents a challenge for development of an effective treatment.

Some natural phenolic compounds extracted from plants exhibit certain anti-amyloid activity in vitro and in vivo [4]. Curcumin, a polyphenol compound, is extracted from the rhizome of *Curcuma longa* and has a long history of use in traditional medicines in some countries in Asia. In in vitro experiments, curcumin has been shown to suppress the aggregation and cytotoxicity of Aβ, αSyn, islet amyloid precursor protein (IAPP), ATTR and prion protein (PrP) [4]. In 2001, the first evidence of the efficacy of curcumin against Aβ amyloidosis in a transgenic model mice was reported [5]. Curcumin was found to suppress amyloid deposition in a mouse model of Alzheimer's disease and improve memory function. It was subsequently demonstrated that the amount of amyloid present in TTR- and tau-transgenic mice were reduced by curcumin supplementation [6-8]. Due to the strong affinity of curcumin for the amyloid structure, it is believed that curcumin inhibits the formation of amyloid fibrils by binding to amyloid protein monomers or aggregates [4, 5, 9, 10]. This curcumin-protein complex exhibits better stability and reduces the tendency to aggregate. However, another mechanism that has been proposed suggests that curcumin inhibits Aβ production by down-regulating the expression of amyloid-beta precursor protein (APP) or beta-site APP cleaving enzyme 1 (BACE1) in vitro [11, 12]. Unfortunately, there are few reports that suggest that curcumin will provide clinical benefit in patients with Alzheimer's disease or AL amyloidosis [4, 13, 14]. In fact, it is unclear how curcumin inhibits amyloid deposition in vivo.

Curcumin is a compound with multiple physiological activities, which include anti-oxidation,
anti-inflammatory, anti-cancer, lipid metabolism regulation and anti-amyloid properties. However, a link between the various physiological activities has not been completely established [15]. Curcumin has been found to exert an influence on multiple signaling pathways [16]. In 2003, curcumin was first shown to inhibit rat hepatic stellate cell growth by activation of PPARγ, suggesting that curcumin might have an effect on the PPAR signaling pathway [17]. In mammals, the PPAR subfamily (PPARs) is a group of nuclear receptor proteins, e.g., transcription factors, and consists of three members, namely PPAR-α, PPAR-β/δ and PPAR-γ, that play essential roles in the regulation of metabolic homeostasis, glucose and energy metabolism, cellular differentiation, inflammation, and ROS metabolism [18-21]. The functions of the three PPAR subtypes are different. PPAR-α regulates fatty acid transport and oxidative decomposition in the liver and muscle in response to energy metabolism levels. PPARγ mainly regulates fatty acid synthesis and fat accumulation in adipose tissue, as well as differentiation of adipose cells and macrophages. PPARβ/δ plays an important role in lipid catabolism, energy homeostasis and cell differentiation, but the mechanism and network of action are not completely clear [21, 22]. Among the three isotypes, the relationship between curcumin and PPARγ is the most extensively studied, while information on α and β/δ remains scarce.

In this study, we sought to determine whether curcumin affects the amyloid deposition process besides directly binding to amyloid proteins, and identify a link between curcumin’s anti-amyloid activity and its various other biological activities. In our previous study, we found that antioxidants (tempol and apocynin) can effectively reduce AApoAII amyloid deposition [23]. It is therefore possible that the anti-oxidative effects of curcumin also play an important role in amyloid formation. We examined the effects of curcumin supplementation in a mouse model of AApoAII amyloidosis, in which mice were induced to develop systemic amyloidosis [24]. In contrast to expectations, our results showed that curcumin significantly promoted AApoAII amyloid deposition by activating the PPAR signaling pathway. Moreover, our results suggest that activation of PPARα plays a major role in the amyloid formation process.
Results:

Degree of AApoAII amyloid deposition and liver weights were significantly increased after supplementation with curcumin.

Two-month-old female R1.P1-Apoa2c mice were divided into 4 groups: the control (Con) and curcumin (Cur) groups are non-amyloid-induced groups and fed a common diet or 2% w/w curcumin diet, respectively. The other two groups were injected with 1 μg AApoAII amyloid fibrils into the tail vein to induce amyloidosis, and were fed a common diet (A-NT group) or 2% curcumin diet (A-Cur group) (see experimental design in Supplementary Fig. 1). After 8 and 12 weeks, we evaluated the effect of curcumin intake on amyloid deposition. Unlike previous studies, the degree of amyloid deposition in the A-Cur group was significantly increased compared to the A-NT group at both 8 and 12 weeks, especially in the liver and spleen (Fig. 1a-d, Supplementary Fig. 2). This is the first evidence that curcumin promotes amyloid deposition in vivo, and suggests that curcumin can affect the amyloid deposition process via a complex mechanism, not just via binding with amyloid protein monomers or aggregates. In addition, no amyloid deposition was observed in the Cur group, suggesting that curcumin does not result in the pathogenesis of amyloidosis without induction by amyloid fibril injection. These results suggest that curcumin accelerates amyloid deposition but does not cause structural changes in amyloidogenic proteins.

