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Tranilast, an anti-fibrogenic agent, ameliorates a dietary rat model of nonalcoholic steatohepatitis

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Abbreviations: ALT, alanine aminotransferase; CPT-1, carnitine O-palmitoyltransferase-1; collagen I, α1(I) procollagen; iNOS, Inducible NO synthase; IL-6, interleukin-6; LETO, Long–Evans Tokushima Otsuka; LPS, lipopolysaccharide; MCD, methionine- and choline-deficient; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OLETF, Otsuka Long–Evans Tokushima Fatty; PPARα, peroxisomal proliferator activated receptor-α; PPARγ, peroxisomal proliferator activated receptor-γ; PAI-1, plasminogen activator inhibitor-1; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VCAM-1 , vascular cell adhesion molecule 1;

Key words: tranilast, nonalcoholic steatohepatitis, steatosis, β-oxidation, TGF-β

Running title: Tranilast prevents progression of NASH

Abstract

Nonalcoholic steatohepatitis (NASH) is the progressive form of nonalcoholic fatty liver disease and is one of the most common liver diseases in the developed world. The histological findings of NASH are characterized by hepatic steatosis, inflammation, and fibrosis. To date, however, the optimal treatment of NASH has not been established. Tranilast, *N*-(3′,4′-dimethoxycinnamoyl)-anthranilic acid, is an anti-fibrogenic agent that inhibits the action of transforming growth factor (TGF)-β. This drug is used clinically for fibrogenesis-associated skin disorders including hypertrophic scars and scleroderma. TGF-β plays a central role in the development of hepatic fibrosis, and tranilast may thus ameliorate the pathogenesis of NASH. We investigated the effects of tranilast by using an established dietary animal model of NASH, obese diabetic Otsuka Long–Evans Tokushima Fatty (OLETF) rats and nondiabetic control Long–Evans Tokushima Otsuka (LETO) rats fed a methionine- and choline-deficient diet. Treatment with 2% tranilast (420 mg $kg^{-1}day^{-1}$) for 8 weeks prevented the development of hepatic fibrosis and the activation of stellate cells, and down-regulated expression of genes for TGF-β and TGF-β-target molecules, including α1 procollagen and plasminogen activator-1. In addition, tranilast attenuated hepatic inflammation and Kupffer cells recruitment, and down-regulated expression of tumor necrosis factor-α (TNF-α). Unexpectedly, tranilast ameliorated hepatic steatosis, and up-regulated expression of genes involved in β-oxidation, such as peroxisome proliferator-activated receptor α and carnitine *O*-palmitoyltransferase-1. Most of these effects were observed in LETO rats and OLETF rats, which suggest that the action of tranilast is mediated through the insulin resistance-independent pathway. Further, TNF-α and interleukin-6 (IL-6) expression in RAW 264.7 macrophages was suppressed by tranilast. *Conclusion:* Our findings suggest that targeting TGF-β with tranilast represents a new mode of therapy for NASH.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases in the United States and other developed countries[1, 2] and involves a wide spectrum of liver disease, ranging from simple steatosis to steatohepatitis and cirrhosis. Nonalcoholic steatohepatitis (NASH), the progressive form of NAFLD, is characterized by steatosis, inflammation, and fibrosis. It has recently been proposed that NAFLD be included as a component of the metabolic syndrome, a cluster of metabolic abnormalities with visceral fat accumulation, hyperglycemia, hypertension, and dyslipidemia.[3–5] Insulin resistance, an underlying factor in these metabolic disorders, may play a critical role in the pathogenesis of NASH. We recently created a rat model of NASH with insulin resistance, abundant visceral fat tissue, hyperglycemia, and hypertension, and demonstrated that pioglitazone, a peroxisomal proliferator-activated receptor (PPAR)-γ agonist, prevents the progression of NASH, mainly through enhancement of insulin resistance.[6] However, it has remained unresolved whether all patients with NASH respond to insulin-sensitizing agents such as PPARγ agonists,[7] and the optimal treatment of NASH has not been established.

Transforming growth factor (TGF)-β is a multifunctional peptide that play a pivotal role in fibrogenesis.[8–11] High levels of TGF-β are often found in hepatic fibrosis and it has been implicated as a mediator of fibrosis in several liver diseases.[12] TGF-β stimulates collagen synthesis in cultured stellate cells, and hepatic overexpression of TGF-β in transgenic mice has been shown to cause hepatic fibrosis.[13, 14] Several lines of evidence have demonstrated that the blockade of TGF-β synthesis or signaling using different experimental strategies prevents liver fibrosis in various animal models.[15–18] TGF-β is also known to play a central role in the development of NASH by activating hepatic fibrosis.[19] Thus, TGF-β signaling can be designated as a potential anti-fibrogenic target for the treatment of fibrosis-associated liver diseases.

Tranilast, *N*-(3′,4′-dimethoxycinnamoyl)-anthranilic acid, was originally developed as an antiallergic drug for the systemic and topical treatment of bronchial asthma, atopic dermatitis, and allergic conjunctivitis because it inhibits the release of chemical mediators from mast cells and cytokines from macrophages.[8] More recently, tranilast has been reported to exert anti-fibrogenic effects by inhibiting the action of TGF-β. In fact, tranilast has been used clinically for the treatment of hypertrophic scars, scleroderma, and skin disorders associated with an excessive fibrogenic response.[20, 21] We previously found that tranilast prevents renal fibrosis in animal models of diabetic nephropathy, through the suppression of TGF-β.[22] To date, however, no reports are available on the effect of tranilast in animal models of liver diseases.

We therefore hypothesized that administration of tranilast would exert a beneficial effect on the pathology of NASH by suppressing TGF-β activity and development of hepatic fibrosis. To test this hypothesis, we investigated the effect of tranilast by using an established dietary animal model of NASH, obese diabetic rats fed a methionine- and choline-deficient (MCD) diet.[6]

Methods

Animal model and experimental design. Male Otsuka Long–Evans Tokushima Fatty (OLETF) rats, an established animal model of obese type 2 diabetes,[23] were used (Otsuka Pharmaceutical, Tokushima, Japan). Spontaneous-onset type 2 diabetic OLETF rats showed obesity and hyperinsulinemia from 8 weeks of age. NAFLD has been studied in OLETF rats because these animals show an age-dependent increase of fat accumulation in the liver. All rats in the OLETF group developed diabetes after 24 weeks of age (data not shown) as described previously.[23, 24] As control animals, 4-week-old OLETF and Long–Evans Tokushima Otsuka (LETO) rats, which originated from the same colony as the OLETF rats by selective mating but do not develop diabetes, were obtained and housed in a room under controlled temperature (25°C), humidity, and lighting (12-h artificial light and dark cycle). Animals were given free access to standard laboratory rat chow and tap water. At 24 weeks of age, OLETF rats were divided into two experimental groups and fed for 8 weeks as follows: MCD diet (Oriental Yeast, Tokyo, Japan; OLETF-MCD, 21% fat, $n = 11$), and MCD-tranilast diet (OLETF-MCD-trani; tranilast content 1%, $n = 3$,1.4%, $n = 3$, 2%, $n = 10$). In addition, at 24 weeks of age, male LETO rats were divided into two groups: MCD diet (LETO-MCD, $n = 9$) and MCD-tranilast diet (LETO-MCD-trani; tranilast content 2%, $n = 5$). The rats were allowed unrestricted access to water and the standard or mixed chow. The weight and food intake in each group of rats were recorded every week. Tranilast was donated by Kissei Pharmaceutical Co. (Nagano, Japan). All animal procedures were performed in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi campus of Kanazawa University.

Blood sampling and analysis. After 8 weeks, the rats were killed, and blood samples were obtained from the heart after a 12-h fast. Blood glucose was determined by the Freestyle Kissei monitor (Kissei pharmaceutical, Matsumoto, Japan). The blood samples were centrifuged, and serum was frozen at −70°C for subsequent measurement of serum alanine aminotransferase (ALT), triglyceride, free fatty acid (FFA), total cholesterol (TC), and HDL-cholesterol (HDL-C) levels. Plasma ALT was

determined by spectrophotometry (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. Plasma FFA levels were determined by a non-esterified fatty acid (NEFA) C-test (Wako, Osaka, Japan). TC and HDL-C concentrations were quantified by colorimetry, as described previously.[21] Plasma levels of insulin and tumor necrosis factor-α (TNF-α) were determined by ELISA (Mercodia, Uppsala, Sweden) (Bender Medsystems, Vienna, Austria).

Measurement of serum glucose levels. Before 3 days of sacrifice, all rats underwent an oral glucose tolerance test after a 12-h fast. Glucose (2 g kg^{-1}) was administered orally. Blood was drawn from a tail vein at 0, 60, and 120 min for measurement of serum glucose concentrations.

Measurement of serum and liver triglyceride levels. After 8 weeks, the rats were killed, and liver weight and triglyceride level were measured. For quantification of the hepatic triglyceride content, the liver was lysed with the buffer in a commercially available kit (TG E-test; Wako).

Morphological analysis and immunohistochemistry. After 8 weeks on each diet, the animals were killed and their livers were fixed in 10% buffered formalin and embedded in paraffin. Severity of hepatic histological changes were assessed by hematoxylin and eosin (H&E) and Azan staining, and blindly scored by a single pathologist. Steatosis, fibrosis, and disease activity were semiquantitatively evaluated according to the standard criteria of grading and staging for NASH, with minor modifications.[25] Degree of steatosis was scored as the percentage of hepatocytes containing lipid droplets. Fibrosis scores were as follows: 1, pericellular and perivenular fibrosis; 2, focal bridging fibrosis; 3, bridging fibrosis with lobular distortion; and 4, cirrhosis. Disease activity was evaluated as the sum of scores (score 0–6) of acinar inflammation (score 0–3) and portal inflammation (score 0–3).

