# Angiotensin Receptor Blockers Potentiate the Protective Effect of Branched-Chain Amino Acids on Skeletal Muscle Atrophy in Cirrhotic Rats

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Scope: This study investigated the combined effect of the angiotensin II (AT-II) receptor blocker losartan and branched-chain amino acids (BCAAs) on skeletal muscle atrophy in rats with cirrhosis and steatohepatitis.
Method and Results: Fischer 344 rats are fed a choline-deficient l-amino acid-defined (CDAA) diet for 12 weeks and treated with oral losartan (30 mg kg−1 day−1) and/or BCAAs (Aminoleban EN, 2500 mg kg−1 day−1). Treatment with losartan and BCAAs attenuated hepatic inflammation and fibrosis and improved skeletal muscle atrophy and strength in CDAA-fed rats. Both agents reduced intramuscular myostatin and pro-inflammatory cytokine levels, resulting in inhibition of the ubiquitin–proteasome system (UPS) through interference with the SMAD and nuclear factor-kappa B pathways, respectively. Losartan also augmented the BCAA-mediated increase of skeletal muscle mass by promoting insulin growth factor-I production and mitochondrial biogenesis. Moreover, losartan decreased the intramuscular expression of transcription factor EB (TFEB), a transcriptional inducer of E3 ubiquitin ligase regulated by AT-II. In vitro assays illustrated that losartan promoted mitochondrial biogenesis and reduced TFEB expression in AT-II-stimulated rat myocytes, thereby potentiating the inhibitory effects of BCAAs on the UPS and caspase-3 cleavage.
Conclusion: These results indicate that this regimen could serve as a novel treatment for patients with sarcopenia and liver cirrhosis.

## 1 Introduction

Sarcopenia, characterized as the loss of skeletal muscle mass, strength, or function, is one of the most common complications in patients with advanced-stage chronic liver disease (CLD).[1] Sarcopenia approximately affects 30–70% of patients with cirrhosis, and it is implicated in higher rates of mortality, frequent hospital admission, reduced quality of life, and the development of other cirrhotic complications.[2] Sarcopenia in patients with cirrhosis results from an imbalance between skeletal muscle protein synthesis and degradation, which is based on a multifactorial pathogenesis involving impaired protein turnover, hyperammonemia, chronic inflammation, endocrine changes, and malnutrition; hence, it is difficult to identify therapeutic targets.[1, 2] Indeed, the efficacy of physical activity and

nutritional supplementation against sarcopenia in cirrhosis has been investigated, but no approved and effective therapeutics to counteract sarcopenia in cirrhosis are available.[3]

Branched-chain amino acids (BCAAs), comprising the essential amino acids leucine, isoleucine, and valine, are known to play an important role in skeletal muscle homeostasis. [4–5] They contribute to both increased protein synthesis and decreased muscle degradation by activating intracellular mammalian target of rapamycin (mTOR) complex 1 (mTORC1) signaling and inhibiting the ubiquitin–proteasome system (UPS).[6] Because lower plasma and muscle levels of BCAA are frequently observed in patients with cirrhosis, the effects of BCAA supplementation on sarcopenia in patients with cirrhosis has been explored.[7] However, most clinical studies suggested that BCAA supplementation partially but not sufficiently improved muscle mass or function as a single treatment.[3, 8] Therefore, we postulated that the combination of BCAAs and another agent with anti-sarcopenic effects would be more beneficial for treating sarcopenia in patients with cirrhosis.

Angiotensin-II (AT-II) acts as a vasoconstrictor with key roles in hypertension, heart failure, and chronic renal injury, and it influences the pathogenesis of CLD beyond vasoconstriction.[9] The AT-II/AT-II type 1 receptor (AT1R) axis has been reported to participate in the development of liver fibrosis by regulating cell proliferation and pro-fibrogenic responses in hepatic stellate cells.[10] In addition, a number of studies demonstrated that inhibition of AT-II by AT1R blockers (ARBs) could exert strong inhibitory effects on the development of liver fibrosis.[11] Notably, increasing evidence also indicates that AT-II hyperactivity induces skeletal muscle degradation.[12-15] In animal models, increased AT-II levels induced skeletal fiber wasting through augmented protein degradation and apoptosis and repressed protein synthesis.[12] Additional animal studies confirmed the protective effects of ARBs on muscle function and mass loss.[13, 14] Moreover, a recent clinical study revealed that ARB treatment potentially prevents muscle strength deficiency in patients undergoing chronic hemodialysis.[15] However, the ability of ARBs to modulate the myotrophic activity of BCAAs in patients with liver cirrhosis-related muscle atrophy has not been elucidated.

In this study, we investigated the effects of the combination of BCAAs and ARBs on skeletal muscle atrophy and analyzed the therapeutic mechanisms using a choline-deficient l-amino acid-defined (CDAA)-fed rat model of cirrhosis and steatohepatitis.[16]

## 2 Results

2.1 Combination of Losartan with BCAAs Sufficiently Suppresses Inflammatory Responses and Liver Fibrosis in CDAA-fed Rats