On the other hand, we noticed that the livers of mice were larger and heavier in those mice that received dietary supplementation with curcumin than in those without supplementation (Fig. 1e-f, Supplementary Table 1). In the subsequent histological observation, it was found that the mice in the curcumin-supplemented groups had hepatocyte hypertrophy and abnormal changes in some hepatocyte nuclei, but no tumors or abnormal organisms were observed (Supplementary Fig. 3). This is somewhat confusing, as it has been demonstrated in many high-fat diet experiments that curcumin effectively reduces liver lipid deposition and liver weight [25, 26]. To exclude the effects of hepatocyte damage or inflammation, plasma levels of aspartate aminotransferase (AST) and alanine transaminase (ALT) and several inflammatory marker cytokines in the liver were detected (Supplementary Figs. 4 and 5). However, we found no support that dietary supplementation with curcumin causes inflammation or cell injury in the liver. To confirm whether different doses will affect the experimental results, we repeated the experiment with a lower dose (0.5%w/w) of curcumin diet and obtained similar changes in the degree of amyloid deposition and liver weight (Supplementary Fig. 6).
Figure 1

(a) Amyloid index (AI) in the 8-week group. (b) Representative Congo red and IHC images of AAp0AII amyloid deposition in the 8-week group. Amyloid deposits (red arrows) were identified by green birefringence in Congo red-stained sections using polarizing light microscopy. Each scale bar indicates 100 μm. (c) Amyloid index in the 12-week group. (d) Representative Congo red and IHC images of amyloid deposition in the 12-week group. (e) Mice in curcumin-supplemented group have larger livers and less adipose tissue in the abdominal cavity than mice without curcumin supplementation (left: commercial diet; right: curcumin diet) (f) Ratio of liver weight / body weight in all groups. Each dot represents an individual mouse (a, c). Data are mean ± SD (f). N = 3 - 5. The Kruskal-Wallis test with the Steel-Dwass test was used for the amyloid index, and the Tukey-Kramer method was used for multiple comparisons of liver weights; *P < 0.05, **P < 0.01, *** P < 0.001.
Curcumin elevated levels of ApoA-II protein and affected lipid metabolism in mice.

It has been reported that overexpression of amyloid protein or precursor protein is one of the most important factors contributing to the pathogenesis of amyloidosis, and results in increased amyloid deposition in transgenic model mice [2, 3, 27]. Apolipoprotein A-I (ApoA-I) and apolipoprotein A-II (ApoA-II) are the major proteins comprising HDL (high-density lipoprotein) particles, and curcumin has been shown to increase HDL levels in some studies of lipid metabolism [28, 29]. We previously demonstrated that overexpression of ApoA-II can significantly aggravate amyloid deposition in Apoa2 transgenic mice [30]. Moreover, the co-deposited proteins in AApAII amyloidosis may also affect the degree of amyloid deposition [31]. We hypothesized that the increase in amyloid deposition in the curcumin diet group was due to upregulation of ApoA-II or other co-deposition proteins. Plasma levels of ApoA-II and the major proteins that were co-deposited with AApA-II amyloids (ApoA-I and ApoE) were measured by Western immunoblot. In line with our expectations, significantly higher plasma levels of ApoA-II at 12 weeks were observed for the Cur group compared with the Con group (Fig. 2a). Following the increase in amyloid deposition, the plasma levels of ApoA-II will gradually decrease upon tissue deposition [31]. As shown in Fig. 2a, plasma levels of ApoA-II in the A-Cur group decreased significantly compared with those in the Cur group, while the decline in the A-NT group was mild compared with that in the Con group. These different degrees of decline resulted in a narrowing difference between the A-NT and A-Cur groups. ApoA-I plasma levels showed a slight increase with curcumin supplementation, but were not significant (Fig. 2b). Consistent with previous results [23], ApoE, which is the most abundant co-deposited protein in AApAII amyloidosis, was significantly increased in the amyloid-induced groups, but was not significantly affected by curcumin (Fig. 2c).

Because ApoA-II, ApoA-I, and ApoE are all constituent proteins of HDL and are related to lipid metabolism, we evaluated the change in lipid metabolism by detecting plasma levels of total cholesterol, HDL cholesterol and triglycerides. Results suggest that curcumin supplementation increased HDL cholesterol levels, but reduced triglycerides levels (Fig. 2e-g), which is consistent with previous experiments of curcumin supplementation. Similar results were also shown in lipid metabolism studies, in which hypolipidemic agents, namely fibrates, were used as PPARα agonists [32, 33].

Further, immunoblotting results of ApoA-I and ApoE in the 8-week group are consistent with those of the 12-week group, but the change in ApoA-II protein did not show a significant difference. These results indicate that the increase in ApoAII levels may have undergone a slow process to adapt to the changes in lipid metabolism (Supplementary Fig. 7).
RNA sequence analysis showed that curcumin regulates many lipid metabolism-related genes via the PPAR signaling pathway, especially by activating PPARα in the liver. Although it has been suggested that curcumin inhibits amyloid fibril formation by binding to amyloid proteins and maintaining protein homeostasis, our results showed that curcumin may also affect the degree of amyloid deposition by other means. Several physiological activities of curcumin are related to the activation of PPARγ, including decreased insulin resistance, anti-inflammatory, and anti-cancer activities [15, 16, 34, 35]. However, ApoAII expression is thought to be upregulated upon activation of PPARα to enhance the delivery of lipids from the periphery tissue to the liver [36, 37]. To identify possible signaling pathways or target proteins that respond to curcumin, we performed a comprehensive analysis of mRNA transcription in the liver using the RNA sequence method. As shown in the Venn diagram (Fig. 3a), 75 genes are changed in mRNA expression by curcumin supplementation regardless of induction of amyloidosis (Supplementary Table 2). The enrichment pathway analysis based on the KEGG database suggested that differentially expressed genes (DEGs) are mainly distributed in lipid metabolism-related signaling pathways centered on the PPAR pathway (Fig. 3b). Analysis of the
non-amyloid induced groups separately showed that 98 genes were upregulated and 93 genes were down-regulated in the Cur group compared with the Con group. Among the 15 genes that were most significantly upregulated, more than 2/3 were related to fatty acid transport and fatty acid synthesis.