Slides were immunostained with monoclonal mouse antihuman α-smooth muscle actin (α-SMA) (Dako, Kyoto, Japan) and anti-rabbit TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ED1 (AbD, SerotecOxford, UK). This was followed by application of the immunoperoxidase technique using an Envision kit (Dako). Peroxidase activity was identified by reaction with 3',3'-diaminobenzidine (Sigma). Two independent observers with no previous knowledge of the experimental design evaluated each section. Positive staining with anti-α-SMA or anti-TGF-β or ED1 was

expressed as a percentage of the field, using WinROOF version 5.71 (Mitani Shoji, Fukui, Japan).

Real-time quantitative PCR. Total RNA was extracted from each liver using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) as described previously.[26] Real-time quantitative PCR was performed for TGF-β, α1(I) procollagen (collagen I), plasminogen activator inhibitor-1 (PAI-1), carnitine *O*-palmitoyltransferase-1 (CPT-1), peroxisomal proliferator activated receptor-α (PPAR-α), TNF-α, vascular cell adhesion molecule 1 (VCAM-1) mRNA using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The sets of primers and TaqMan probes for collagen I were proprietary to Applied Biosystems (TaqMan Gene Expression Assays product). To control for variation in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18S rRNA (TaqMan Control Reagent Kit; Applied Biosystems). The PCR conditions were one cycle at 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 58°C for 1 min.

Western blot analysis of 4-HNE-modified proteins. Livers were homogenized in RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholic acid, and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The following procedures for Western blot analysis have been described previously.[27]

Cell culture model and experimental design. RAW 264.7 macrophages were grown in DMEM (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum in a humidified 5% CO_2 atmosphere at 37°C until they were 90% confluent. Cells were treated with lipopolysaccharide (LPS, Escherichia coli 05:B5, Sigma−Aldrich, St. Louis, USA) (1 μg/ml) and tranilast (30 μmol/l, 300μmol/l) for 6 hour. After treatment, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) as described previously.[26] Gene expression of TNF-α, IL-6 and inducible NO synthase (iNOS) was determined by realtime-PCR. In the next experiment, the cells were treated tranilast alone(30 μmol/l, 300 μmol/l) for 6 hour. After treatment, concentration of IL-6 and TNF-α in culture medium was determined by ELISA (R&D systems, Minneapolis, USA and Bender Medsystems, Vienna, Austria).

Statistical analysis. All results are shown as means or means ± SEM. Data were analyzed by one-way analysis of variance, and $P < 0.05$ was considered statistically significant. All calculations were performed with SPSS version 11.0 software for Windows (SPSS Inc., Chicago, IL, USA).

Results

Tranilast was well tolerated by rats fed the MCD diet. Spontaneous-onset type 2 diabetic OLETF rats were fed with the MCD diet for 8 weeks. The MCD diet caused intense lobular inflammation, and prominent perivenular and pericellular fibrosis in zone 3 of the liver (Fig. 1A, B) as previously described.[6] To ascertain the most effective doses of tranilast, we treated the OLETF-MCD rats with tranilast at three concentrations, 1, 1.4, and 2%, which provided an average dose of 160, 190, and 420 mg $kg^{-1}day^{-1}$, respectively. These are reasonable daily doses for animal experiments, as previously determined.[22, 28, 29] Tranilast did not affect physical appearance, stool composition, coat maintenance, or food or water intake, which indicated that tranilast was well tolerated. Treatment with tranilast dose-dependently ameliorated the liver pathology in MCD-OLETF rats (Fig. 1C). The effects were more prominent in the 2% tranilast-treated group, and we thus performed the following investigations in this group.

Metabolic parameters. Metabolic parameters in OLETF rats and nondiabetic control LETO rats fed the MCD diet, with or without 2% tranilast, for 8 weeks are shown in Table 1. Tranilast significantly decreased serum ALT and increased liver weight of both OLETF-MCD and LETO-MCD rats. Blood glucose and triglycerides were unaffected by tranilast. In addition, an insulin loading test showed that tranilast-treated rats exhibited no improvement in insulin sensitivity (data not shown).

Tranilast inhibited activation of stellate cells and development of hepatic fibrosis in a dietary animal model of NASH. Histological analysis with Azan staining revealed that treatment with 2% tranilast dramatically prevented the development of hepatic fibrosis in LETO-MCD and OLETF-MCD rats (Fig. 2A, B). Activation of hepatic stellate cells is known to play a central role in development of hepatic fibrosis. Thus, to examine the effect of tranilast on activation of stellate cells, we performed immunohistochemical analysis of α-SMA, a marker for stellate cell activation. α-SMA-positive cells significantly increased in the liver of OLETF-MCD rats compared

to that in LETO-MCD rats. Tranilast reduced the numbers of these cells in both LETO-MCD and OLETF-MCD rats (Fig. 2C, D).

Tranilast prevented development of hepatic steatosis. To date, little has been reported on the effect of tranilast on intracellular lipid accumulation. However, unexpectedly, our histological analysis showed that treatment with tranilast clearly improved hepatic steatosis in both LETO-MCD and OLETF-MCD rats (Fig. 3A). Consistent with the histological findings, tranilast significantly reduced hepatic triglyceride content in these two groups of rats (Fig. 3B).

Tranilast suppressed TGF-β expression in the liver in the dietary animal model of NASH. To clarify the mechanism by which tranilast prevents hepatic fibrosis in rats, the expression of TGF-β, a master regulator of fibrogenesis in the liver, was estimated by quantitative real-time PCR (Fig. 4A). Treatment with tranilast down-regulated mRNA for TGF-β in the liver of OLETF-MCD rats, and also had a tendency to down-regulate it in the liver of LETO-MCD rats. Moreover, tranilast suppressed the expression of TGF-β target genes involved in fibrogenesis, collagen I, and PAI-1 (Fig. 4B, C). Consistent with the findings for mRNA, immunohistochemical analysis showed suppression of TGF-β protein expression in the liver of tranilast-treated rats (Fig. 4D).

Tranilast up-regulated mRNA expression of genes involved in fatty acid β-oxidation. To clarify the mechanism by which tranilast improves hepatic steatosis in the rats, the expression of genes involved in fatty acid β-oxidation was measured by quantitative real-time PCR (Fig. 5A, B). mRNA expression for PPAR-α, a transcriptional factor central to the regulation of β-oxidation, was significantly up-regulated by tranilast in the liver of OLETF-MCD and LETO-MCD rats. In addition, tranilast also induced mRNA expression for CPT-1, a rate-limiting enzyme for β-oxidation that controls fatty acid entry into mitochondria.

Tranilast reduced numbers of Kupffer cells and TNF-α production in a dietary animal model of NASH. To clarify the mechanism by which tranilast improves hepatic inflammation in the rats, we evaluated the effect of tranilast on Kupffer cells, the resident liver macrophages. We performed immunohistochemical analysis using a monoclonal antibody specific for ED1 antigen, a marker of rat monocytes/macrophages and Kupffer cells. While ED1-positive cells significantly increased in the liver of

OLETF-MCD rats compared to that in LETO-MCD rats, tranilast reduced the numbers of these cells in both LETO-MCD and OLETF-MCD rats (Fig.6A, B). As Kupffer cells are the primary source of hepatic TNF-α,[30] we measured hepatic TNF-α mRNA levels. Real-time PCR analysis showed that tranilast dramatically down-regulated mRNA for TNF-α (Fig.6C). Serum levels of TNF-α were also decreased by treatment with tranilast (Table 1). In addition, tranilast down-regulated mRNA for VCAM-1, the adhesion molecules facilitating leucocyte recruitment, in the liver of OLETF-MCD rats, and also had a tendency to down-regulate it in the liver of LETO-MCD rats (Fig.6D).

Tranilast inhibited LPS-induced TNF-α and IL-6 production in RAW 264.7 macrophages. To determine whether tranilast has a direct action on the activated macrophages, we performed the experiments by RAW 264.7 cells. Incubation of RAW 264.7 cells with LPS for 6 hours resulted in a remarkable increase of TNF-α, IL-6, and iNOS mRNA expression (Fig.7A, B, C). Concomitant administration of tranilast significantly suppressed LPS-induced expresson of these genes. In addition, the release of TNF-α and IL-6 from RAW 264.7 cells into the culture medium was also significantly decreased by tranilast treatment (Fig. 7D E).

Tranilast attenuated intrahepatic oxidative stress in the dietary animal model of NASH. To clarify the anti-oxidative effect of tranilast, we estimated 4-HNE-modified proteins, a marker of oxidative stress, in the liver lysates by Western blot analysis. 4-HNE was significantly decreased by tranilast administration in the liver of OLETF-MCD and LETO-MCD rats (Fig.8A-D). In addition, thiobarbituric acid reactive substances (TBARS), another marker of oxidative stress, was also estimated by western blot analysis. Hepatic accumulation of TBARS was significantly reduced by treatment with tranilast (data not shown).

Discussion

Hepatic fibrosis is a well-known histological and biochemical hallmark of NASH. Progressive fibrosis causes liver insufficiency and portal hypertension, and is a risk factor for developing hepatocellular carcinoma. Thus, hepatic fibrosis is an important therapeutic target for NASH, and various inhibitors of fibrosis are now being investigated as potential candidate drugs.[31] In the current study, we demonstrated that tranilast, an anti-fibrogenic agent, improved hepatic fibrosis in an animal model of NASH. Administration of tranilast inhibited the activation of hepatic stellate cells and

reduced expression of genes for TGF-β and its target molecules, which probably led to the prevention of fibrosis. The present study provides *in vivo* evidence that anti-fibrogenic treatment by tranilast is a beneficial and promising treatment approach for NASH.