Initially, we investigated the effects of losartan and BCAAs on hepatic inflammation and fibrosis development in rats with CDAA diet-induced steatohepatitis (Figure 1A). Twelve weeks of CDAA diet feeding induced remarkable body weight loss and relative liver weight gain in rats, and treatment with losartan and BCAAs did not prevent these changes (Figure 1B). CDAA-fed rats displayed a marked elevation of serum alanine transaminase (ALT) levels as well as a decline of serum albumin levels (Figure 1C,D). Neither losartan nor BCAAs significantly affected ALT levels (Figure 1C). Meanwhile, either treatment with losartan or BCAAs suppressed the progression of hypoalbuminemia in CDAA-fed rats which was augmented by combining both agents (Figure 1D). Serum levels of insulin-like growth factor-1 (IGF-1), known as a myotrophic factor, were lower in CDAA-fed rats than in choline-sufficient amino acid-defined (CSAA) fed rats, and remarkably these were increased by either treatment with losartan or BCAAs which was augmented by both combination (Figure 1E). Histological assessment illustrated that CDAA diet-induced steatosis was not significantly altered by treatment with losartan and BCAAs (Figure 1F). Meanwhile, both treatments reduced the hepatic mRNA expression of pro-inflammatory genes including tumor necrosis factor-$\alpha$ (Tnfa), Interleukin-1$\beta$ (Il1b), and Il6 in CDAA-fed rats (Figure 1G). In addition, 12 weeks of CDAA diet feeding sufficiently induced the development of liver fibrosis according to Sirius Red staining (Figure 1F). Treatment with either losartan or BCAAs inhibited CDAA-induced liver fibrosis, and the combination of the agents exhibited enhanced anti-fibrotic effects (Figure 1F,H). The observed suppression of hepatic fibrosis mediated by losartan and BCAAs coincided with a decline in the hepatic mRNA expression of pro-fibrotic genes including Actin alpha 2 (Acta2) and Collagen Type1 Alpha 1 Chain (Col1a1) (Figure 1I). These findings indicate that the combination of losartan and BCAAs efficiently suppresses hepatic inflammatory responses and fibrosis development in CDAA-fed rats.

2.2 Inhibitory Effects of Losartan and BCAAs on the Atrophy of Psoas Muscle and the Decline of Forelimb Grip Strength in CDAA-fed Rats

Given the significant loss of body weight in CDAA-fed rats, we assumed that skeletal muscle atrophy developed in this model. To evaluate the chronological changes of skeletal muscle mass, we measured the psoas muscle area on axial imaging at the L-spine level and calculated the psoas muscle mass index (PMI) at each time point during the experimental period (Figure 2A). As illustrated in Figure 2B, CDAA-fed rats were shorter in length than CSAA-fed rats at the end of the experiment, and treatment with losartan and BCAAs did not reverse this change. PMI was significantly lower after 4 weeks of feeding in the CDAA-fed group than in the

CSAA-fed group (Figure 2C,D). Of note, treatment with losartan and BCAAs inhibited the CDAA-induced reduction of PMI after 8 weeks of administration, and these effects were markedly augmented by the combined treatment at the end of the experiment (Figure 2C,D).

Next, we assessed the skeletal muscle strength in the experimental groups by measurement of the forelimb grip strength. As shown in Figure 2E,F, the CDAA-fed rats showed a significant decline in the forelimb grip strength as compared with CSAA-fed control rats after 3 weeks of feeding. Although either treatment with losartan or BCAAs did not affect the declined muscle strength in the CDAA-fed rats, combination of both agents significantly enforced the forelimb grip strength at 12 weeks of treatment (Figure 2E,F).

2.3 Losartan and BCAAs Promote Gastrocnemius Muscle Protein Synthesis and Inhibit its Degradation of in CDAA-Fed Rats

To uncover the mechanism of the anti-atrophic effects of losartan and BCAAs, we next assessed protein synthesis and degradation in the soleus muscle tissues of CDAA-fed rats. As presented in Figure 3A–C,E,H staining revealed atrophic changes in gastrocnemius muscle fiber in CDAA-fed rats, in line with the decreases of fiber length and density, and these changes were efficiently suppressed by treatment with losartan and BCAAs. These suppressive effects were more prominent in the concurrent treatment group than each monotherapy group (Figure 3A–C). The phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling is a key pathway in muscle protein synthesis. Thus, we examined the phosphorylation status of p70S6 kinase (p70S6K), a downstream target of mTOR, in gastrocnemius muscle tissues.[17] p-p70S6K (Thr389) protein expression was remarkably lower in CDAA-fed rates than in CSAA-fed rats, suggesting the repression of mTOR signaling (Figure 3D). p-p70S6K expression in CDAA-fed rats was strikingly increased by BCAA treatment and mildly by losartan treatment, and the combination of both agents could recover the phosphorylation rate to that in CSAA-fed rats (Figure 3D). Next, we evaluated the intramuscular expression of the E3 ubiquitin ligases atrogin-1 and Muscle RING-finger protein-1 (MuRF-1), crucial regulators of ubiquitin-mediated skeletal muscle protein degradation. The intramuscular mRNA expression of Fbxo32 and Trim63, which encode atrogin-1 and MuRF-1, respectively, was increased in CDAA-fed rats, and this upregulation was significantly suppressed by treatment with losartan and BCAAs (Figure 3E).

2.4 Intramuscular Myostatin and Pro-inflammatory Cytokine Pathways Are Suppressed by Losartan and BCAAs

We next investigated several myokine-regulating pathways to assess whether the anti-

myopenic effects of losartan and BCAAs were associated with the improvement of hepatic inflammation and fibrosis. Myostatin, a transforming growth factor (TGF)-$\beta$ superfamily protein, is a myokine produced and released by myocytes that acts on muscle cells to inhibit muscle cell growth.[18] Myostatin levels are reported to be elevated in patients with cirrhosis.[19] Correspondently to the development of liver fibrosis, the intramuscular concentrations of myostatin were remarkably higher in CDAA-fed rats than in CSAA-fed rats, and this increase was reduced by treatment with losartan and BCAAs (Figure 4A). In accordance with intramuscular myostatin levels, the increase of SMAD2/3 phosphorylation in CDAA-fed rats was attenuated by treatment with both agents (Figure 4B,C). We further examined pro-inflammatory nuclear factor-kappa B (NF-$\kappa$B) signaling in gastrocnemius muscle tissue. The intramuscular mRNA levels of pro-inflammatory cytokines including Tnfa, Il1b, and Il6 were upregulated in correspondence with the intrahepatic expression of these genes in CDAA-fed rats, and this upregulation was significantly repressed by combined treatment with losartan and BCAAs (Figure 4D). In accordance with the mRNA expression of these pro-inflammatory cytokines, the combination of both agents efficiently attenuated p65 phosphorylation, indicating the suppression of NF-$\kappa$B activation (Figure 4E,F). These findings indicated that the anti-myopenic effects of both agents partially result from the suppression of hepatic inflammation and fibrosis.