**Figure 3**

![Venn diagram](image)

**Table 1**

<table>
<thead>
<tr>
<th>Rank</th>
<th>Term</th>
<th>Sample number/Background</th>
<th>Corrected P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PPAR signaling pathway</td>
<td>17/82</td>
<td>1.22e-10</td>
</tr>
<tr>
<td>2</td>
<td>Retinol metabolism</td>
<td>17/88</td>
<td>1.65e-10</td>
</tr>
<tr>
<td>3</td>
<td>Fatty acid degradation</td>
<td>10/49</td>
<td>2.64e-06</td>
</tr>
<tr>
<td>4</td>
<td>Metabolic pathways</td>
<td>53/1256</td>
<td>2.64e-06</td>
</tr>
<tr>
<td>5</td>
<td>Chemical carcinogenesis</td>
<td>12/02</td>
<td>5.92e-06</td>
</tr>
<tr>
<td>6</td>
<td>Steroid hormone biosynthesis</td>
<td>11/06</td>
<td>1.92e-05</td>
</tr>
<tr>
<td>7</td>
<td>Desynthesis of unsaturated fatty acids</td>
<td>6/25</td>
<td>2.57e-04</td>
</tr>
<tr>
<td>8</td>
<td>Peroxisome</td>
<td>9/81</td>
<td>4.16e-04</td>
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</table>

Figure 3. RNA sequence analysis showed that curcumin regulates many lipid metabolism-related genes via the PPAR signaling pathway.

(a) The Venn diagram shows that 75 genes are changed in the liver by supplementation with curcumin for 12 weeks. (b) Enrichment pathway analysis based on the KEGG database. (c-d) The Volcano plot diagram shows that DEGs affected by curcumin are related to lipid metabolism and the PPARa pathway. (e) Regulated genes were identified by real-time qPCR. Histograms show fold changes relative to the Con group. Data are mean ± SD. The Tukey-Kramer method was used for multiple comparisons of gene changes; *P < 0.05, **P < 0.01, ***P < 0.001.
acid oxidation (Fig. 3c). Even considering the possible interference of AApoAll amyloidosis on gene expression in amyloid-induced groups, most of these genes had still been promoted in the A-Cur group (Fig. 3d).

As a transcription factor, PPARα is a major regulator of lipid metabolism in the liver. When the body is in an energy-deprived state, activated PPARα can mobilize fatty acids to the liver and promote fatty acid β oxidation to produce energy by upregulation of genes involved in fatty acid transport, fatty acid binding, and peroxisomal and mitochondrial fatty acid β-oxidation [18-20]. During lipid metabolism, the physiological function of PPARγ mainly involves synthesis and elongation of fatty acids and the differentiation of adipocytes for energy storage [19, 20, 34]. Upon analysis of the DEGs (Supplementary Table 2), we found that most of the genes were related to fatty acid oxidation, which suggests that curcumin regulates gene transcription by activated PPARα in the liver.

We further confirmed the elevated mRNA expression levels of Ppara, Apoa2, Apoa1 and some genes related to fatty acid metabolism in curcumin-supplemented groups by real-time qPCR (Fig. 3e). Although Pparγ mRNA expression was confirmed to be upregulated by curcumin (Supplementary Fig. 8), its levels were much lower than Ppara in the liver (data not shown).

These results suggest that curcumin is a PPARα/γ dual activator, and that the various physiological activities of curcumin may be derived from the complex regulation of PPARα and PPARγ. Because expression of PPARα and PPARγ varies largely in different tissues and organs, curcumin exhibits diverse physiological activities in different studies depending on the organs that are evaluated [19-21], while Ppara should be the major target of curcumin owing to its abundance in mouse liver.

PPARα levels in the liver were increased and showed more intranuclear localization in mice supplemented with curcumin.

As a transcription factor, activated PPARα transferred to the nucleus and formed heterodimers with retinoid X receptor (RXR). The heterodimers bind to the peroxisome proliferator response element (PPRE), a specific DNA sequence present in the promoter region of PPAR-regulated genes [17]. To elucidate how curcumin affects PPARα, we evaluated intracellular localization and PPARα levels in the liver. Compared with mice without curcumin supplementation, the fluorescence signal of PPARα in the Cur and A-Cur groups was concentrated in the nucleus, resulting in a higher signal intensity (Fig. 4a). Moreover, as shown in Fig. 4b, PPARα protein levels were increased in curcumin-supplemented mice, which is consistent with real-time qPCR results. These results confirm that the synthesis and activation of PPARα are promoted by curcumin and also explain the DEGs related to lipid metabolism in the liver.
Curcumin induced a higher abundance of peroxisomes and elevated a variety of peroxisome proteins downstream of PPARα.

Proliferation of peroxisomes provides additional evidence that PPARα is a target protein of curcumin. The mitochondria and peroxisomes are the most important organelles responsible for fatty acid oxidation. However, very long chain fatty acids (VLCFAs) exhibiting >22 carbons are too long to be metabolized in the mitochondria, and must be metabolized in peroxisomes [38]. Activated PPARα is known to promote peroxisomes in mice, and increase levels of fatty acid oxidation [38-40]. We noticed that peroxisome is ranked 8th in the analysis of enrichment pathways (Fig. 3b). Many enzymes related to fatty acid ß oxidation that are located in peroxisomes, including Acox, Acaa1, Ehhadh, Crat, Crot, are known to be upregulated (Supplementary Table 2). Another important upregulated protein is Pex11, a protein that regulates peroxisome division to increase peroxisome abundance [41, 42].