Among the numerous pro-fibrogenic mediators, a functional hierarchy might exist, which points to TGF-β as being the most effective.[32] Concomitant with increased activity of TGF-β during hepatic fibrosis, hepatic stellate cells increase the production and deposition of collagen. It is well-known that blockade of TGF-β synthesis or signaling using different experimental strategies prevents liver fibrosis in various animal models.[15–18] To date, however, it is unclear whether an oral inhibitor of TGF-β activity is effective in preventing liver fibrosis in animal models. We demonstrated that administration of tranilast reduced TGF-β gene expression and α-SMA-positive cells in the liver of MCD-fed rats. The current data suggest that treatment with tranilast ameliorates hepatic fibrosis and liver damage through the direct suppression of TGF-β expression. These findings are supported by a previous *in vitro* study that demonstrated that tranilast inhibits the activation of cultured rat stellate cells through the suppression of TGF-β mRNA.[10] In addition, tranilast also down-regulated hepatic expression of PAI-1, one of the TGF-β target genes. PAI-1 has been reported to be a therapeutic target of metformin for hepatic injury caused by ethanol, [33] which suggests that tranilast might also be effective treating in this disease.

The current study is the first to demonstrate that tranilast exerts broad and profound effects on Kupffer cells, the resident liver macrophages. Previous reports showed that tranilast inhibits cytokine release from human monocytes/macrophages,[34, 35] however, it was unresolved whether tranilast acts upon Kupffer cells. In liver injury, activated Kupffer cells are a major source of inflammatory mediators including cytokines, superoxides, nitric oxide, and chemokines.[36] Particularly, TNF-α, derived from activated Kupffer cells, plays a critical role in the development of various liver diseases including nonalcoholic steatohepatitis.[37] TNF-α activates Kupffer cells through autocrine and paracrine mechanisms,[30] and promotes Kupffer cells recruitment by enhancing adhesion molecules such as VCAM-1 on endothelial cells, creating a vicious cycle.[36] Our experiments indicated that TNF-α production in Kupffer cells/macrophages are suppressed by tranilast treatment. Tranilast may attenuate MCD-induced hepatic inflammation and accumulation of oxidative stress probably by breaking the vicious cycle of Kupffer cells activation and TNF-α production.

Obesity, type 2 diabetes, and dyslipidemia frequently coexist with NAFLD. Insulin resistance is an underlying factor in these metabolic disorders and may play a

critical role in the pathogenesis of NAFLD. Several lines of evidence have indicated that insulin-sensitizing agents, such as pioglitazone, improve histological findings and liver injury in both animals and patients with NASH, the progressive form of NAFLD.[6, 38] In the current study, insulin sensitivity in rats was unaffected by treatment with tranilast. In addition, unlike the action of pioglitazone,[6, 38] tranilast was beneficial in preventing hepatic fibrosis in the LETO-MCD rats, which mimics animal models of NASH, without insulin resistance. These results suggest that the inhibitory action of tranilast is mediated through an insulin-resistance-independent pathway. Therefore, nonresponders to insulin-sensitizing agents might benefit from treatment with tranilast for preventing hepatic fibrosis in NASH.

Surprisingly, we found that tranilast prevented the development of not only hepatic fibrosis, but also liver steatosis in MCD-induced NASH. This finding was reinforced by the reduction of liver triglyceride content. In addition, these findings were unexpected because little has been reported on the effect of tranilast on intracellular lipid accumulation. Real-time PCR revealed that expression of genes involved in fatty acid β-oxidation, such as CPT-I and PPAR-α, were up-regulated, which suggests that tranilast-induced enhancement of β-oxidation may have attenuated hepatic steatosis in rats. Our results were consistent with a previous study showing that TGF-β signaling pathways suppress PPAR-α activity, a transcriptional factor central to the regulation of β-oxidation, and reduce fatty acid β-oxidation in cardiomyocytes.[39] Therefore, tranilast-mediated inhibition of TGF-β action might enhance β-oxidation through the up-regulation of PPAR-α. However, one limitation of our study is that the analysis was performed only at the gene transcriptional levels. Further studies evaluating the alteration of β-oxidation capacity are needed to clarify the mechanism by which tranilast attenuates hepatic steatosis.

In the current study, the OLETF-MCD rats treated with tranilast had a significant body weight loss. Because tranilast significantly reduced body weight in MCD-OLETF rats but not in MCD-LETO rats, this action is unlikely due to a toxic effect. Certainly, we cannot rule out the possibility that body weight reduction might, at least in part, contribute to the tranilast-induced attenuation of hepatic steatosis and up-regulation of gene expression involved in fatty acid β-oxidation. However, our data indicated the direct inhibitory effects of tranilast on activated macrophages both *in vivo* and *in vitro*, suggesting that tranilast ameliorated the liver pathology independently of its weight reducing effects. The critical parameters contributing to body-weight maintenance include caloric intake, physical activity, and adaptive thermogenesis.[40, 41] As both food intake and physical appearance were unaffected by tranilast, we should examine

the effect of tranilast on adaptive thermogenesis and energy expenditure in future. Tranilast might inhibit activated macrophages infiltrating into the adipose tissues leading to inhibition of adipose tissue hyperplasia. These unraveled effects of tranilast are under investigation in MCD-untreated animal models of obesity and type 2 diabetes without cachexia.

Serological analysis revealed that all the animals treated with tranilast showed improvement of serum ALT levels compared to those without tranilast treatment. These findings indicated that treatment with tranilast for 8 weeks did not induce hepatic toxicity, as evidenced by the histomorphology of the liver. Tranilast has long been used safely for the treatment of patients with bronchial asthma and atopic dermatitis. However, since TGF-β-mediated fibrogenesis is involved in an active wound-healing process that is essential for tissue remodeling, long-term treatment with a TGF-β inhibitor may produce adverse effects.[31] To determine the safety of administering tranilast for patients with NASH, further investigations of the long-term outcome are needed.

In conclusion, our experiments demonstrated that tranilast prevented the development of NASH in a dietary rat model, possibly through an insulin-resistance-independent pathway. Our findings suggest that targeting TGF-β with tranilast is a novel approach to the prevention and treatment of NASH.

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References

1. Reid AE. Nonalcoholic steatohepatitis. Gastroenterology 2001;121:710–723.
2. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med 2002;346:1221–1231.
3. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, Natale S, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 2003;37:917–923.
4. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCullough AJ, et al. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes 2001;50:1844–1850.
5. Marceau P, Biron S, Hould FS, Marceau S, Simard S, Thung SN, Kral JG. Liver pathology and the metabolic syndrome X in severe obesity. J Clin Endocrinol Metab 1999;84:1513–1517.
6. Ota T, Takamura T, Kurita S, Matsuzawa N, Kita Y, Uno M, Akahori H, et al. Insulin resistance accelerates a dietary rat model of nonalcoholic steatohepatitis. Gastroenterology 2007;132:282–293.
7. Ota T, Takamura T, Kaneko S. Pioglitazone in nonalcoholic steatohepatitis. N Engl J Med 2007;356:1068; author reply 1068–1069.
8. Suzawa H, Kikuchi S, Ichikawa K, Koda A. Inhibitory action of tranilast, an anti-allergic drug, on the release of cytokines and PGE2 from human monocytes–macrophages. Jpn J Pharmacol 1992;60:85–90.
9. Bonnet F, Cao Z, Cooper ME, Cox AJ, Kelly DJ, Gilbert RE. Tranilast attenuates vascular hypertrophy, matrix accumulation and growth factor overexpression in experimental diabetes. Diabetes Metab 2003;29:386–392.
10. Ikeda H, Inao M, Fujiwara K. Inhibitory effect of tranilast on activation and transforming growth factor beta 1 expression in cultured rat stellate cells. Biochem Biophys Res Commun 1996;227:322–327.
11. Miyazawa K, Kikuchi S, Fukuyama J, Hamano S, Ujiie A. Inhibition of PDGF- and TGF-beta 1-induced collagen synthesis, migration and proliferation by tranilast in vascular smooth muscle cells from spontaneously hypertensive rats. Atherosclerosis 1995;118:213–221.
12. Liu X, Hu H, Yin JQ. Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. Liver Int 2006;26:8–22.
13. Chapman HA. Disorders of lung matrix remodeling. J Clin Invest 2004;113:148–157.

14. Casini A, Pinzani M, Milani S, Grappone C, Galli G, Jezequel AM, Schuppan D, et al. Regulation of extracellular matrix synthesis by transforming growth factor beta 1 in human fat-storing cells. Gastroenterology 1993;105:245–253.
15. George J, Roulot D, Koteliansky VE, Bissell DM. *In vivo* inhibition of rat stellate cell activation by soluble transforming growth factor beta type II receptor: a potential new therapy for hepatic fibrosis. Proc Natl Acad Sci U S A 1999;96:12719–12724.
16. Arias M, Sauer-Lehnen S, Treptau J, Janoschek N, Theuerkauf I, Buettner R, Gressner AM, et al. Adenoviral expression of a transforming growth factor-beta1 antisense mRNA is effective in preventing liver fibrosis in bile-duct ligated rats. BMC Gastroenterol 2003;3:29.
17. Qi Z, Atsuchi N, Ooshima A, Takeshita A, Ueno H. Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. Proc Natl Acad Sci U S A 1999;96:2345–2349.
18. Nakamura T, Sakata R, Ueno T, Sata M, Ueno H. Inhibition of transforming growth factor beta prevents progression of liver fibrosis and enhances hepatocyte regeneration in dimethylnitrosamine-treated rats. Hepatology 2000;32:247–255.
19. Williams EJ, Gaca MD, Brigstock DR, Arthur MJ, Benyon RC. Increased expression of connective tissue growth factor in fibrotic human liver and in activated hepatic stellate cells. J Hepatol 2000;32:754–761.
20. Shigeki S, Murakami T, Yata N, Ikuta Y. Treatment of keloid and hypertrophic scars by iontophoretic transdermal delivery of tranilast. Scand J Plast Reconstr Surg Hand Surg 1997;31:151–158.
21. Yamada H, Tajima S, Nishikawa T, Murad S, Pinnell SR. Tranilast, a selective inhibitor of collagen synthesis in human skin fibroblasts. J Biochem (Tokyo) 1994;116:892–897.
22. Akahori H, Ota T, Torita M, Ando H, Kaneko S, Takamura T. Tranilast prevents the progression of experimental diabetic nephropathy through suppression of enhanced extracellular matrix gene expression. J Pharmacol Exp Ther 2005;314:514–521.
23. Kawano K, Hirashima T, Mori S, Saitoh Y, Kurosumi M, Natori T. Spontaneous long-term hyperglycemic rat with diabetic complications. Otsuka Long–Evans Tokushima Fatty (OLETF) strain. Diabetes 1992;41:1422–1428.
24. Kahn C. [Chapter title]. In: Joslin's diabetes mellitus, 14th ed. Boston, Lippincott Williams & Wilkins;2005:p. 1224.

25. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999;94:2467–2474.
26. Takamura T, Sakurai M, Ota T, Ando H, Honda M, Kaneko S. Genes for systemic vascular complications are differentially expressed in the livers of type 2 diabetic patients. Diabetologia 2004;47:638–647.
27. Matsuzawa N, Takamura T, Kurita S, Misu H, Ota T, Ando H, Yokoyama M, et al. Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. Hepatology 2007;46:1392-1403.
28. Saiura A, Sata M, Hirata Y, Nagai R, Makuuchi M. Tranilast inhibits transplant-associated coronary arteriosclerosis in a murine model of cardiac transplantation. Eur J Pharmacol 2001;433:163–168.
29. Izawa A, Suzuki J, Takahashi W, Amano J, Isobe M. Tranilast inhibits cardiac allograft vasculopathy in association with p21(Waf1/Cip1) expression on neointimal cells in murine cardiac transplantation model. Arterioscler Thromb Vasc Biol 2001;21:1172–1178.
30. Su GL. Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. Am J Physiol Gastrointest Liver Physiol 2002;283:G256-265.
31. Gressner AM, Weiskirchen R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. J Cell Mol Med 2006;10:76–99.
32. Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. Front Biosci 2002;7:d793–d807.
33. Bergheim I, Guo L, Davis MA, Lambert JC, Beier JI, Duveau I, Luyendyk JP, et al. Metformin prevents alcohol-induced liver injury in the mouse: critical role of plasminogen activator inhibitor-1. Gastroenterology 2006;130:2099–2112.
34. Suzawa H, Kikuchi S, Ichikawa K, Koda A. Inhibitory action of tranilast, an anti-allergic drug, on the release of cytokines and PGE2 from human monocytes-macrophages. Jpn J Pharmacol 1992;60:85-90.
35. Capper EA, Roshak AK, Bolognese BJ, Podolin PL, Smith T, Dewitt DL, Anderson KM, et al. Modulation of human monocyte activities by tranilast, SB 252218, a compound demonstrating efficacy in restenosis. J Pharmacol Exp Ther 2000;295:1061-1069.
36. Kolios G, Valatas V, Kouroumalis E. Role of Kupffer cells in the pathogenesis of liver disease. World J Gastroenterol 2006;12:7413-7420.

37. Tomita K, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, Kitamura N, et al. Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. Gut 2006;55:415-424.
38. Belfort R, Harrison SA, Brown K, Darland C, Finch J, Hardies J, Balas B, et al. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. N Engl J Med 2006;355:2297–2307.
39. Sekiguchi K, Tian Q, Ishiyama M, Burchfield J, Gao F, Mann DL, Barger PM. Inhibition of PPAR-alpha activity in mice with cardiac-restricted expression of tumor necrosis factor: potential role of TGF-beta/Smad3. Am J Physiol Heart Circ Physiol 2007;292:H1443–H1451.
40. Lowell BB, Spiegelman BM. Towards a molecular understanding of adaptive thermogenesis. Nature 2000;404:652-660.
41. Lin J, Wu PH, Tarr PT, Lindenberg KS, St-Pierre J, Zhang CY, Mootha VK, et al. Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. Cell 2004;119:121-135.

Figure legends

Fig. 1. Tranilast dose-dependently ameliorated liver pathology in OLETF rats fed the MCD diet. Paraffin-embedded sections of the liver were stained with H&E (A) or Azan (B) stain (original magnification, ×100). Histological changes in the liver were semiquantitatively expressed as histological scores (C). The MCD diet caused marked macrovesicular steatosis, intense lobular inflammation, and prominent perivenular and pericellular fibrosis in zone 3 of the liver of OLETF rats. Compared to the MCD diet alone, the 1.4 and 2% tranilast-mixed diet significantly improved liver steatosis, fibrosis, and acinar and portal inflammation (Values are means ± SEM. $*P < 0.05$ versus the control diet).

Fig. 2. Tranilast inhibited activation of stellate cells and development of hepatic fibrosis. (A) Paraffin-embedded sections of the liver were stained with Azan stain (original magnification, ×100). (B) Percentages of fibrous area (blue area in Azan stain) were calculated under a microscope using an image analysis system. (C) Immunohistochemical staining using a monoclonal mouse antihuman α-SMA antibody (original magnification, ×100). (D) The α-SMA-positive area was quantified morphometrically in the liver sections. Compared to the MCD diet alone, the tranilast-mixed diet significantly improved liver fibrosis and activation of stellate cells (Values are means ± SEM. $**P < 0.01$ versus the MCD diet).

Fig. 3. Tranilast prevented development of hepatic steatosis. (A) Paraffin-embedded sections of the liver were stained with H&E (original magnification, ×100). (B) Tranilast significantly reduced hepatic triglyceride content in these two groups of rats (Values are means ± SEM. $*P < 0.05$ versus the MCD diet).

Fig. 4. Tranilast suppressed hepatic expression of TGF-β. (A) Treatment with tranilast down-regulated mRNA for TGF-β in the liver of OLETF-MCD rats. (B, C) Tranilast suppressed expression of TGF-β target genes, genes for procollagen and PAI-1 (Values are means ± SEM. $*P < 0.05$ versus MCD diet; $**P < 0.01$ versus MCD diet). (D) Paraffin-embedded sections of the liver were immunostained with a TGF-β antibody. Immunohistochemical analysis showed that TGF-β expression was down-regulated in the liver of tranilast-treated rats (original magnification, ×100).

Fig. 5. Tranilast up-regulated the genes involved in fatty acid β-oxidation. (A) mRNA expression for PPAR-α, a transcriptional factor central to the regulation of β-oxidation, was significantly up-regulated by tranilast in the liver of OLETF-MCD and LETO-MCD rats. (B) Tranilast induced mRNA expression for CPT-1, a rate-limiting enzyme for β-oxidation that controls fatty acid entry into mitochondria (Values are means ± SEM. **P* < 0.05, ***P* < 0.01 versus the MCD diet).

Fig. 6. Tranilast inhibited intrahepatic recruitment of Kupffer cells and TNF-α production in a dietary animal model of NASH. (A) Immunohistochemical staining using a ED1 antibody (original magnification, ×100). (B) The ED1-positive area was quantified morphometrically in the liver sections. Compared to the MCD diet alone, the tranilast-mixed diet significantly inhibited the permeation of the macrophage to the liver (Values are means ± SEM. ***P* < 0.01 versus the MCD diet). (C) Tranilast suppressed mRNA expression for TNF-α (Values are means ± SEM. **P* < 0.05 versus the MCD diet). (D) mRNA expression for VCAM, the adhesion molecules, was significantly down-regulated by tranilast in the liver of OLETF-MCD rats.

Fig. 7. Tranilast inhibited TNF-α, IL-6 and iNOS production in RAW 264.7 macrophages. (A) TNF-α mRNA expression was significantly down-regulated by tranilast in RAW 264.7 cells stimulated by LPS. (B) IL-6 expression was significantly down-regulated by tranilast in RAW cells. (C) iNOS expression was significantly down-regulated by tranilast in RAW cells (Values are means ± SEM. **P* < 0.05 versus LPS(+), tranilast(-)). (D) The concentration of TNF-α in medium was decreased by treatment of tranilast (E) The concentration of IL-6 in medium was decreased by treatment of tranilast (Values are means ± SEM. **P* < 0.05, ***P* < 0.01 versus tranilast(-)).

