Synthetic liver X receptor agonist T0901317 inhibits semicarbazide-sensitive amine oxidase gene expression and activity in apolipoprotein E knockout mice

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Semicarbazide-sensitive amine oxidase (SSAO) catalyzes oxidative deamination of primary aromatic and aliphatic amines. Increased SSAO activity has been found in atherosclerosis and diabetes mellitus. We hypothesize that the anti-atherogenic effect of liver X receptors (LXRs) might be related to the inhibition of SSAO gene expression and its activity. In this study, we investigated the effect of LXR agonist T0901317 on SSAO gene expression and its activity in apolipoprotein E knockout (apoE−/−) mice. Male apoE−/− mice (8 weeks old) were randomly divided into four groups: basal control group; vehicle group; prevention group; and treatment group. SSAO gene expression was analyzed by real-time quantitative polymerase chain reaction and its activity was determined. The activity of superoxide dismutase and content of malondialdehyde in the aorta and liver were also determined. In T0901317-treated mice, SSAO gene expression was significantly decreased in the aorta, liver, small intestine, and brain. SSAO activities in serum and in these tissues were also inhibited. The amount of superoxide dismutase in the aorta and liver of the prevention group and treatment group was significantly higher compared with the vehicle group (P <0.05). Malondialdehyde in the tissues of these two groups was significantly lower compared with the vehicle group (P <0.05). Our results showed that T0901317 inhibits SSAO gene expression and its activity in atherogenic apoE−/− mice. The atheroprotective effect of LXR agonist T0901317 is related to the inhibition of SSAO gene expression and its activity.

Keywords: semicarbazide-sensitive amine oxidase; nuclear receptor; liver X receptor; T0901317; atherosclerosis

Semicarbazide-sensitive amine oxidase (SSAO) is a kind of enzyme sensitive to semicarbazide that contains copper and quinone [1]. The enzyme is present in either soluble or membrane-bound form in the vascular system, such as blood vessels, retina, and brain microvessels, and adipocytes [2,3]. Methylamine [4,5] and aminoacetone [6,7] are readily deaminated by SSAO in vitro and in vivo, leading to production of toxic formaldehyde and methylglyoxal, respectively, as well as hydrogen peroxide and ammonia. These toxic products could be responsible, at least in part, for protein modification, oxidative stress, and vascular cell damage. Many clinical reports have further implicated that elevated levels of vascular adhesion protein-1/SSAO in several disease states were found in the circulating plasma. Such diseases include diabetes mellitus [8,9], atherosclerosis [10,11], heart failure [12,13], obesity [14], and inflammatory liver disease [15,16]. Karadi et al reported that serum SSAO activity might be a candidate biochemical marker of early atherosclerosis and diabetic microvascular complications [10].

Liver X receptors (LXRs) are members of the nuclear hormone receptor superfamily and activated by oxysterol ligands [17]. LXRs heterodimerize with retinoid X receptors. Synthetic LXR agonists promote cholesterol efflux [18], inhibit inflammation in vivo [19], and inhibit the development of atherosclerosis in animal models [20–22]. LXRs directly induce the expression of ATP-binding cassette transporter (ABC) A1, ABCG1 [23,24], and apolipoprotein E (apoE), which mediate cellular cholesterol export in the presence of acceptors, such as high-density lipoprotein. Our previous studies have shown that oxidized low density lipoprotein, apoA-I, increased ABCA1 mRNA and protein expression in THP-1 macrophage-derived foam cells and promoted cholesterol efflux from these cells, whereas oleate reduced cholesterol efflux and the level of ABCA1 protein in THP-1 macrophage-derived...
foam cells [25–27]. In contrast, LXR agonists negatively regulate macrophage inflammatory gene expression [19], and recent data indicated that pathogens that contribute to the initiation and progression of atherosclerosis interfere with macrophage cholesterol metabolism by inhibition of the LXR signaling pathway [28]. The ability of LXRs to integrate metabolic and inflammatory signaling makes them attractive targets for intervention in human metabolic disease, such as atherosclerosis and diabetes mellitus.