Catalase is one of the most important enzymes involved in protecting the cell from oxidative damage by catalyzing the decomposition of hydrogen peroxide. Due to localization in peroxisomes, it is usually used as a marker for peroxisomes [43, 44]. We detected catalase in the liver by immunohistochemistry (IHC) and Western immunoblotting to confirm peroxisome
abundance. Results obtained for catalase by IHC and immunoblotting suggest a higher abundance of peroxisomes after curcumin supplementation (Figs. 5a-b). The overexpression of catalase also reflects an increase in fatty acid metabolic activity and oxidative stress in hepatocytes.

**Figure 5**

<table>
<thead>
<tr>
<th>#</th>
<th>Identified Proteins (369)</th>
<th>Gene Name</th>
<th>Con</th>
<th>Cur</th>
<th>A-NT</th>
<th>A-Cur</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Peroxosomal bifunctional enzyme</td>
<td>Ehhadh</td>
<td>123</td>
<td>581</td>
<td>107</td>
<td>577</td>
</tr>
<tr>
<td>2</td>
<td>Carbamoyl-phosphate synthase</td>
<td>Cps1</td>
<td>154</td>
<td>190</td>
<td>225</td>
<td>169</td>
</tr>
<tr>
<td>3</td>
<td>Sterotransferin</td>
<td>Tf</td>
<td>151</td>
<td>121</td>
<td>163</td>
<td>134</td>
</tr>
<tr>
<td>4</td>
<td>Peroxosomal multifunctional enzyme type 2</td>
<td>Hsd17b4</td>
<td>58</td>
<td>81</td>
<td>56</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>Long-chain-fatty-acid–CoA ligase 1</td>
<td>Acs1</td>
<td>39</td>
<td>53</td>
<td>44</td>
<td>64</td>
</tr>
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</table>

In addition, we unexpectedly found that the protein band observed at a molecular weight of 75 kD is significantly increased in SDS PAGE of the liver extracts stained by Coomassie brilliant blue (Fig. 5c). We analyzed the proteins in this band by proteomic LC-MS/MS analysis and determined that it was comprised of several peroxisomal proteins related to fatty acid oxidation, including Ehhadh, Hsd17b4 and Acs1 (Supplementary Table 3). These results demonstrate that curcumin regulates peroxisome abundance via PPARα activation.
Discussion:

In previous studies, curcumin was found to exert physiological activities involved in the regulation of several transcription factors (PPARr, NFκB, AP-1, STAT, etc.) and their signaling pathways [15, 16]. In the present experiment, we found that the gene expression changes in mouse liver after curcumin supplementation are centered on the PPAR signaling pathway. In the enrichment pathway analysis, most of the DEGs involved in retinol metabolism, metabolic pathways, fatty acid degradation and the peroxisome pathway are also downstream of PPARα.

Until now, research on curcumin has mainly focused on the activation of PPARr, and it has been demonstrated that curcumin participates in glucose and lipid metabolism, inflammatory cytokine production, and inhibiting tumor cell proliferation via PPARr [16]. Although some studies have found that PPARα expression is increased after curcumin intake [45, 46], there is currently a lack of further experimental results revealing any connection between curcumin and PPARα pathway activation. Our results complement the theoretical system suggesting that curcumin regulates the transcription of many genes in the liver depending centrally on PPARα activation and broadly affects fatty acid transport and catabolism. These changes in genes and proteins levels may be involved, in which curcumin regulates the occurrence and development of various diseases or phenotypes, such as amyloidosis, changes in HDL and triglycerides, oxidative stress, peroxisome proliferation and hepatocyte hypertrophy, etc. (Fig. 6).

**Figure 6**

Figure 6. Schematic diagram of the effect of curcumin mediated by PPARα in mouse liver. Curcumin regulates gene expression via PPARα activation and exhibits biological activities involved in amyloidosis, peroxisome proliferation, lipid metabolism, and hepatocyte hypertrophy.
Due to the unexpected promotion of AApoAII amyloid deposition by curcumin in this study, we suspect that the anti-amyloid effect of curcumin is not applicable to all types of amyloidosis. To better understand the findings of the present study, we suggest that the effects of curcumin on progression of amyloidosis should be divided into two aspects, one of which is a pro-amyloidosis effect. In this experiment, the plasma concentration of ApoA-II increased almost three-fold (Fig. 2a) via PPARα pathway activation. Levels of amyloid protein are the most important factor for progression of amyloidosis, and suppression of amyloid protein levels is the main target for treatment of various systemic amyloidosis, including AA, AL, ATTR and dialysis-related Aβ3M amyloidosis [2, 3, 27, 47, 48]. In ApoA-II transgenic mice, the serum concentration of ApoA-II increased two-fold and AApoAII amyloid deposition was notably accelerated [30]. However, calorie restriction (diet) decreased the ApoA-II/ApoA-I ratio in serum and suppressed amyloidosis [49]. Thus, we believe that the pro-amyloid effect of curcumin observed here is mainly caused by increased ApoA-II levels.

The other aspect is the anti-amyloid effect of curcumin. Curcumin has shown general affinity for the amyloid protein structure and it has been demonstrated that this binding is efficient to maintain stability of the amyloid protein and inhibit the formation of insoluble amyloid fibrils [4, 9, 10]. In addition to direct binding to amyloid proteins, it has also been shown that curcumin activates autophagy and macrophages in some studies to reduce the dysfunction caused by amyloid proteins [50, 51]. Moreover, it was reported that some molecules elevate lysosome biosynthesis via activation of PPARα and accelerate the clearance of amyloid proteins and protein aggregates [52, 53]. Compared with previous data, we noticed that amyloid deposition in Apoa2-/- transgenic mice with twice the serum concentration of ApoA-II is more severe than in those with greater ApoA-II concentrations in this study [30]. In transgenic mice injected with 1 μg AApoAII fibrils for 8 and 12 weeks, the average amyloid score in the liver and spleen at 12 weeks and the amyloid index at 8 weeks was 3.4, 4 and 3, respectively, compared with values of 2, 3.4 and 1.5, respectively, in curcumin-supplemented mice. We believe that this difference demonstrates that curcumin may also exert a certain anti-amyloid ability in this experiment, but it is difficult to evaluate and further analyses should be carried out in the future.