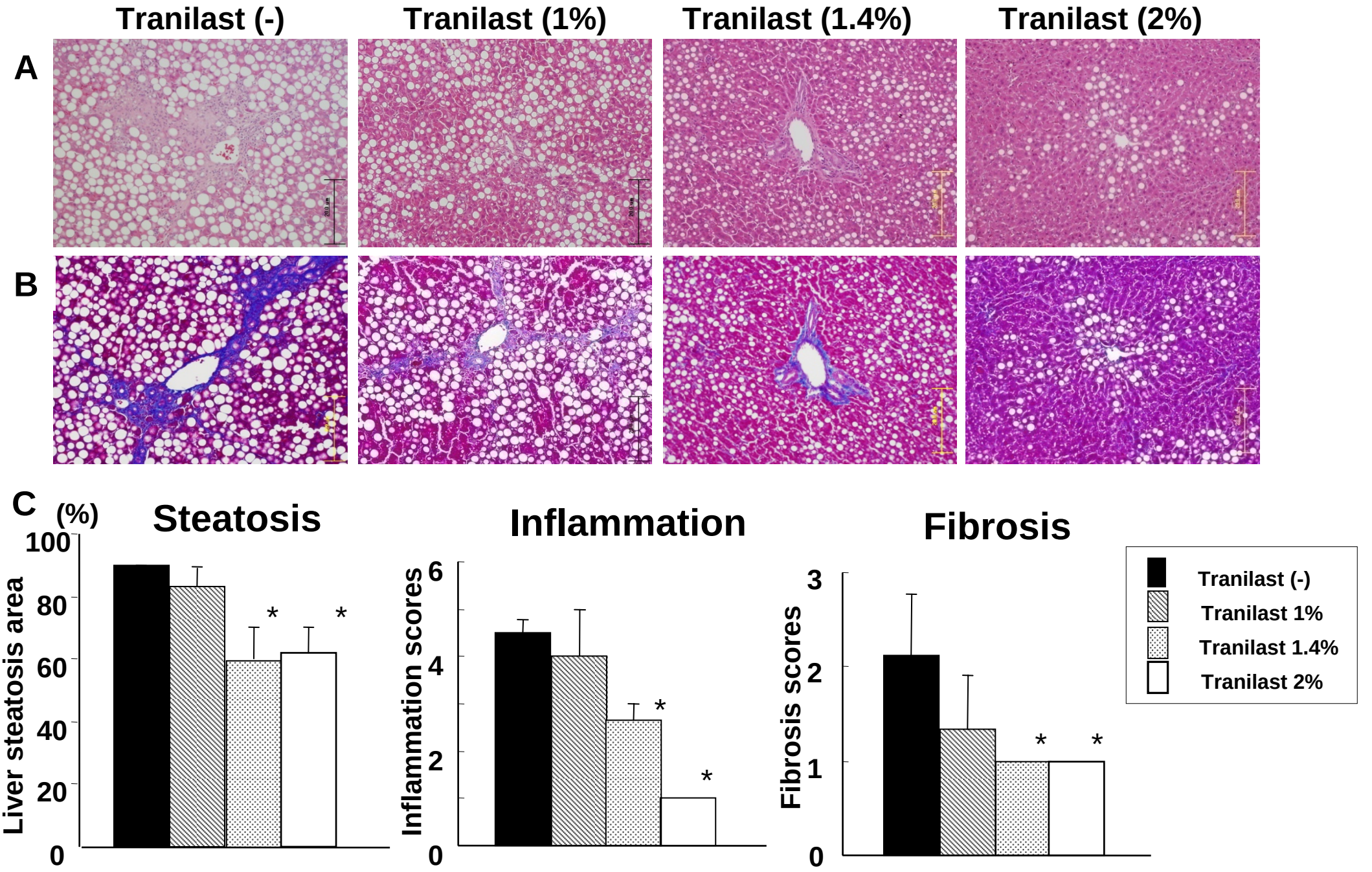
Fig. 8. Tranilast suppressed oxidative stress. Western blot analysis of 4-HNE-modified proteins (A), (C), and these quantification (B), (D), showed that tranilast significantly decreased 4-HNE in the liver of OLETF-MCD and LETO-MCD rats (Values are means ± SEM. **P* < 0.05 versus the MCD diet).

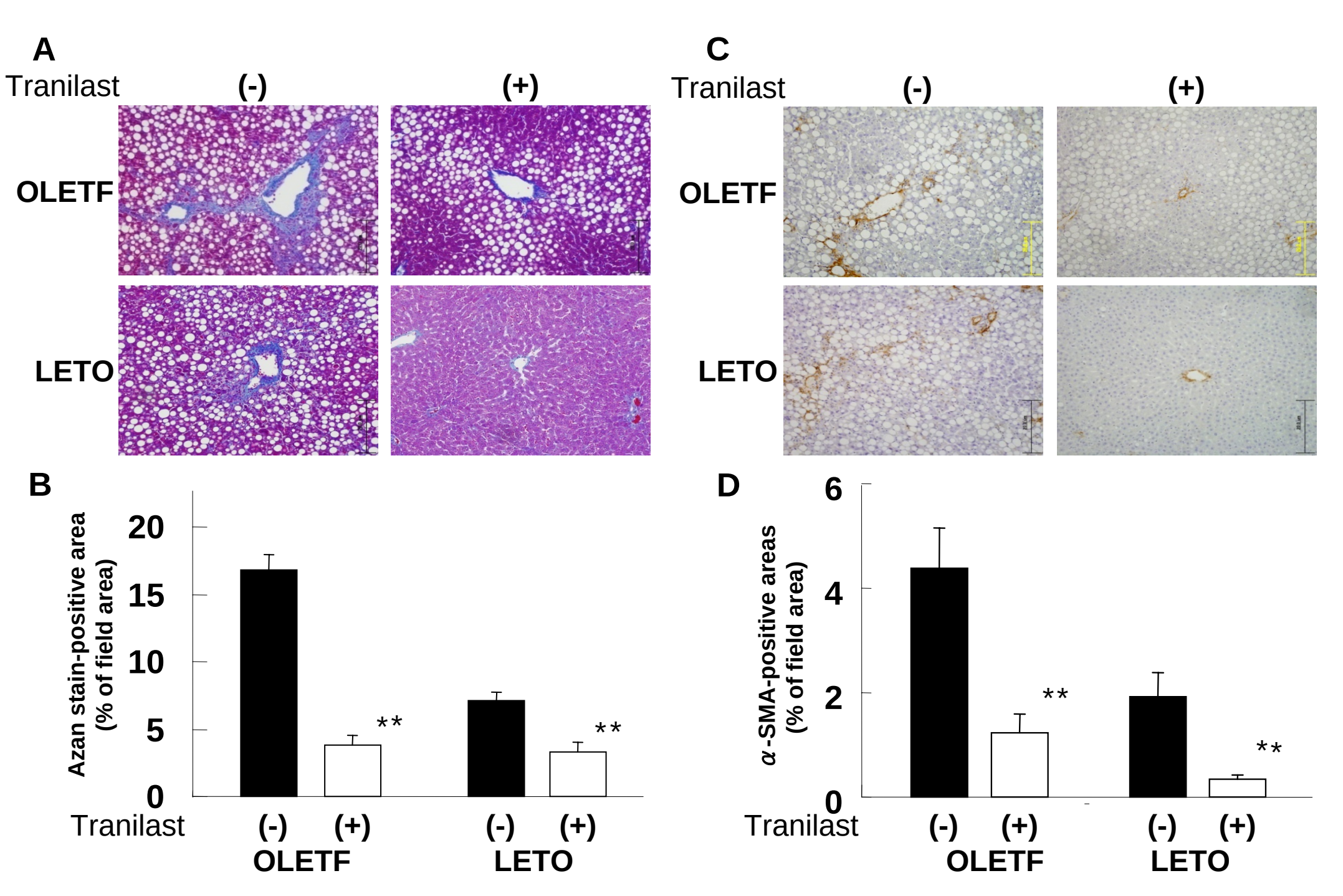
Table 1. Effects of MCD and MCD+tranilast diet on metabolic parameters at 8 weeks of treatment

	OLETF		LETO	
Treatment	(－)	Tranilast (2%)	(－)	Tranilast (2%)
Body weight (g)	402 ± 5.4	359 ± 4.5*	384 ± 4.6+	366 ± 6.7
Food intake (kcal/day)	31.1 ± 2.9	36.5 ± 7.1	43.7 ± 1.7+	41.6 ± 0.8+
Tranilast intake (mg/kg BW/day)	(–)	423 ± 99	(–)	482 ± 21
Liver weight (g)	14.2 ± 0.3	15.8 ± 0.5*	9.9 ± 0.4+	12.6 ± 0.2*+
Serum glucose (fasting) (mg/dL)	107 ± 7.1	138 ± 5.0	118 ± 9.6	122 ± 4.1+
60 min (mg/dL)	222 ± 24.3	238 ± 30.0	172 ± 8.6	150 ± 5.6
120 min (mg/dL)	166 ± 16.9	246 ± 43.8	141 ± 6.0	144 ± 8.2+
Plasma ALT levels (U/L)	165 ± 22.1	80.8 ± 9.9*	118.4 ± 11.0	59.2 ± 3.2*
Serum TNF-**α** levels (pg/ml)	4.0 ± 3.0	unmeasurable	0.8 ± 0.7	unmeasurable

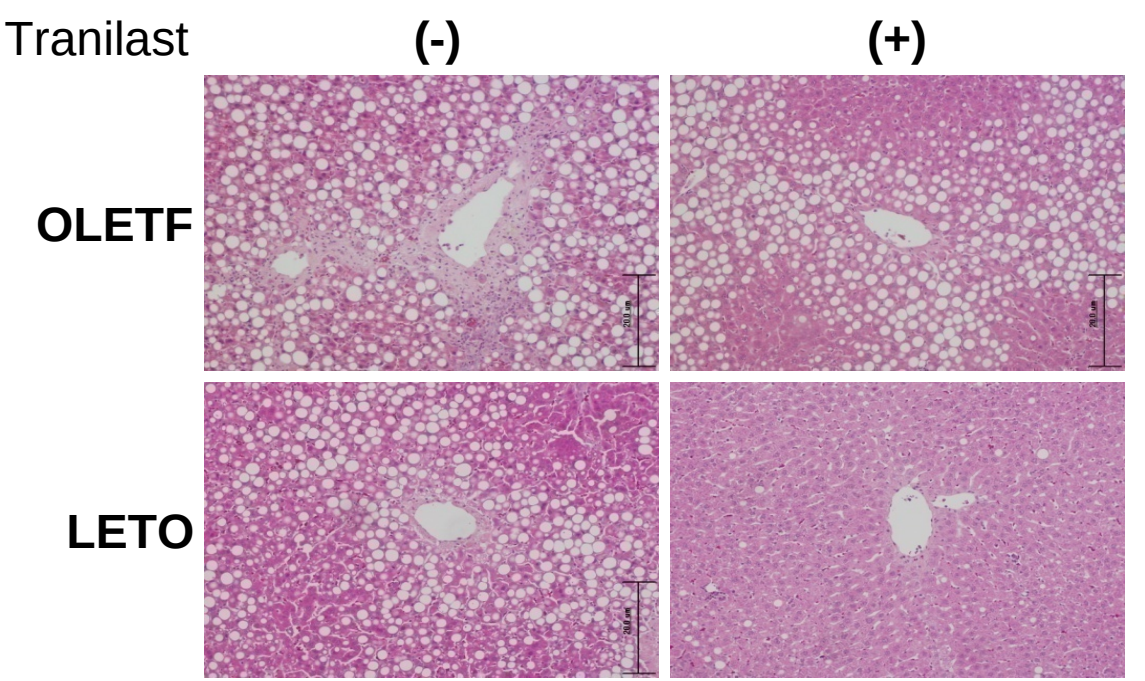
Data are means ± SEM (n = 9–11).