2.5 Losartan and BCAAs Restore Mitochondrial Biogenesis and Interfere with FoxO1 and FoxO3a Transcription

Skeletal muscle mitochondrial dysfunction has also been implicated in muscle fiber atrophy.[20] Therefore, we analyzed the effects of losartan and BCAAs on mitochondrial biogenesis in gastrocnemius muscle tissue in the current model. As illustrated in Figure 5A, genes related to mitochondrial biogenesis, including peroxisome proliferator-activated receptor $\gamma$ coactivator-1$\alpha$ (PGC-1$\alpha$; Ppargc1a), citrate synthetase, sirtuin 1, and mitochondrial transcription factor A (TFAM) (Tfam), were downregulated in the gastrocnemius muscle of CDAA-fed rats, and this downregulation was significantly restored by treatment with both agents. We also found that treatment with both agents upregulated the protein levels of PGC-1$\alpha$ and mtTFA in the muscle of CDAA-fed rats in parallel with the mRNA expressions (Figure 5B). Moreover, the amount of mitochondrial DNA/nuclear DNA (mtDNA/nDNA) in the experimental groups was measured in the gastrocnemius muscle tissues since the increase in the mtDNA copy number was considered the index of mitochondrial biogenesis. Figure 5C exhibits a significant reduction in the mitochondrial copy number in the CDAA-fed rats as compared with that of CSAA-fed control rats. Furthermore, the mitochondrial copy number in in the CDAA-fed rats treated with losartan and BCAAs

increased significantly in comparison with that in the vehicle-treated CDAA-fed rats. These results potently support that losartan and BCAAs enhance the mitochondrial biogenesis in the cirrhotic rats.

Meanwhile, atrogin-1 and MuRF-1 are known to be transcriptionally regulated by Forkhead box Os (FOXOs).[21] Thus, we next performed immunohistochemical analysis to evaluate the intracellular localization of FoxO1 and FoxO3a in gastrocnemius muscle tissue to identify whether the losartan- and BCAAs-mediated downregulation of both genes is implicated in the regulation of FoxOs. In the gastrocnemius muscle of CSAA-fed control rats, both FoxO1 and FoxO3a mainly localized to the cytoplasm and limitedly to the nuclei, whereas these proteins exhibited remarkably increased intranuclear localization in CDAA-fed rats, indicating the transcriptional activation of FoxO1 and FoxO3a (Figure 5D,E). It was noteworthy that treatment with losartan and BCAAs efficiently interfered with the intranuclear translocation of FoxO1 and FoxO3a in the gastrocnemius muscle of CDAA-fed rats (Figure 5D,E). Indeed, western blotting showed that the protein levels of FOXO1 and FOXO3a were increased in the nuclei and conversely decreased in the cytosol of gastrocnemius muscle tissues in CDAA-fed rats which was suppressed by treatments with losartan and BCAAs (Figure 5F). These findings suggest that the losartan- and BCAA-induced attenuation of muscle protein degradation is mediated by the inhibition of FoxO1 and FoxO3a transcription. These molecular changes demonstrate that losartan and BCAAs interfere with FoxO1 and FoxO3a transcription by restoring mitochondrial biogenesis.

2.6 Direct Effects of Losartan and BCAAs on AT-II-Stimulated Skeletal Muscle Cells

A recent report found that the lysosomal hydrolase-coordinating transcription factor EB (TFEB) is a regulator of AT-II/AT1R-mediated MuRF-1 expression.[22] In the current model, the mRNA expression of Tfeb was increased in parallel with the elevated levels of AT-II in the gastrocnemius muscle of CDAA-fed rats (Figure 6A,B). Treatment with losartan did not affect AT-II levels, but it reduced intramuscular Tfeb expression (Figure 6A,B). These results suggest that losartan-mediated anti-atrophic effects are also partially attributed to Tfeb downregulation via AT1R blockade. To further analyze this sequence, we examined the effects of losartan and BCAAs on in vitro AT-II-stimulated rat myocytes, which were differentiated from L6 myoblasts as confirmed by increased mRNA expressions of MyoD1 and MyoG (Figure 6C and Figure S1A, Supporting Information). AT-II stimulated the upregulation of Fbxo32 and Trim63 in a dose- and time-dependent manner (Figure S1B, Supporting Information, and Figure 6D). Notably, treatment with losartan attenuated the AT-II–stimulated upregulation of both genes (Figure 6E). As expected, losartan significantly

reduced the AT-II–stimulated upregulation of Tfeb in rat myocytes (Figure 6F). This indicates that the losartan-mediated inhibition of the ubiquitin–proteasome system (UPS) is associated with blockade of the AT1R/TFEB pathway. Conversely, treatment with BCAAs also unexpectedly decreased AT-II–induced Fbxo32 and Trim63 expression, although it did not affect Tfeb expression (Figure 6E,F). Interestingly, BCAAs and losartan potently increased the expression of Ppargc1a and Tfam, which was reduced by AT-II stimulation in rat myocytes (Figure 6G). Consistently, both losartan and BCAAs suppressed caspase-3 cleavage and NF-$\kappa$B activation (Figure 6H). These observations suggest that BCAAs also reversed the AT-II–induced impairment of mitochondrial biogenesis.