Curcumin interacts with various cellular metabolic pathways by activating PPARs. Most biological process, such as metabolic control and defect, involve complex molecular interactions and are regulated via various signaling pathways. When amyloid proteins play functional roles in certain metabolic pathways, progression of amyloidosis may be accelerated or decelerated via treatments that modulate such metabolic pathways. As ApoA-II interacts particularly with lipid metabolism, it is likely that amyloid deposition was increased by metabolic changes caused by curcumin, including acceleration of β-oxidation of fatty acids, reduction of lipogenesis and increased synthesis of apolipoproteins.
In addition, we think the peroxisome proliferation is the most important factor that explains the hepatocyte and liver hypertrophy. There was no significant change in food intake between the four groups investigated in our experiments (Supplementary Fig. 9), and no significant lipid deposition was observed in the liver (Supplementary Fig. 3). Peroxisomes in liver parenchymal cells are very few, and represent <2% of cytoplasmic volume under physiological conditions and contribute about 35% of the $\text{H}_2\text{O}_2$-production levels. However, peroxisomes in the liver in the presence of peroxisome agonists may occupy as much as 25% of the cytoplasmic volume [54]. In previous studies, long-term administration of PPARα agonists was shown to induce disordered peroxisome proliferation, liver hypertrophy and liver tumors in mice [55]. In our experiments, overexpression of catalase and peroxisomal proteins suggests that curcumin promotes peroxisome proliferation mediated by PPARα. The hypertrophic changes in the hepatocyte nucleus we observed in histological sections are consistent with the early pathological changes of hepatocyte heterogeneity.

Peroxisomes are a conserved organelle and play a key role in lipid metabolism and redox homeostasis in both plants and mammals [54, 56, 57]. According to the proliferation mode of peroxisomes, the process of peroxisome fission is a three-step process involving peroxisome elongation, constriction, and scission. Pex11 is essential for this process and its overexpression causes peroxisome proliferation, while its deletion causes a decrease in the number of peroxisomes [58]. Overexpression of Pex11 in curcumin-supplemented mice suggests that Pex11 may play a key role in peroxisome proliferation mediated by PPARα and curcumin. Moreover, proteins such as Acsl1, Acaa1, and Ehhadh upregulated by curcumin in this experiment are localized in the peroxisome matrix and may be involved in this process.

A hallmark of eukaryotic cells is the presence of membrane-bound organelles, such as endoplasmic reticulum (ER), mitochondria, and peroxisomes. Such distinct compartments create special micro-environments for more efficient metabolic reactions. To coordinate complex metabolic processes and signal transduction, there are functional interplays between various organelles. Due to the central metabolic role, it was shown that peroxisomes interact with many organelles involved in cellular lipid metabolism, such as the ER, mitochondria, lysosomes and lipid droplets [59-61]. There is also functional interplay between peroxisomes and the nucleus, which may also involve signaling via $\text{H}_2\text{O}_2$ [62, 63].

Other means by which peroxisome proliferation affects ER or mitochondrial functions are also known. However, the mechanism by which curcumin activates PPARs is not yet clear. There are two means of activating PPARs, namely ligand-dependent and ligand-independent. In the ligand-dependent manner, the molecular shape of PPARs is modified by ligand binding in the cytoplasm, and PPARs enter into the nucleus. In a ligand-independent manner, PPARs can be phosphorylated by protein kinases to induce a structural change of phosphorylated PPARs, even
in the absence of ligands [20-22]. Further experiments are needed to confirm whether curcumin directly binds and activates PPARs as an exogenous ligand, or whether activation involves a ligand-independent pathway.

PPARα and PPARγ involve different aspects of metabolic pathways, such as decomposition or storage of fatty acids, fatty acid-based energy production or glucose-based energy production. PPARα agonists (e.g., fibrates) or PPARγ agonists (e.g., thiazolidinedione) play important roles in the treatment of hyperlipidemia and type 2 diabetes in the clinic. PPARα/γ dual agonists are also under development to treat more complex metabolic diseases, but some exhibit side effects and cause liver or cardiac dysfunction [64, 65]. In clinical trials, it has been demonstrated that supplementation with curcumin at a high dose is safe in humans [66, 67]. Improving the molecular structure of drugs based on that of curcumin offers the possibility to produce dual or specific agonists without side effects.

Taken together, our results demonstrate the novel agonistic effect of curcumin on PPARα. We identified specific effects of curcumin on mice, including promotion of AApoAII amyloidosis and peroxisome proliferation. Curcumin is involved in various physiological activities mediated by PPAR activation, leading to regulation of genes participating in the PPAR pathway. The beneficial use of curcumin based on these particular abilities requires further consideration. The development of derivative agents based on curcumin with high bioavailability or specific effects may have far-reaching significance for the treatment of diseases such as amyloidosis, hyperlipidemia, type 2 diabetes and other metabolic disorders.

**Materials and Methods:**

**Animals & Drug Administration**

R1.P1-Apoa2c congenic mice were used in this study, which carry the amyloidogenic type c allele (Apoa2c) of amyloidosis-susceptible SAMP1 strain on a genetic background of the SAMR1 strain. R1.P1-Apoa2c mice exhibit a normal aging process and develop accelerated AApoAII amyloidosis by oral or intravenous administration of AApoAII fibrils [24]. Mice were maintained under SPF conditions at 24 ± 2°C with a light-controlled regimen (12-hour light/dark cycle) in the Division of Animal Research, Research Center for Supports to Advanced Science, Shinshu University. The mice were fed a commercial diet (Con group and A-NT group) or curcumin diet (Cur group and A-Cur group) and tap water ad libitum. The commercial diet is a MF diet (Oriental Yeast, Tokyo, Japan) and the curcumin diet is the MF diet supplemented with 0.5% or 2% w/w curcumin (Wako, Osaka, Japan).