*P < 0.05 versus rats fed the MCD diet. +P < 0.05 versus the OLETF rats.

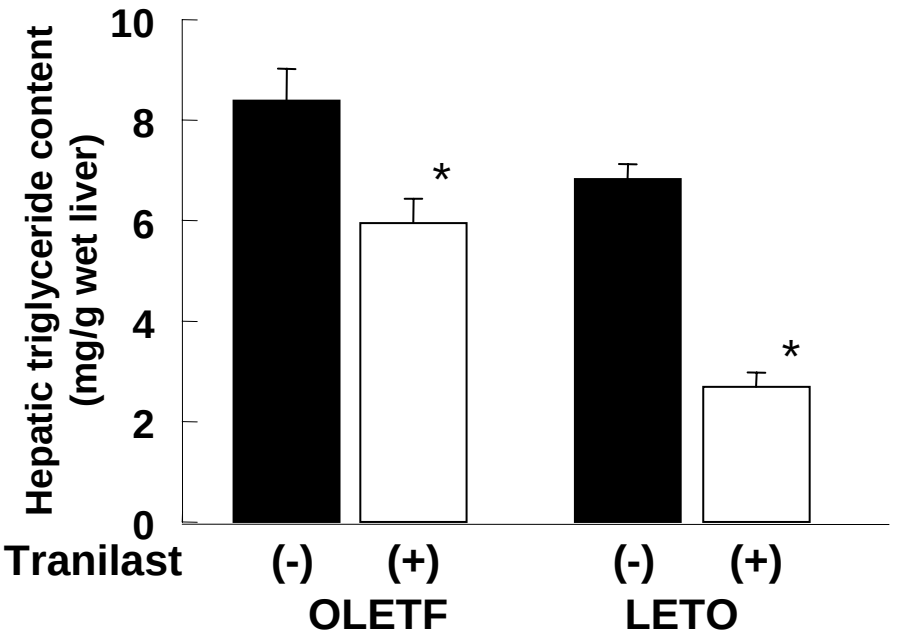

Tranilast (-)
Tranilast (1%)
Tranilast (1.4%)
Tranilast (2%)
A
B
C
(%)
Steatosis
Liver steatosis area
100
80
60
40
20
0
*
*
Inflammation
Inflammation scores
6
4
2
0
*
*
Fibrosis
Fibrosis scores
3
2
1
0
*
*
Tranilast (-)
Tranilast 1%
Tranilast 1.4%
Tranilast 2%

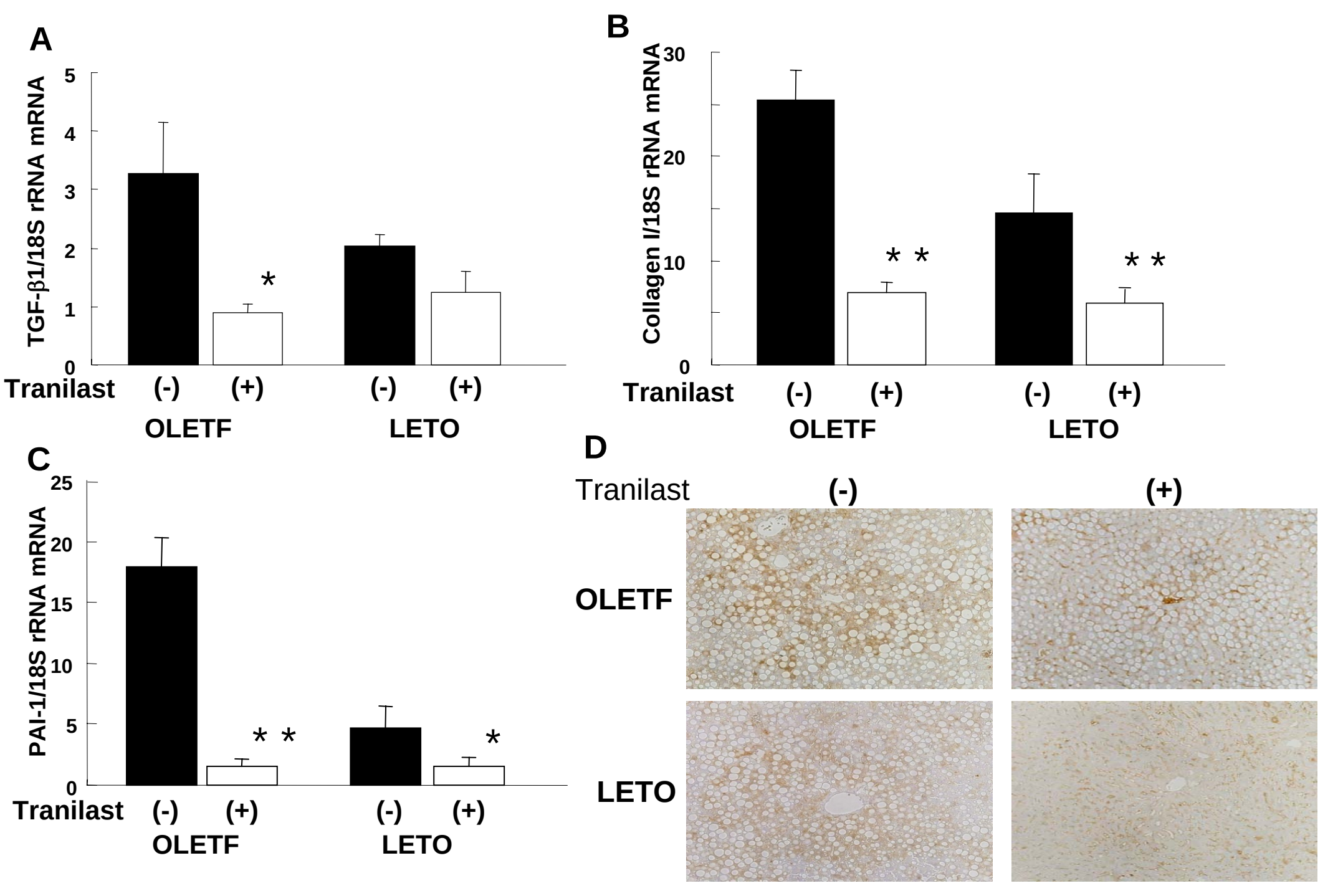

A
Tranilast
(-)
(+)
OLETF
LETO
C
Tranilast
(-)
(+)
OLETF
LETO
B
Azan stain-positive area
(% of field area)
20
15
10
5
0
**
**
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO
D
α-SMA-positive areas
(% of field area)
6
4
2
0
**
**
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO

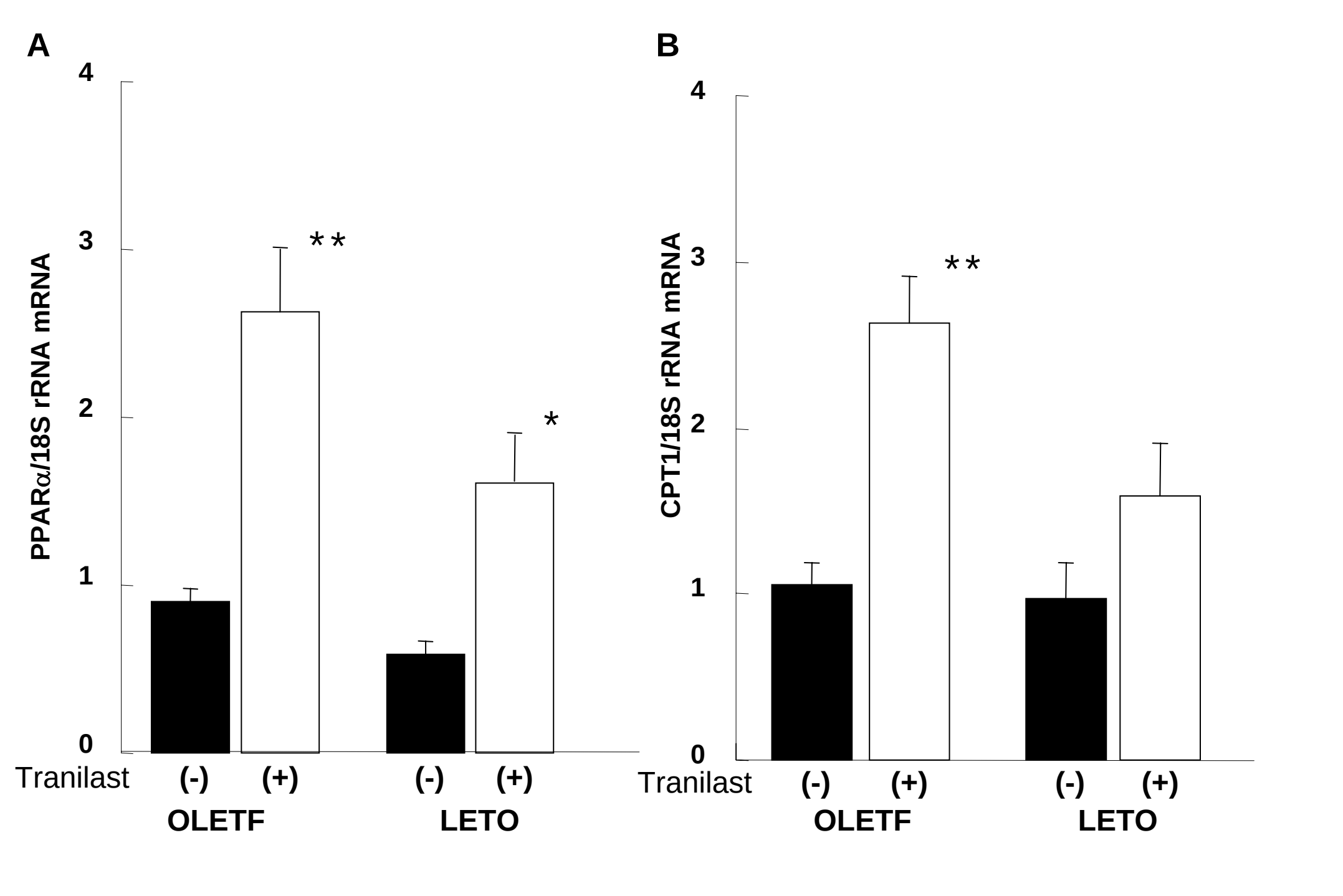
A

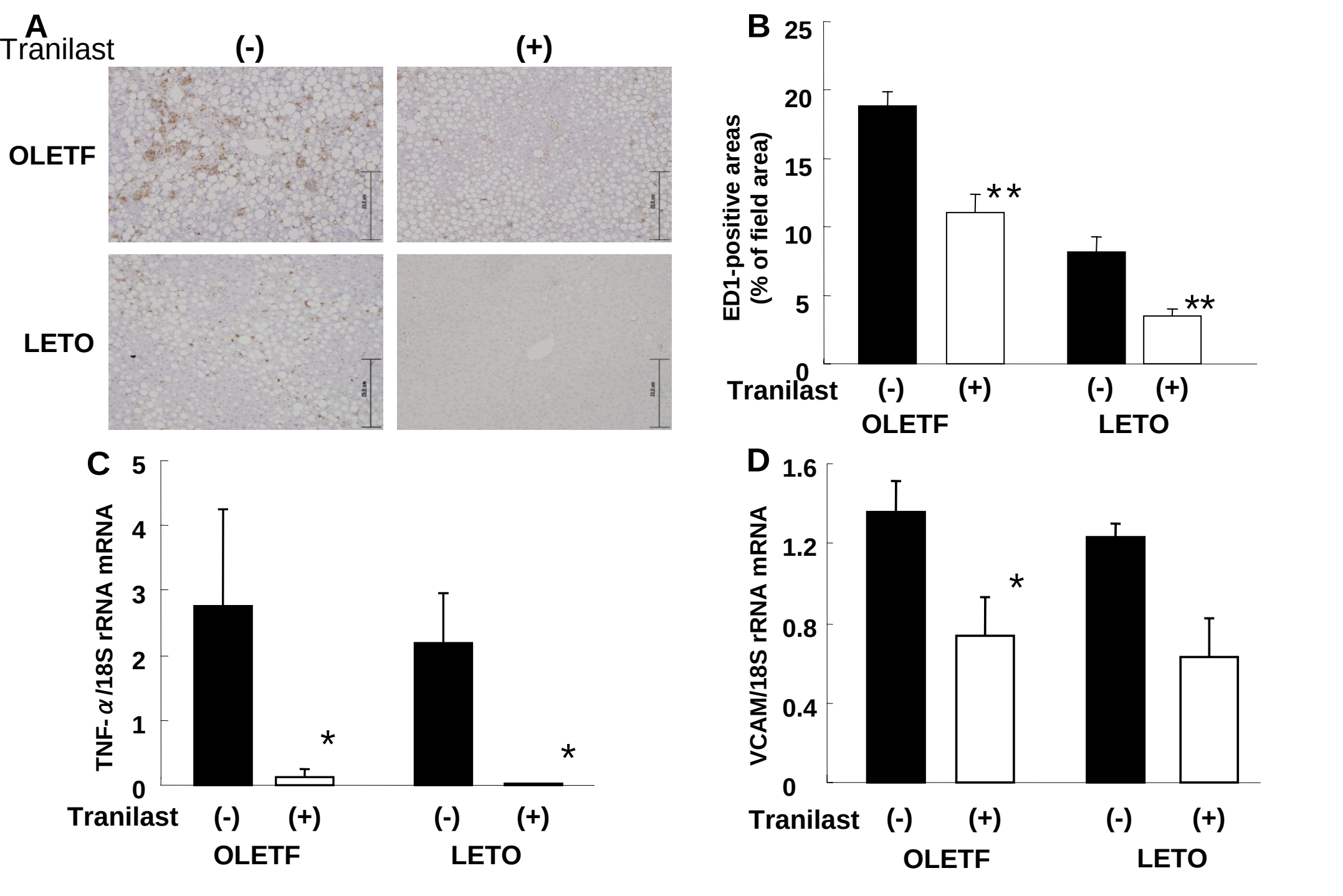

Tranilast
(-)
(+)
OLETF
LETO

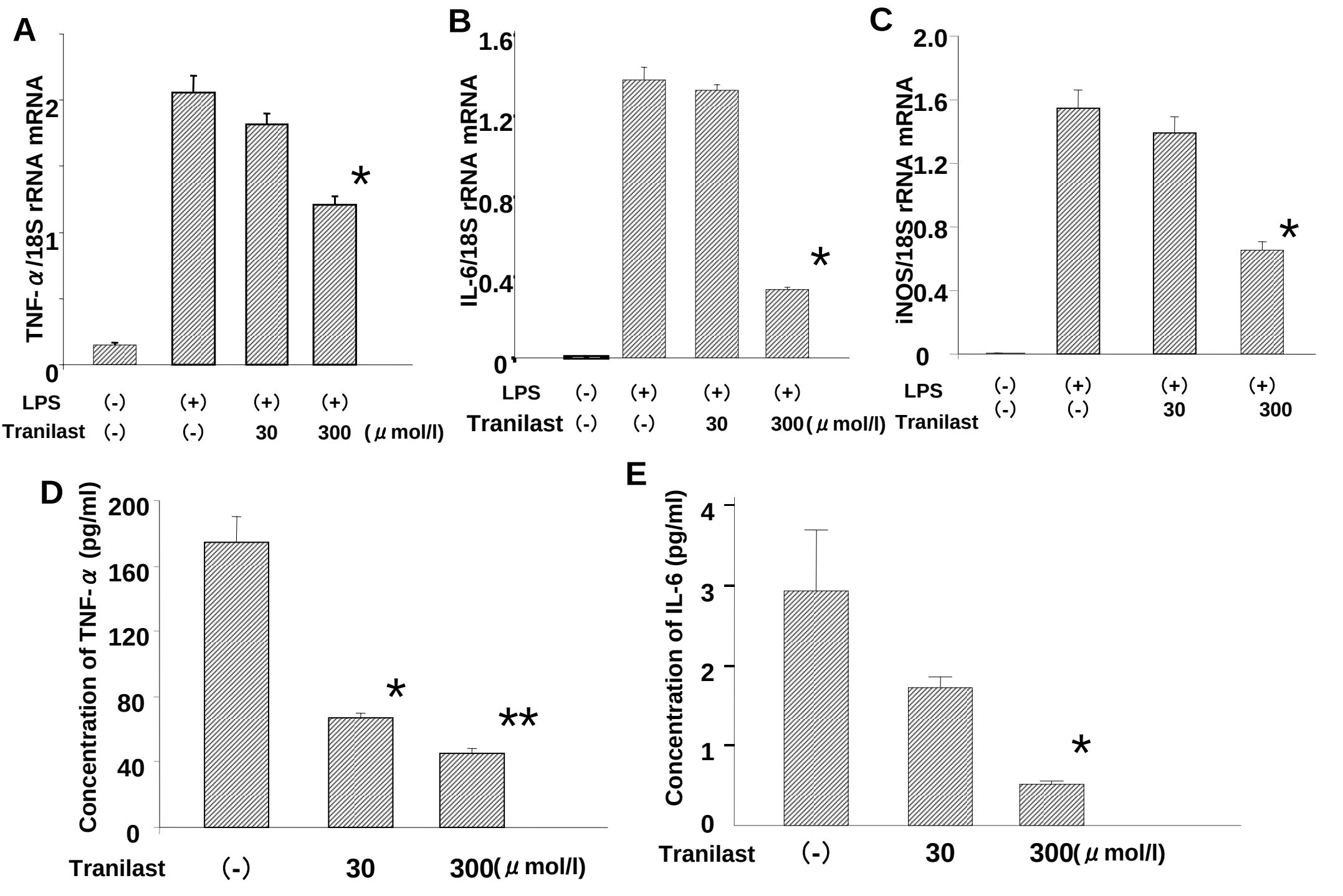
B

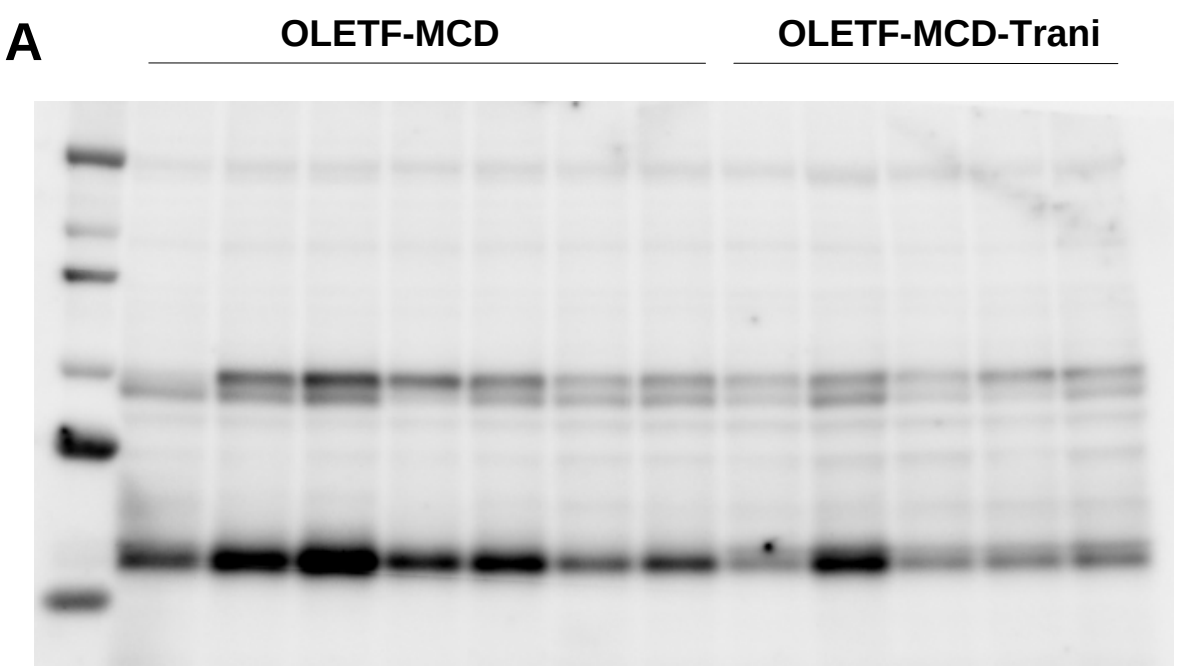

Hepatic triglyceride content
(mg/g wet liver)
10
8
6
4
2
0
*
*
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO

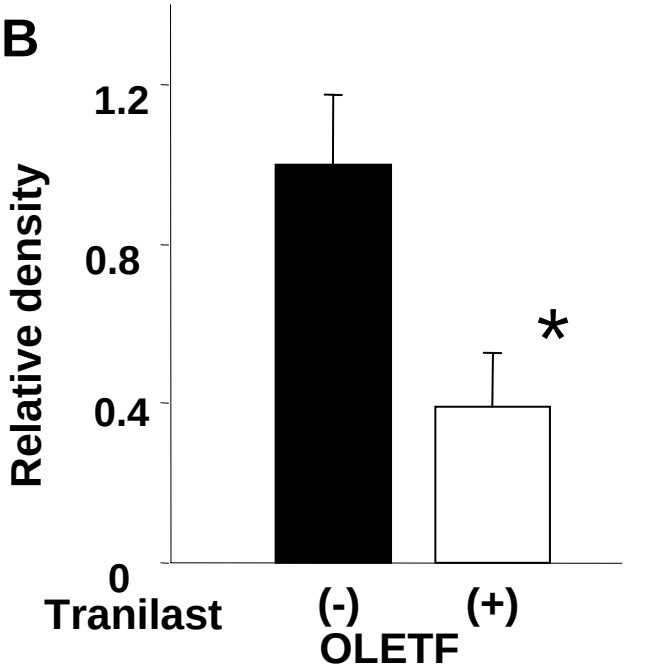

A
TGF-β1/18S rRNA mRNA
0
1
2
3
4
5
*
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO
B
Collagen I/18S rRNA mRNA
0
10
20
30
* *
* *
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO
C
PAI-1/18S rRNA mRNA
0
5
10
15
20
25
* *
*
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO
D
Tranilast
(-)
(+)
OLETF
LETO

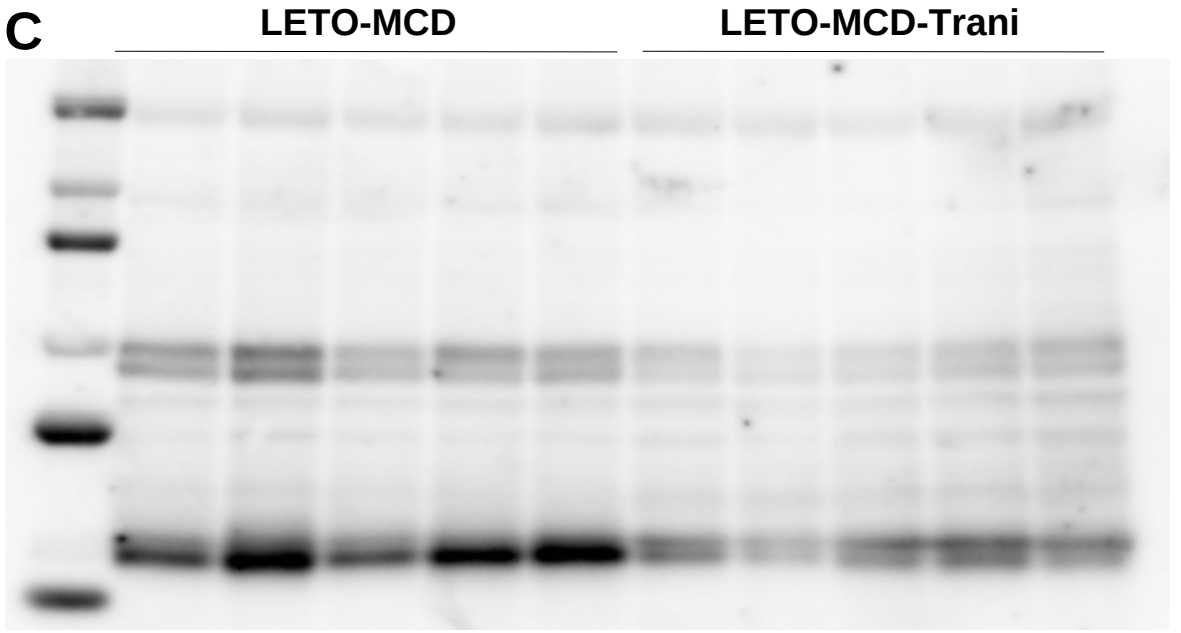

A
PPARα/18S rRNA mRNA
4
3
2
1
0
**
*
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO
B
CPT1/18S rRNA mRNA
4
3
2
1
0
**
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO

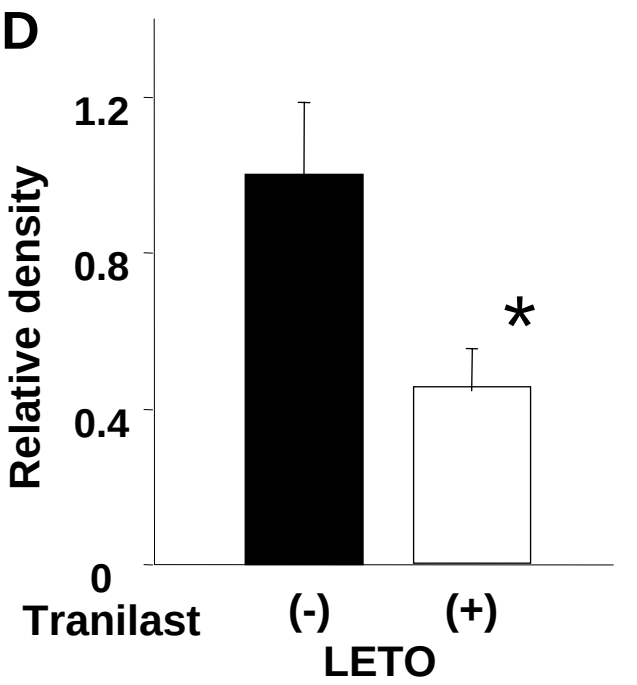