## 3 Discussion

In the present study, we provided the initial evidence that combination treatment with ARBs efficiently augments the inhibitory effects of BCAAs on skeletal muscle atrophy in a rat liver cirrhosis model. First, it was a novel finding that CDAA-fed cirrhotic rats exhibited prominent atrophic changes in their skeletal muscle. Recent animal studies demonstrated skeletal muscle wasting in several murine liver fibrosis models including bile duct ligation, carbon tetrachloride, and the 5-diethoxycarbonyl-1,4-dihydrocollidine models.[23] Meanwhile, it has been reported that a choline is essential for the homeostasis in the skeletal muscle growth and function.[24] Moreover, a choline and methionine deficiency has been suggested to induce hepatic overexpression of AT-II promoting skeletal muscle degradation.[25] Based on these evidences, we heuristically found that CDAA-fed rats exhibited both physiological and pathological evidence of skeletal muscle atrophy, suggesting that this model can also be used to examine cirrhosis-based myopenia.

In our findings, both ARBs and BCAAs attenuated hepatic inflammation and fibrosis in cirrhotic rats, in accordance with the results of previous basic studies, and intramuscular myostatin levels were decreased in parallel to the improvement of hepatic fibrosis.[11, 19, 26] Myostatin overproduction has been recognized as a key factor in the progression of sarcopenia in cirrhosis.[27, 28] Qiu et al.[27] demonstrated that hyperammonemia in cirrhosis stimulated myostatin expression via NF-$\kappa$B activation. Nishikawa et al. [28] observed that serum myostatin concentrations increased with worsening liver disease as measured using the Child–Pugh score. Moreover, the intramuscular expression of pro-inflammatory cytokines was also reduced in accordance with their expression in the liver. These observations indicated that the anti-myopenic effects of both agents are at least partially associated with the improvement of hepatic pathological phenotypes.

In addition to the hepatic function-dependent effects of ARBs, our pathological and molecular analyses also indicated the multifunctional pathways through which these drugs could suppress skeletal muscle atrophy in CDAA-fed rats. First, CDAA-fed rats exhibited lower serum IGF-1 levels, and losartan treatment suppressed these changes. This finding suggests that the restoration of IGF-1 expression by losartan contributes to the suppression of muscle wasting. Song et al. reported that AT-II induced muscle wasting by blocking the action of IGF-1 in skeletal muscle through decreasing its expression and signaling, leading to stimulation of the UPS and apoptosis.[29] In addition, Burks et al. indicated that losartan has beneficial effects on skeletal muscle remodeling in response to injury and protects against disuse atrophy in sarcopenia by modulating IGF-1/Akt/mTOR signaling cascades.[14] However, further investigation is required to elucidate the underlying mechanism of losartan-mediated IGF-1 production in CDAA-fed rats. Second, CDAA-fed rats exhibited marked suppression of mitochondrial biogenesis in their skeletal muscle, and this suppression was efficiently relieved by treatment with losartan. Inoue et al. demonstrated that that AT-II–induced oxidative stress leads to mitochondrial dysfunction in skeletal muscle.[30] Mitsuishi et al. reported that AT-II reduces mitochondrial content and impairs mitochondrial biogenesis in cultured myocytes and skeletal muscle in mice.[31] In accordance with these findings, our in vitro assay also demonstrated that losartan inhibited the AT-II–induced downregulation of PGC-1 $\alpha$ and TFAM in rat myocytes. Third, Bois et al.[22] suggested that the basic helix-loop-helix TFEB is a potent MuRF-1 inducer and found that TFEB activity is regulated via the AT-II/protein kinase D1/histone deacetylase-5 signal transduction pathway. In this context, our results revealed that losartan potently reduced intramuscular TFEB expression in both CDAA-fed rats and AT-II-stimulated myocytes. Thus, TFEB downregulation is relevant to losartan-mediated anti-myopenic effects.

Interestingly, we found that BCAAs suppressed muscle wasting by both promoting protein synthesis and preventing protein degradation. BCAAs mainly contribute to protein synthesis through the different pharmacological actions of their components (leucine, isoleucine, and valine). Leucine acts on mTORC1 kinase to initiate translation and protein synthesis.[32] Valine also plays a valuable role in muscle protein synthesis enhancement.[33] Isoleucine regulates the immunity, inflammation, and protein metabolism and promotes muscle protein synthesis.[5] Conversely, a recent clinical study reported that BCAA ingestion reduced atrogin-1 mRNA expression and prevented the exercise-induced increase of MuRF-1 total protein expression in both the resting and exercising legs.[34] It has been reported that BCAA-induced mTORC1 activation is positively correlated with cell oxidative capacity, and mTORC regulates PGC-1 $\alpha$ coactivation of its own promoter and mitochondrial gene expression in muscle cells.[35] Another report illustrated that PGC-1 $\alpha$ protects skeletal

muscle from atrophy by suppressing FoxO3 activity.[36] Consistently, our results indicated that BCAA supplementation induced the expression of mitochondrial biogenesis-related genes and inhibited FoxO activation in the skeletal muscle of CDAA-fed rats. Moreover, Yamanashi et al.[37] recently demonstrated that BCAA supplementation ameliorated AT-II–induced muscle atrophy. To support this evidence, our in vitro experiments elucidated that BCAAs decreased atrogin-1 and MuRF-1 expression and protected against apoptosis and inflammation by improving mitochondrial biogenesis in AT-II–mediated rat myocytes.