Increase in SSAO activity has been found in atherosclerosis [10,11], diabetes mellitus, and congestive heart failure. Yu et al suggested that blocking of SSAO activity with a selective inhibitor significantly reduced lipopoly saccharide-induced pulmonary inflammation [1]. It is well known that there is a strong relationship between atherosclerosis and oxidative damage [29–31]. According to our hypothesis, the anti-atherogenic effect of LXRs might be related to the inhibition of SSAO gene expression and its activity. In the present study, we investigated the effect of LXR agonist T0901317 on SSAO gene expression and its activity in apoE knockout (apoE−/−) mice.

Materials and Methods

Materials

T0901317 was synthesized by Cayman Chemical (Ann Arbor, USA) and dissolved in vehicle (PEG400:Tween-80=4:1). RevertAid first-strand cDNA synthesis kit (#k1622) was purchased from Fermentas (Glen Burnie, USA). DyNamo SYBR green qPCR kit was obtained from Finnzymes (Espoo, Finland). [7-14C]-Benzyllamine hydrochloride (59 mCi/mM) was purchased from Amersham Life Science (Little Chalfont, UK).

Animals and diets

Eight-week-old apoE−/− male mice (purchased from the Laboratory Animal Center of Peking University, Beijing, China) were randomly divided into four groups: basal control group (n=10); vehicle group (n=14); prevention group (n=14); and treatment group (n=14). All of the mice were fed with a high-fat/high-cholesterol diet [15% fat (W/W); 0.25% cholesterol (W/W)]. Animals in the basal control group treated with vehicle were killed after 8 weeks to evaluate atherosclerotic lesions. The vehicle group and the prevention group were treated with either vehicle or LXR agonist T0901317 (10 mg/kg body weight) [21] daily by oral lavage (0.2 ml/mouse) for 14 weeks. The treatment group was treated with vehicle for 8 weeks, then treated with the agonist T0901317 for an additional 6 weeks (as described above). At week 14, the mice were killed, and blood was obtained and tissues collected for further analysis. All animal experiments were carried out in accordance with the Institutional Animal Ethics Committee and the University of South China (Hengyang, China) animal care guidelines for use of experimental animals.

RNA isolation and real-time quantitative polymerase chain reaction (PCR) analysis

Total RNA from aortas, livers, and intestines of apoE−/− mice was extracted using Trizol reagent (BBI, Kitchener, Canada). Briefly, tissue samples were homogenized in 1 ml Trizol reagent per 50–100 mg tissue, then incubated for 5 min at 25 ºC to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml chloroform per 1 ml Trizol reagent was added, and the samples were centrifuged at 12,000 g for 15 min at 4 ºC. The aqueous phase was transferred to a fresh tube, and the organic phase was saved. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. Samples were incubated at 25 ºC for 10 min and centrifuged at 12,000 g for 10 min at 4 ºC. The supernatant was removed. The RNA pellet was washed once with 75% ethanol, with at least 1 ml of 75% ethanol added per 1 ml Trizol reagent used for the initial homogenization. The sample was mixed by vortexing and centrifuged at 7500 g for 5 min at 4 ºC. At the end of the procedure, the RNA pellet was dried for 5–10 min. RNA was dissolved in RNase-free water by passing the solution a few times through a pipette tip, and incubated for 10 min at 60 ºC.

Real-time quantitative PCR, using SYBR green detection chemistry, was carried out on a 7900HT fast real-time PCR system (Applied Biosystems, Foster City, USA). The primers used for quantification of mouse SSAO mRNA levels were 5′-TGCACATCCCTCATGCGAAG-3′ (forward) and 5′-GGAGGAAGAAGCCCACTGAGT-3′ (reverse) [32]. The reaction was carried out in a 96-well plate in a 30 μl amplification system containing 13.5 μl deionized water, 15 μl Master Mix, 1 μl cDNA, and 0.5 μl primer. The thermal profile for reverse transcription-PCR was 42 ºC for 30 min and 95 ºC for 2 min, followed by 40 cycles of 95 ºC for 10 s and 60 ºC for 30 s, and annealing at 60 ºC for 20 s [33]. Melt curve analysis of all real-time PCR products was carried out and shown to produce a single DNA duplex. Quantitative measurements were determined using the ΔΔCt method and β-actin was used as the internal control.