Three to 5 R1.P1-Apoa2c congenic mice were housed in a single cage. Female mice were used for experiments to avoid the anticipated adverse impacts due to fighting among male mice. Mice were sacrificed by cardiac puncture under deep sevoflurane anesthesia after 8 weeks and 12
weeks of curcumin intake. Plasma and half of the major organs (heart, liver, spleen, stomach, small intestine, tongue, skin, lung and kidney) were snap-frozen by liquid nitrogen and stored at -80°C for biochemical analysis. The remaining organs were fixed in 10% neutral buffered formalin followed by embedding in paraffin for histochemical analysis. All experiments were approved by the Committee for Animal Experiments of Shinshu University (Approval No. 280086).

**Induction of AApoAII amyloidosis**

AApoAII amyloid fibrils were isolated using Pras’ method [68] from the livers of R1.P1-Apoa2c mice having severe amyloid deposits. Mice in the amyloid-induced groups were injected with 1 μg amyloid fibrils into the tail vein for induction of AApoAII amyloidosis at 8 weeks of age. AApoAII fibrils were sonicated before use and the injection was performed immediately.

**Evaluation of amyloid deposition**

Amyloid deposits were detected in paraffin organ sections stained with a saturated solution of 1% Congo red dye. An amyloid score (from 0 to 4) in each organ was determined semi-quantitatively as described previously [69] under polarizing light microscopy (LM) (Axioskop 2, Carl Zeiss, Tokyo, Japan). Two observers, with no information of the Congo red stained tissues, graded the degree of amyloid deposition in each mouse, separately. The degree of amyloid deposition in each mouse was represented by an amyloid index (AI), which is the average of the amyloid scores in seven organs (heart, liver, spleen, stomach, small intestine, tongue, and skin).

**Hepatocyte size in each group**

To analyze the hepatocyte size in each mouse quantitatively, we captured 5 images of each section selected randomly at 400× magnification and determined the average hepatocyte size in each image by calculating the total area divided by the cell counts using an image processing program (NIH ImageJ software, version 1.61). We determined the average hepatocyte size of each mouse and then performed a statistical analysis between all four groups.

**Immunohistochemistry and immunofluorescence analysis**

We detected AApoAII deposition and catalase by immunohistochemistry (IHC) following a previously described method [49]. Antiserum against mouse ApoA-II was produced against guanidine hydrochloride-denatured AApoAII in our laboratory [70] and applied at a dilution ratio of 1:3000. Catalase antibody was applied (1:500, GTX110704, GeneTex Inc., CA, USA) to reveal the degree of peroxisome change in the liver. After incubation overnight at 4°C with the
primary antibody, the sections were incubated with the biotinylated secondary antibody (1:300, DAKO, Glostrup, Denmark) for 1 h at room temperature. Target proteins were identified by the horseradish peroxidase-labeled streptavidin-biotin method (1:300, DAKO). In the immunofluorescence experiments, the sections were incubated with the PPARα antibody (1:500, GTX101098, GeneTex Inc., CA, USA) overnight and incubated with Alexa Fluor™ 488 goat anti-rabbit antibody (1:500, Thermo Fisher Scientific, Japan) for 1 h at room temperature and incubated with DAPI for 10 min. Images were captured immediately using a confocal laser fluorescence microscope (LSM 880 with Airyscan, Carl Zeiss, Germany). In a negative control section, the primary antibody was omitted to confirm the specificity of staining.

**Lipid metabolism analysis**

Lipid metabolism levels were determined using quantitative assay kits by means of HDL cholesterol, total cholesterol and triglycerides concentrations in the plasma with the instructions provided by the manufacturer (HDL-cholesterol E test, 431-52501; Total-cholesterol E test, 439-17501; TG E test, 432-40201, FUJI FILM Wako, Osaka, Japan).

**AST and ALT detection**

Two hundred microliters (200 μL) of frozen mouse plasma per mouse was sent to Nagahama life science laboratory (Oriental Yeast, Tokyo, Japan) for determination of AST and ALT levels. The laboratory provided a test report.

**Immunoblot analysis**

We measured proteins levels by western blotting as described previously [30, 49]. To determine plasma levels of ApoA-II, ApoA-I, ApoE, 0.5 μL samples from each mouse were separated by Tris-Tricine/SDS–16.5% or 15% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, 0.2 μm pore, Millipore Corp., MA, USA) and incubated overnight at 4°C with primary antibody solution containing polyclonal rabbit anti-mouse ApoA-II antiserum (diluted 1:3000) or the ApoA-I antiserum (diluted 1:4000) produced in our laboratory, or ApoE antibody (1:500, Santa Cruz, San Francisco, CA, USA). Next, horseradish peroxidase-conjugated anti-rabbit IgG (Code #7074, Cell Signaling Technology Inc., Danvers MA, USA) (1:3000) was used for 1-h incubation at room temperature and target proteins were detected by the enhanced chemiluminescence (ECL) method. Thirty micrograms (30 μg) of liver lysates were separated on Tris-Tricine/SDS–12% PAGE to determine levels of PPARα (1:3000, GTX101098, GeneTex Inc.), β-actin (1:3000, GTX110564, GeneTex Inc.) and catalase (1:3000, GTX110704, GeneTex Inc., CA, USA). Target protein levels were analyzed using the NIH ImageJ software.
RNA sequence analysis