Several limitations were apparent in the present study. First, we observed that ARB and BCAA treatment decreased intramuscular myostatin levels with the concomitant improvement of liver inflammation and fibrosis, but the factors responsible for these effects were not identified. In this context, CDAA feeding and/or both agents did not change blood ammonia levels (data not shown). A recent study indicated that p65 NF-$\kappa$B binding to the myostatin gene promoter was also increased in response to TNF-$\alpha$ treatment as well as ammonia in myocytes.[27] Therefore, the downregulation of myostatin by both agents was potentially associated with attenuated inflammatory responses rather than ammonia levels in this model. Further investigation is required to address the underlying mechanisms.

Second, we consider that the combination therapy with losartan and BCAAs exert an additive effect but not a synergistic effect against cirrhosis-related skeletal muscle atrophy. Our in vitro study elucidated that BCAAs could attenuate AT-II-induced Atrogin-1 and MuRF-1 upregulations, mitochondrial dysfunction and apoptosis in myocytes cells as with the results in the previous report. [37] On the other hand, recent study has shown that valsartan, another ARB, administration in rats resulted in decreased plasma BCAA concentrations as well as increased branched chain keto-acid dehydrogenase activity. [38] These findings suspect that the effects of losartan with BCAA are not necessarily complementary. Finally, we found that losartan and BCAAs suppressed a decline in forelimb grip strength in cirrhotic rats. However, further investigation by quantifying mechanical properties of isolated muscle is required to in detail analyze muscle strength.[39]

Taken together, ARBs potentiated the protective effects of BCAAs on skeletal muscle atrophy in CDAA-fed cirrhotic rats. This enhancing effect is based on multiple regulatory functions including the inhibition of myokine signaling pathways, prevention of hepatic inflammation and fibrosis, restoration of IGF-1 expression, and promotion of mitochondrial biogenesis and the AT-II/AT1R/TFEB pathway. It is noteworthy that these inhibitory effects were achieved at clinically relevant low doses without causing any serious adverse events. Therefore, this

combination regimen may eventually emerge as a potential new therapeutic strategy for treating sarcopenia in patients with cirrhosis.

## 4 Experimental Section

### Animals and Experimental Protocol

Male Fischer 344 rats aged 6 weeks (CLEA Japan, Tokyo, Japan) were housed at 23 ± 3 ° C and with 50 ± 20% humidity and a 12 h light/12 h dark cycle. All experiments were performed over a 12-week period. The rats were randomly divided into five groups and treated as follows (n = 10): 1) CSAA diet (Research Diets Inc., New Brunswick, NJ, USA) plus vehicle; 2) CDAA diet (Research Diets Inc.) plus vehicle; 3) CDAA diet with oral losartan dissolved in water (30 mg kg−1 day−1); 4) CDAA and BCAA (Aminoleban EN, 2500 mg kg−1 day−1, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) diet; and 5) CDAA and BCAA diet plus oral losartan.[40] The same amount of lactose hydrate was used as vehicle. All rats underwent abdominal computed tomography every 4 weeks using CosmoScan FX (Rigaku, Tokyo, Japan). The psoas muscle mass index (PMI, cross-sectional area/height2) was assessed on a single computed tomography slice (image) at the level of the L3 pedicle using Slice-O-Matic (Tomovision, Montreal, Canada).[41] Body weight was measured in each rat every 4 weeks. At the end of the 12-week experimental period, the rats were anesthetized, their abdominal cavities were opened, blood samples were drawn via an aortic puncture to measure serum ALT, albumin, and IGF-1 levels. Liver and gastrocnemius were harvested and immediately fixed in neutral-buffered formalin for histological findings. The animal care and experimental procedures were approved by the ethics committee of Experimental Animal Care in Nara Medical University, Kashihara, Japan (No. 12639, No. 12750).

### Rat Myoblast Culture

L6 rat skeletal muscle myoblasts were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (1% penicillin and streptomycin) at 37 ° C in a 5% CO2 air environment. To induce differentiation into myocytes, cells were further cultured in DMEM containing 2% horse serum for 8 days.[42] Cells were fed fresh medium every 48 h and used at the stage of myotubes (60–70%). Myogenic differentiation into myotubes was confirmed by microscopy based on the morphological alignment, elongation, and fusion. After terminating cell culture, the differentiated myotubes were stimulated with AT-II (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at different concentrations (1 × 10−8−1 × 10−5 M) for 0−48 h at 1 × 10−6 M for optimization. For each assay, L6 myotubes was

treated with AT-II (1 × 10−6 M), losartan (1 × 10−6 M), and/or BCAAs (2 mM) for 48 h.

Plasma rat IGF-1 Measurement

Serum rat IGF-1 concentrations were measured using a Mouse/Rat IGF-I/IGF-1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). All samples were processed and assayed according to the manufacturer's protocol.

Measurement of Forelimb Grip Strength

Forelimb grip strength was measured by using a commercial grip strength test meter (MK-380Si; Muromachi Kikai, Tokyo, Japan) as previously described.[43] During the grip strength test, the rats were allowed to use their front paws to grab a horizontal bar mounted on the gauge, and the tail was slowly pulled back. The peak tension was recorded at the time the mouse released the grip on the bar. Measurements were repeated three times, and the mean of the three measurements was recorded. Grip strength was measured before and 3, 6, 9, and 12 weeks after the start of CDAA feeding and/or losartan/BCAAs treatments.

Histological and Immunohistochemical Analyses

Liver and gastrocnemius specimens were fixed in 10% formalin and embedded in paraffin. Sections of 5 μm in thickness were stained with hematoxylin and eosin (H&E) and Sirius Red. Primary antibodies, including FoxO1 (#2880, Cell Signaling Technology, Danvers, MA, USA) and FoxO3a antibodies (#12829, Cell Signaling Technology), were used for immunofluorescence analyses of gastrocnemius specimens, and staining was performed according to the manufacturer's instructions. Detection of the primary antibodies was performed using Alexa Fluor-conjugated secondary antibodies (Invitrogen, Waltham, MA, USA). Images were captured using a BX53 (Olympus, Tokyo, Japan) for histology and a BZ-X700 (Keyence, Osaka, Japan) for immunofluorescence. Semi-quantitative analysis was performed using ImageJ software version 64 (National Institutes of Health, Bethesda, MD, USA).