Measurement of SSAO activity

The enzyme activity assay was carried out according to the procedure described by McCaman [34] with our own
T0901317 inhibits SSAO gene expression and activity modifications. Briefly, serum or 5% tissue homogenate (W/V) was incubated with clorgyline (10^{-6} M) and (-)-deprenyl (10^{-6} M) at 37 °C for 20 min to ensure that any monoamine oxidase activity was completely blocked. Aliquots of the enzyme preparation were then incubated with 50 μl [7-14C]-benzylamine (4×10^{-4} M, 1 μCi/ml) in a final volume of 200 μl in phosphate buffer (0.1 M, pH 7.4) at 37 °C for 30 min. The enzyme reaction was terminated by adding 250 μl of 2 M citric acid. The oxidized products were extracted into 1 ml toluene:ethyl acetate (1:1; V/V), of which 600 μl was transferred to a counting vial containing 10 ml ACS scintillation cocktail (Amersham Radiolabeled Chemicals, St. Louis, USA). Radioactivity was assessed in an FJ-2107P liquid scintillation counter (State-owned 262 Factory, Xi’an, China). Radioactivity was assessed in an FJ-2107P liquid scintillation counter (State-owned 262 Factory, Xi’an, China). Results are expressed as nanomoles of benzaldehyde per hour per milliliter for serum and nanomoles of benzaldehyde per minute per milligram of protein for tissue homogenate. Protein concentrations were determined by the Lowry method [35].

Measurement of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content

SOD activity and MDA content in the aorta and liver tissues were assayed by assay kit strictly according to the manufacturer’s instruction (Jiancheng Biotech, Nanjing, China) [36].

Statistical analysis
Data are expressed as the mean±SD. Results were analyzed by one-way ANOVA and Student’s t-test, using SPSS 13.0 software (SPSS, Chicago, USA). Statistical significance was obtained when P values were less than 0.05.

Results
LXR agonist T0901317 down-regulated SSAO gene expression in different tissues of apoE^{−/−} mice
To examine the effect of the LXR agonist T0901317 on SSAO gene expression in different tissues of apoE^{−/−} mice, we collected aorta, liver, small intestine, and brain of apoE^{−/−} mice fed with a high-fat/high-cholesterol diet to determine SSAO gene expression using real-time quantitative PCR. As shown in Fig. 1, SSAO gene expressions in these tissues were markedly inhibited by

Fig. 1 Semicarbazide-sensitive amine oxidase (SSAO) gene expression regulated by liver X receptor agonist T0901317 in different tissues from apolipoprotein E knockout mice fed a high-fat/high-cholesterol diet

Animals in the basal control group (n=10) were treated with vehicle and killed after 8 weeks. The vehicle group (n=14) and prevention group (n=14) were treated with vehicle or T0901317 (10 mg/kg body weight), respectively, daily by oral lavage (0.2 ml/mouse) for 14 weeks. The treatment group (n=14) was treated with vehicle for 8 weeks, then treated with T0901317 for 6 weeks. Mice in the latter three groups were killed at week 14. Gene expression was measured by real-time quantitative polymerase chain reaction assays. Data are presented as mRNA expression relative to the vehicle control. The results are expressed as the mean±SD from three independent experiments, and each carried out in triplicate. *P<0.05 versus vehicle; **P<0.01 versus vehicle.
T0901317 compared with vehicle group mice. It is interesting that the changes in SSAO gene expression in the aorta of prevention and treatment groups were the most significant, with expression down-regulated by 417.32% and 324.69%, respectively.

**LXR agonist T0901317 reduced SSAO activity in different tissues of apoE−/− mice**

To investigate whether T0901317-mediated changes in SSAO gene expression could result in corresponding changes in SSAO activity, we measured SSAO activities in the different tissues. Consistent with the data on gene expression, treatment with T0901317 resulted in decreased SSAO activities in serum (0.13±0.01 nmol/h·ml in prevention group or 0.18±0.01 nmol/h·ml in vehicle group versus 0.84±0.06 nmol/h·ml in vehicle group, *P*<0.01), aorta [0.21±0.02 nmol/(min·mg protein) in prevention group or 0.30±0.02 nmol/(min·mg protein) in vehicle group versus 0.80±0.07 nmol/(min·mg protein) in vehicle group, *P*<0.01], liver [0.20±0.02 nmol/(min·mg protein) in prevention group or 0.21±0.02 nmol/(min·mg protein) in treatment group versus 0.34±0.03 nmol/(min·mg protein) in vehicle group, *P*<0.05], small intestine [0.07±0.01 nmol/(min·mg protein) in prevention group or 0.09±0.01 nmol/(min·mg protein) in treatment group versus 0.21±0.02 nmol/(min·mg protein) in vehicle group, *P*<0.01], and brain [0.14±0.01 nmol/(min·mg protein) in prevention group or 0.22±0.02 nmol/(min·mg protein) in treatment group versus 0.46±0.04 nmol/(min·mg protein) in vehicle group, *P*<0.01] (Fig. 2). SSAO activity was most significantly inhibited in the aorta treated with T0901317, consistent with the data from real-time quantitative PCR analysis.