A
Tranilast
(-)
(+)
OLETF
LETO
B
ED1-positive areas
(% of field area)
25
20
15
10
5
0
**
**
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO
C
TNF-α/18S rRNA mRNA
5
4
3
2
1
0
*
*
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO
D
VCAM/18S rRNA mRNA
1.6
1.2
0.8
0.4
0
*
Tranilast
(-)
(+)
(-)
(+)
OLETF
LETO


A
TNF-α/18S rRNA mRNA
0
1
2
*
LPS (-) (+) (+) (+)
Tranilast (-) (-) 30 300 (μmol/l)
B
IL-6/18S rRNA mRNA
0
0.4
0.8
1.2
1.6
*
LPS (-) (+) (+) (+)
Tranilast (-) (-) 30 300 (μmol/l)
C
iNOS/18S rRNA mRNA
0
0.4
0.8
1.2
1.6
2.0
*
LPS (-) (+) (+) (+)
Tranilast (-) (-) 30 300
D
Concentration of TNF-α (pg/ml)
0
40
80
120
160
200
*
**
Tranilast (-) 30 300(μmol/l)
E
Concentration of IL-6 (pg/ml)
0
1
2
3
4
*
Tranilast (-) 30 300(μmol/l)


A
OLETF-MCD
OLETF-MCD-Trani


B
Relative density
1.2
0.8
0.4
0
*
Tranilast
(-)
(+)
OLETF


C
LETO-MCD
LETO-MCD-Trani


D
Relative density
1.2
0.8
0.4
0
*
Tranilast
(-)
(+)
LETO