RNA Extraction and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from liver and muscle tissues and cultured L6 cells. A RNeasy Mini Kit (Qiagen, Hilden, Germany) is used for liver tissues and L6 cells and a RNeasy Fibrous Tissue Mini Kit (Qiagen) for muscle tissues. The resulting RNA concentrations were determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). A High-Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City,

CA, USA) was used for RT to generate cDNA. RT-qPCR was performed using the primer pairs described in supporting information. Table S1, Supporting Information, and SYBR Green PCR Master Mix (Applied Biosystems), and an Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems). Relative expression was normalized to GAPDH/Gapdh expression, estimated using the $2-\Delta\Delta CT$ method, and presented as fold changes relative to controls.

Mitochondrial DNA (mtDNA) Copy Number

Total DNA was obtained from the gastrocnemius muscle tissue using a DNA Extractor TIS Kit (FUJIFILM, Wako Pure Chemical Corporation, Osaka, Japan). The mtDNA copy number was assessed using real-time PCR as described in above RNA extraction and RT-qPCR according to mtDNA (Rnr2)/nDNA (Gapdh). Primer sequences were as follows: RNR2: 5′ -AGCTATTAATGGTT CGTTTGT-3′ and 5′ -AGGAGGCTCCATTTCTCTTGT-3′ , and nuclear-encoded GAPDH: 5′ -GGAAAG ACAGGTGTT TTGCA-3′ and 5′ -AGGTCAGAGTGAGCAGGACA-3′ .

Protein Extraction and Western blotting

Whole cell lysate proteins were extracted from muscle tissues and cultured L6 cells. For this purpose, T-PER Tissue Protein Extraction Reagent supplemented with proteinase and phosphatase inhibitors (Thermo Fisher Scientific) was used as lysis buffer. Nuclear proteins and cytosol proteins were prepared by using an EpiQuik Nuclear Extraction Kit I (EpiGentek, Farmingdale, NY, USA) following manufacturer's directions from muscle tissues. The protein concentration was measured by a protein assay (Bio-Rad, Hercules, CA, USA), and whole cell lysates or nuclear proteins were normalized to 100 or 50 μg, respectively. Western blotting was performed as described previously.[44] The membranes were incubated overnight with antibodies against p70S6K (#9202, Cell Signaling Technology), phospho-p70S6K (Thr389) (#9205), NF-κB p65 (#8242), phospho-NF-κB p65 (Ser536) (#3033), SMAD2/SMAD3 (#3102), phospho-SMAD2 (Ser465/467)/SMAD3 (Ser423/425) (#8828), caspase 3 (#9662), cleaved caspase 3 (Asp175) (#9661), FoxO1 (#2880) and FoxO3a (#12829), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#2118), TATA-binding protein (TBP) (#8515), PGC-1α (#ab106814, Abcam, Cambridge, UK) and mtTFA (#ab131607). Densitometric analysis was performed using ImageJ software version 64.

Muscle Rat Myostatin Measurements

The levels of myostatin and AT-II in 100 mg of rat gastrocnemius muscle tissue homogenate were measured using a myostatin ELISA Kit (R&D Systems) following the manufacturer's

instructions.

Muscle Rat AT-II Measurement

AT-II levels in the rat gastrocnemius muscle tissues were measured as described previously[45] with minor modification. Briefly, 100mg of gastrocnemius muscle tissues were homogenized in lysis buffer. After centrifugation (15 000 × g for 20 min at 4 ° C), the supernatant was loaded onto an equilibrated Sep-Pak C18 cartridge (Millipore), eluted with buffer (60% acetonitril in 1% trifluoroacetic acid), and collected in a centrifuge tube. The collected eluant was then evaporated to dryness using a rotary evaporator (CVE-2200; EYELA, Tokyo, Japan), freeze-dried overnight using a lyophilizer (FDU-12AS; AS ONE, Osaka, Japan), and used later on to detect by using the RayBio Angiotensin II Enzyme Immunoassay (EIA) Kit (RayBiotech, Inc., Peachtree Corners, GA, USA) following the manufacturer's instructions.

Statistical Analyses

Statistical analyses were performed using Prism, version 9 (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± standard deviation and were subjected to Student's t-test or one-way analysis of variance followed by Bonferroni's multiple-comparison test, as appropriate. Statistical variance between each experimental group was analyzed using analysis of variance. Bartlett's test was used to determine the homogeneity of variance. All tests were two-tailed, and $p < 0.05$ was statistically significant.