**Changes in SOD and MDA levels in aorta and liver**

The levels of SOD and MDA in the basal control group were not significantly changed, compared with those in the vehicle group. The level of SOD in the prevention group

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**Fig. 2** Semicarbazide-sensitive amine oxidase (SSAO) activity in serum and different tissues from apolipoprotein E knockout mice treated with or without liver X receptor agonist T0901317 for 14 weeks. Animals in the basal control group (*n*=10) were treated with vehicle and killed after 8 weeks. The vehicle group (*n*=14) and prevention group (*n*=14) were treated with vehicle or T0901317 (10 mg/kg body weight), respectively, daily by oral lavage (0.2 ml/mouse) for 14 weeks. The treatment group (*n*=14) was treated with vehicle for 8 weeks, then treated with T0901317 for 6 weeks. Mice in the latter three groups were killed at week 14. The results are expressed as the mean±SD from five animals. *P*<0.05 versus vehicle.
and treatment group was significantly higher than in the vehicle group (P<0.05; Table 1). Whereas the level of MDA in these two groups was significantly lower than in the vehicle group (P<0.05; Table 2).

Table 1 Changes in superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in aorta of apolipoprotein E knockout mice treated with liver X receptor agonist T0901317

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/mg protein)</th>
<th>MDA (nM/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal control</td>
<td>9.76±1.31</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>Vehicle</td>
<td>10.11±1.47</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td>Prevention</td>
<td>19.47±2.13 *</td>
<td>0.11±0.01 *</td>
</tr>
<tr>
<td>Treatment</td>
<td>18.89±2.08 *</td>
<td>0.12±0.02 *</td>
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</table>

Mice were fed a high-fat/high-cholesterol diet. Animals in the basal control group (n=10) were treated with vehicle and killed after 8 weeks. The vehicle group (n=14) and prevention group (n=14) were treated with vehicle or T0901317 (10 mg/kg body weight), respectively, daily by oral lavage (0.2 ml/mouse) for 14 weeks. The treatment group (n=14) was treated with vehicle for 8 weeks, then treated with T0901317 for 6 weeks. Mice in the latter three groups were killed at week 14. *P=0.05 versus vehicle. The data shown are the mean±SD.

Table 2 Changes in superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in liver tissue of apolipoprotein E knockout mice treated with liver X receptor agonist T0901317

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/mg protein)</th>
<th>MDA (nM/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal control</td>
<td>83.34±8.71</td>
<td>2.10±1.93</td>
</tr>
<tr>
<td>Vehicle</td>
<td>86.21±9.67</td>
<td>2.24±2.17</td>
</tr>
<tr>
<td>Prevention</td>
<td>169.73±19.45 *</td>
<td>1.13±0.17 *</td>
</tr>
<tr>
<td>Treatment</td>
<td>161.89±18.69 *</td>
<td>1.26±0.18 *</td>
</tr>
</tbody>
</table>

Mice were fed a high-fat/high-cholesterol diet. Animals in the basal control group (n=10) were treated with vehicle and killed after 8 weeks. The vehicle group (n=14) and prevention group (n=14) were treated with vehicle or T0901317 (10 mg/kg body weight), respectively, daily by oral lavage (0.2 ml/mouse) for 14 weeks. The treatment group (n=14) was treated with vehicle for 8 weeks, then treated with T0901317 for 6 weeks. Mice in the latter three groups were killed at week 14. *P=0.05 versus vehicle. The data shown are the mean±SD.

Discussion

In the present study, we established a chronic atherosclerotic marine animal model through feeding apoE−/− mice (a classic animal model developing severe aortic atherosclerosis similar to that of human) with a high-fat/high-cholesterol diet with or without T0901317 for 8 or 14 weeks, then observed for the first time the effect of an LXR agonist T0901317 on SSAO gene in apoE−/− mice. We did not use wild-type mice as controls because they are naturally resistant to atherosclerosis and an array of previous studies about atherosclerosis only used gene knockout mice [37–39].