We selected liver samples from the 12-week group for RNA sequence analysis, which exhibit more obvious amyloid deposition in amyloid-induced animals and no abnormal changes in AST and ALT levels. Ten milligrams (10 mg) of each mouse liver stored at -80°C was homogenized in TRIzol RNA isolation reagent (Invitogen-Thermo Fisher, Tokyo Japan) and pooled into 4 sample tubes (Con, Cur, A-NT and A-Cur groups, N = 3-5), and the samples were sent to Filgen (Nagoya, Japan). Total RNA was extracted and the RNA purity and integrity were confirmed using a Bioanalyzer 2100 system (Agilent Technology, Santa Clara, CA USA). mRNA sequencing analysis was performed with an Illumina next generation sequencing platform. Sequencing count data were analyzed using the DESeq2 software to determine the significant DEGs among the different groups. Biological functions into which DEGs accumulated were analyzed using an annotation database (Gene Ontology and KEGG Pathway Database) to elucidate the mechanism of the effects of curcumin supplementation.

Gene expression analysis

We followed a previously described method to confirm mRNA expression levels [71]. Quantitative real-time qPCR analysis was carried out using an ABI PRISM 7500 Sequence Detection system (Applied Biosystems, New York, USA) with SYBR Green (TaKaRa Bio, Tokyo, Japan). The β-actin gene was used to normalize gene expression. The forward and reverse primer sequences for real-time PCR are listed in Supplementary Table 4. Chemical reagents used in the experiments, unless otherwise specified, were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Nano-flow liquid chromatography-ion trap mass spectrometry (LC-MS/MS)

Thirty micrograms (30 μg) of liver lysates were separated on Tris-Tricine/SDS–12% PAGE and stained with Coomassie brilliant blue for 20 min. The stained bands near 75 kD were excised and soaked in 50 mM Tris-HCl, pH 8.0, containing 50% acetonitrile for 30 min. The gel was dried in a Speed-Vac (Savant) and incubated in 50 mM triethylammonium bicarbonate containing proteomics grade trypsin (Sigma-Aldrich, Tokyo, Japan) at 37°C for 20 h. The digests were extracted from the gel with 100-200 μl of 0.1% TFA containing 60% acetonitrile. These extracts were evaporated in a Speed-Vac and stored at −80°C until assayed. Samples were resuspended in 0.1% formic acid and introduced into a nano-flow HPLC system, EASY-nLC 1200 (Thermo Fisher Scientific Inc., Waltham, MA, USA). A packed nano-capillary column, NTCC-360/75-3-123 (0.075 mm I.D. × 125 mm L, particle diameter 3 μm, Nikkyo Technos Co., Ltd., Tokyo, Japan), was used at a flow rate of 300 nl / min with a 2–80% linear
gradient of acetonitrile for 80 min. Eluted peptides were directly detected with an ion trap mass spectrometer (QExactive HF; Thermo Fisher Scientific Inc., Waltham, MA, USA). For ionization, a spray voltage of 2.0 kV and capillary temperature of 250°C was used. The mass acquisition method consisted of one full MS survey scan with an Orbitrap resolution of 60,000, followed by an MS/MS scan of the most abundant precursor ions from the survey scan with an Orbitrap resolution of 15,000. Dynamic exclusion for the MS/MS was set to 30 sec. An MS scan range of 350–1800 m/z was employed in the positive ion mode, followed by data-dependent MS/MS using the HCD operating mode on the top 15 ions in order of abundance. The data were analyzed with Proteome Discoverer (Thermo Fisher Scientific Inc., Waltham, MA, USA), Mascot software (Matrix Science Inc., Boston, MA, USA) and Scaffold software (Proteome Software, Inc., Oregon, USA). Swissprot and GenBank databases were used.

Statistical analyses
For comparison of parametrical data, one-way analysis of variance (ANOVA) with Tukey’s test was performed using the SPSS 26.0 software package (Abacus Concepts, Berkley, CA USA). For comparison of nonparametric data, the Kruskal-Wallis test with the Steel-Dwass test was performed. P values <0.05 were considered to be statistically significant.

Data Availability
The data used to support the findings of this study are included within the article. All other data supporting the findings of this study will be made available upon reasonable request to the corresponding authors.

Conflicts of Interest
The authors declare that they have no competing interests.

Authors’ Contributions
J.D. and K.H. conceived and designed the experiments. J.D., Y.L., F.K., X.C., Y.I and J.H. performed the experiments and were responsible for data acquisition and analysis. J.D., H.M., and K.H. analyzed the data. M.M. interpreted the data and experimental methods. J.D. and K.H. wrote the manuscript. All authors reviewed the manuscript and approved the manuscript for publication.

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Supplementary Data

Supplementary Figure 1

Non-amyloid induced groups

Distilled water

Con

Cur
(Curcumin diet)

R1.P1-Apoa2c congenic mice
(female, 8-week-old)

Curcumin diet

Amyloid induced groups

AApoAII fibrils
1 μg/mouse

A-NT
(Induction)

A-Cur
(Induction + Cur)

R1.P1-Apoa2c congenic mice
(female, 8-week-old)

Con

Cur

MF diet

2% w/w Cur diet

8 weeks

12 weeks

analysis

Supplementary Figure 1. Experimental design

Eight-week-old female R1.P1-Apoa2c mice were divided into 4 groups: control (Con) group, curcumin (Cur) group, amyloid and no-treatment (A-NT) group, and amyloid and curcumin (A-Cur) group. The A-NT and A-Cur groups were injected with 1 μg / mouse of AApoAII fibrils to induce amyloidosis, and the Con and Cur groups were injected with distilled water instead of amyloid fibrils. Mice in the Con and A-NT groups were fed a commercial diet from 8 weeks of age, while mice in the Cur and A-Cur groups were fed a 2% w/w curcumin diet. Mice were sacrificed for biochemical analysis and histochemical analysis after 8 weeks or 12 weeks (N = 3 - 5 in each group).
Supplementary Figure 2