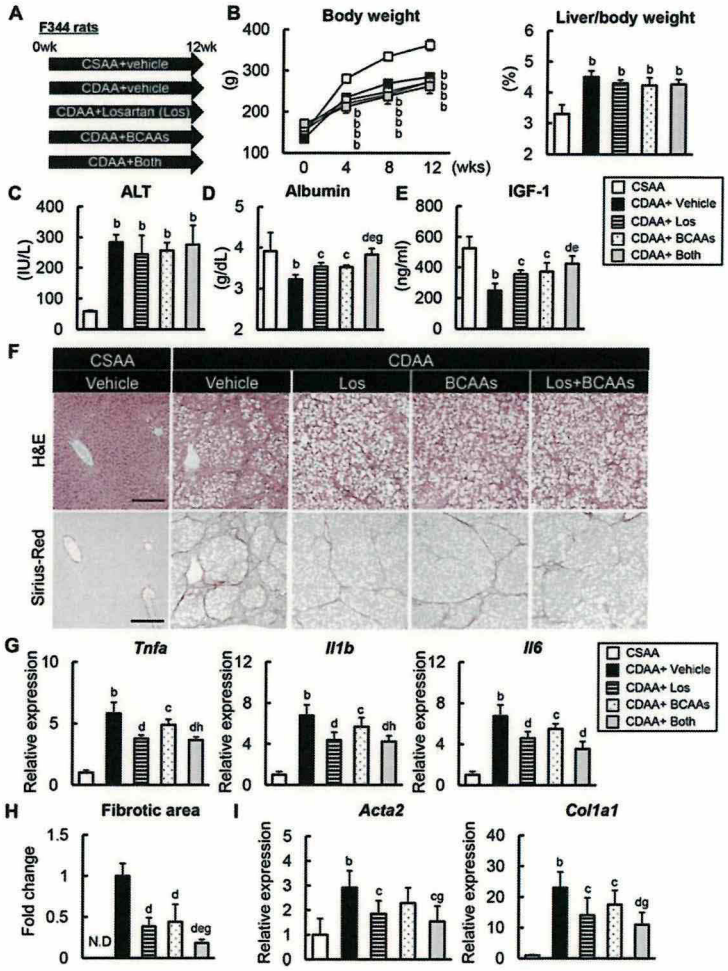


Figure 1

Losartan and BCAAs on hepatic phenotypes in CDAA-fed rats. A) Experimental protocols. B) Changes in body weights during experimental period (left panel). Ratio of liver weight to body weight at the end of experiment (right panel). C–E) Serum levels of C) alanine aminotransferase (ALT), D) albumin, and E) insulin-like growth factor-1 (IGF-1). F) Representative microphotographs of liver sections stained with hematoxylin-eosin (H&E) (upper panels) and Sirius-red (lower panels) in the experimental groups. Scale Bar; 50 μm. G) Relative mRNA expression levels of Tnfa, Il1b, and Il6 in the liver of experimental rats. H) Semi-quantitation of Sirius-Red-stained fibrotic area in high-power field by NIH imageJ software. I) Relative mRNA expression levels of Acta2 and Col1a1 in the liver of experimental rats. The mRNA expression levels were measured by qRT-PCR, and Gapdh was used as internal control for qRT-PCR. Quantitative values are indicated as fold changes to the values of CSAA group G and I) or CDAA+vehicle group H), respectively. Histochemical quantitative

analyses included five fields per section H). Data are mean ± SD (n = 10), a $p < 0.05$ and b $p < 0.01$ versus CSAA+vehicle group, c $p < 0.05$ and d $p < 0.01$ versus CDAA+vehicle group, e $p < 0.05$ and f $p < 0.01$ versus CDAA+Losartan group, g $p < 0.05$ and h $p < 0.01$ versus CDAA+BCAAs group. N.S, not significant; N.D, not detected.

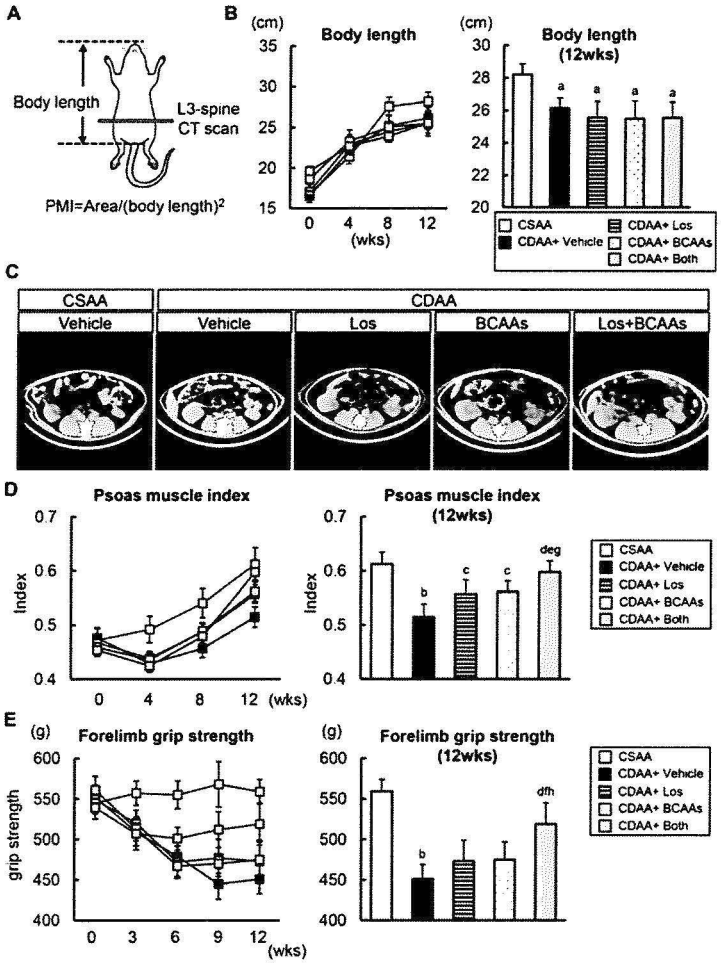


Figure 2

Losartan and BCAAs on psoas muscle mass index (PMI) in CDAA-fed rats. A) PMI (cross sectional area/height2) was assessed on a single CT slice at the level of L3 pedicle with image analysis system. B) Changes in body lengths during experimental period (left panel). Body length at the end of experiment (right panel). C) Representative images on a single CT slice at the level of L3 pedicle. D) Chronological changes in the calculated PMI during experimental period (left panel). PMI at the end of experiment (right panel). E) Chronological changes in the forelimb grip strength during experimental period (left panel).