The primary finding in this work is that T0901317 inhibited both SSO gene expression and its activity in serum and several different tissues, such as aorta, liver, small intestine, and brain (Figs. 1 and 2). The moderate reduction in SSO gene expression and its activity in liver compared with aorta or brain might result from the tissue-specific effect of T0901317.

In broad outline, atherosclerosis can be considered to be a form of chronic inflammation resulting from interaction among modified lipoproteins, monocyte-derived macrophages, T cells and the normal cellular elements of the arterial wall. This inflammatory process can ultimately lead to the development of complex lesions, or plaques, that protrude into the arterial lumen. Plaque rupture and thrombosis result in the acute clinical complications of myocardial infarction and stroke [40,41]. Among many genetic and environmental risk factors that have been identified by epidemiologic studies, elevated levels of serum cholesterol are probably unique in being sufficient to drive the development of atherosclerosis in humans and experimental animals, even in the absence of other known risk factors [42,43].

LXRs are nuclear receptors that play central roles in the transcripational control of lipid metabolism [44]. LXRs function as nuclear cholesterol sensors that are activated in response to elevated intracellular cholesterol levels in multiple cell types [45,46]. Once activated, LXRs induce the expression of an array of genes involved in cholesterol absorption, efflux, transport, and excretion. In addition to their function in lipid metabolism, LXRs have also been found to modulate immune and inflammatory responses in macrophages [47,48]. Synthetic LXR agonists promote cholesterol efflux and inhibit inflammation in vivo, and inhibit the development of atherosclerosis in animal models [28,49]. Our recent study showed that T0901317 could inhibit the initiation of atherogenesis, induce the regression of preexisting atherosclerotic lesions, promote LXR target gene LRα and ABCA1 gene expression, and inhibit inflammatory gene expression in apoE−/− mice [50]. The ability of LXRs to integrate metabolic and inflammatory signaling makes them particularly attractive targets for intervention in human metabolic disease. SSAO constitutes a group of enzymes containing copper and quinone and sensitive to semicarbazide. SSAO-mediated deamination of methylamine and aminoacetone leads to
production of toxic formaldehyde and methylglyoxal, respectively [51]. These toxic products could be responsible, at least in part, for protein cross-linkage, oxidative stress, and cytotoxicity. The inhibition of SSAO activity might be associated with a reduced toxic or oxidative damage in tissues. We also analyzed the SSAO-mediated oxidative damage in aorta and liver of apoE$^{-/-}$ mice. SOD and MDA are important biomarkers of oxidative stress. Many previous studies have used them to quantify oxidative damage [52–54]. Our results showed that the amount of SOD in the prevention group and treatment group was significantly higher than in the vehicle group ($P<0.05$; Tables 1 and 2), whereas MDA in the two groups was significantly lower than in the vehicle group ($P<0.05$; Tables 1 and 2).

Although rodents are generally very resistant to atherosclerosis, some strains of mice, for example, inbred C57BL/6, fed with an atherogenic high-cholesterol diet, will develop atherosclerotic lesions [55,56]. C57BL/6 mice, known to be vulnerable to atherosclerosis, show significantly higher SSAO activity [57]. These findings suggest that SSAO-mediated deamination is probably (at least in part) involved in atherogenesis and vascular disorders. KKAy is a strain of mice possessing features closely resembling those of diabetes mellitus type 2. It has recently been found that both selective mechanism-based SSAO inhibitor and amino-guanidine effectively reduced oxidative stress, as shown by reduction of MDA excretion, inhibitor and amino-guanidine effectively reduced oxidative damage in aorta and liver of apoE$^{-/-}$ mice. SOD and MDA are important biomarkers of oxidative stress. Many previous studies have used them to quantify oxidative damage [52–54]. Our results showed that the amount of SOD in the prevention group and treatment group was significantly higher than in the vehicle group ($P<0.05$; Tables 1 and 2), whereas MDA in the two groups was significantly lower than in the vehicle group ($P<0.05$; Tables 1 and 2).

In summary, the synthetic LXR agonist T0901317 inhibits SSAO gene expression and its activity in atherogenic apoE$^{-/-}$ mice, and also reduces oxidative damage. The antiatherogenic effect of LXR agonist T0901317 could be related to these beneficial effects.

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