(a) Amyloid Score

Liver  Spleen

(b) Amyloid Score

Heart  Stomach  Intestine  Tongue  Skin  Lung  Kidney

(c) Amyloid Score

Liver  Spleen
### Supplementary Figure 2. Amyloid score in various organs

(a) Amyloid deposition in the liver and spleen is increased by supplementation with curcumin for 8 weeks. (b) Amyloid score in other organs (heart, stomach, intestine, tongue, skin, lung, kidney) in the 8-week group. (c) Amyloid deposition in the liver and spleen is increased by supplementation with curcumin for 12 weeks. (d) Amyloid score in other organs (heart, stomach, intestine, tongue, skin, lung, kidney) in the 12-week group. Each dot represents an individual mouse. The Kruskal-Wallis test with the Steel-Dwass test was used for comparisons of the amyloid score; *P < 0.05.

### Supplementary Table 1 Liver and Body Weight

<table>
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<th>2% w/w Cur exp.</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Liver/body weight</th>
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<td><strong>Con</strong> (8-week group)</td>
<td>27.178 ± 2.604</td>
<td>1.249 ± 0.085</td>
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<td><strong>Cur</strong> (8-week group)</td>
<td>25.965 ± 1.107</td>
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<td><strong>A-NT</strong> (8-week group)</td>
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<td><strong>A-Cur</strong> (8-week group)</td>
<td>26.804 ± 1.728</td>
<td>1.458 ± 0.124</td>
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<td><strong>Con</strong> (12-week group)</td>
<td>25.044 ± 1.729</td>
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<td>0.0455 ± 0.003</td>
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<tr>
<td><strong>Cur</strong> (12-week group)</td>
<td>23.570 ± 0.182</td>
<td>1.443 ± 0.161</td>
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<td><strong>A-NT</strong> (12-week group)</td>
<td>24.878 ± 2.044</td>
<td>1.217 ± 0.147</td>
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<td><strong>A-Cur</strong> (12-week group)</td>
<td>26.295 ± 2.881</td>
<td>1.872 ± 0.162</td>
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Supplementary Figure 3

(a) Representative HE stained image of hepatocytes. Large nuclei (black arrows) were identified in curcumin intake groups. Each scale bar indicates 20 μm. (b) Average hepatocyte size in each group. Data represent the mean ± SEM. N = 3 - 5. The Tukey-Kramer method was used for multiple comparisons of hepatocyte size; ** P < 0.001.

Supplementary Figure 3. Hepatocyte hypertrophy in curcumin intake groups
Supplementary Figure 4. No differences in AST and ALT plasma concentrations after curcumin intake. AST and ALT plasma levels were detected to evaluate hepatocyte injury. (a) AST and ALT concentrations in the 8-week group. (b) AST and ALT plasma concentrations in the 12-week group. Data are mean ± SD. N = 3 - 5. The Tukey-Kramer method was used for multiple comparisons; *P < 0.05.

Supplementary Figure 5. Inflammation-related gene expression in the liver in the 12-week group. Representative genes related to inflammation were measured by real-time qPCR. Results show that curcumin does not affect the expression of inflammatory genes, but Tnfα was increased in the A-Cur group, in which mice exhibit severe amyloid deposition. Data are mean ± SD. N = 3 - 5. The Tukey-Kramer method was used for multiple comparisons; *P < 0.05.
Supplementary Figure 6

Degree of AApOAll amyloid deposition and liver weights were significantly increased after supplementation with low dose curcumin diet (0.5% w/w).

(a) Amyloid index (AI) in the 12-week group supplemented with 0.5% curcumin diet. Each dot represents an individual mouse. (b) ratio of liver weight / body weight in the 12-week group. Data are mean ± SD. N = 4 - 5. The Kruskal-Wallis test with the Steel-Dwass test was used for the amyloid index, and the Tukey-Kramer method was used for multiple comparisons of liver weights; *P < 0.05, **P < 0.01.

Supplementary Figure 7

ApoA-II, ApoA-I and ApoE plasma levels after supplementation with 2% w/w curcumin diet for 8 weeks.

(a-c) Plasma levels of ApoA-II, ApoA-I, and ApoE proteins were determined by Western immunoblot. There were no obvious changes in ApoA-II and ApoA-I plasma concentrations; the concentration of ApoE increased significantly in the amyloid-induced groups. Histograms show fold changes relative to the Con group and represent the means ± S.D. P indicates the pooled plasma of female R1.P1-Apoa2c mice at 2 months of age (N = 4) that did not have AAapoAll amyloid deposits, as the positive control of these proteins. N = 3 - 5. The Tukey-Kramer method was used for multiple comparisons; *P < 0.05, **P < 0.01.
Supplementary Figure 8

Pparγ expression in the liver is upregulated in curcumin intake groups. Histograms show fold changes relative to the Con group. Data are mean ± SD. The Tukey-Kramer method was used for multiple comparisons; ***P < 0.001.

Supplementary Figure 9
Supplementary Figure 9. Weekly body weight and food intake measurements. (a) Body weight and (b) food intake in the 8-week group. Each column and bar represent the mean ± S.D. (N = 3 - 5). (c) Body weight and (d) food intake in the 12-week group. Data are represented in g/mouse · day (food intake).
**Supplementary Table 4. Specific primers used in real-time qPCR.**

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