Forelimb grip strength at 6 weeks of treatment (right panel). Data are mean ± SD (n = 10), a $p < 0.05$ and b $p < 0.01$ versus CSAA+vehicle group, c $p < 0.05$ and d $p < 0.01$ versus CDAA+vehicle group, e $p < 0.05$ and f $p < 0.01$ versus CDAA+Losartan group, g $p < 0.05$ and h $p < 0.01$ versus CDAA+BCAAs group.

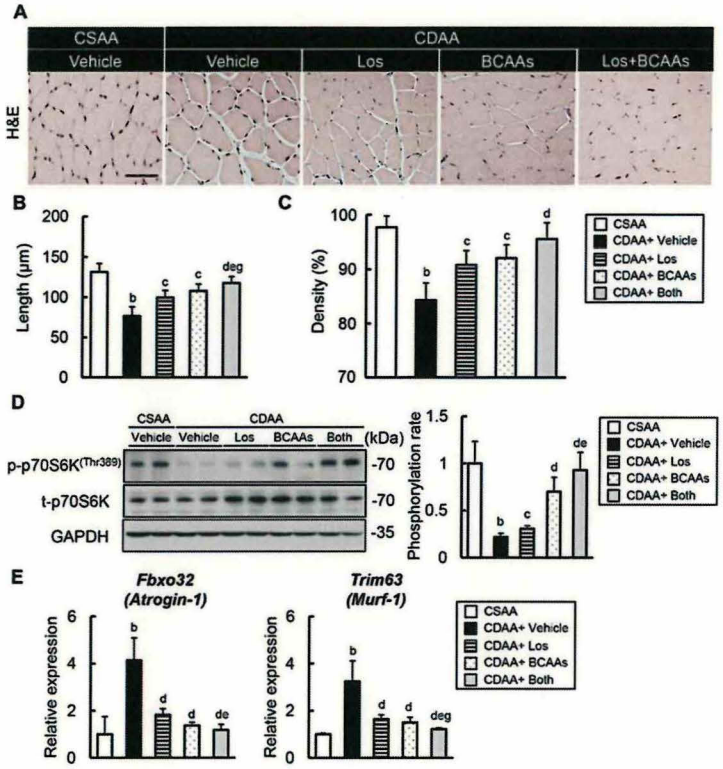


Figure 3

Losartan and BCAAs on protein synthesis and degradation of gastrocnemius muscle in CDAA-fed rats. A) Representative microphotographs of gastrocnemius muscle sections stained with hematoxylin-eosin (H&E) in the experimental groups. Scale Bar; 50 μm. B and C) The summary data of myocyte cross-sectional length B) and density C). D) Western blots for p70S6K phosphorylation in gastrocnemius muscle tissues (left panel) and quantitative phosphorylation rate of phosphorylated p70S6K/p70S6K (right panel). GAPDH was used as the loading control. E) Relative mRNA expression levels of Fbxo32 and Trim63 in the gastrocnemius muscle of experimental rats. The mRNA expression levels were measured by qRT-PCR, and Gapdh was used as internal control for qRT-PCR. Histochemical quantitative analyses included five fields per section B and C). Quantitative values are indicated as fold changes to the values of CSAA group D and E). Data are mean ± SD B, C and E; n = 10, D; n = 4, respectively), a $p < 0.05$ and b $p < 0.01$ versus CSAA+vehicle group, c $p < 0.05$ and d

p < 0.01 versus CDAA+vehicle group, e p < 0.05 and f p < 0.01 versus CDAA+Losartan group, g p < 0.05 and h p < 0.01 versus CDAA+BCAAs group.

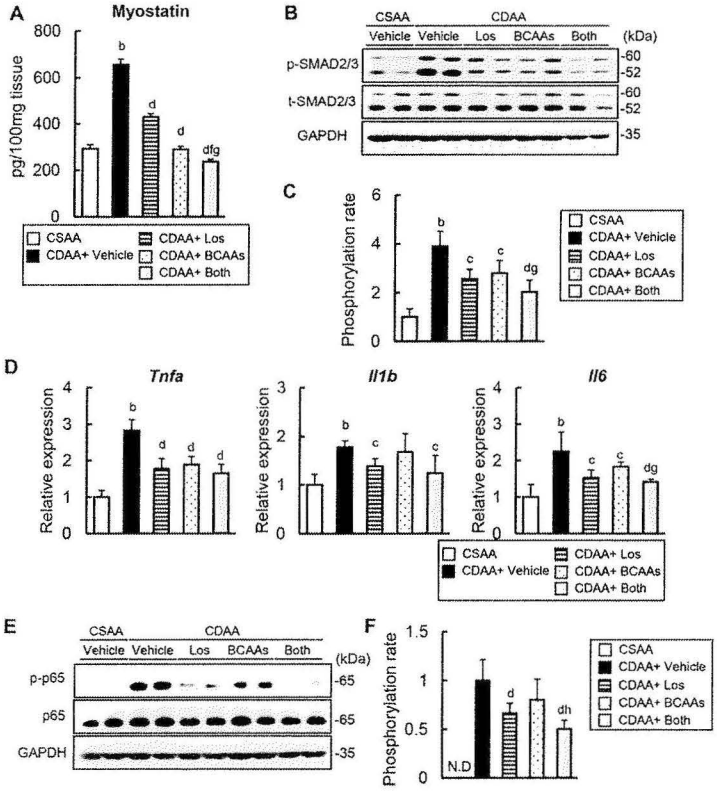


Figure 4

Losartan and BCAAs on intramuscular myokines signaling in CDAA-fed rats. A) Myostatin concentrations in gastrocnemius muscle tissues (100 mg) of experimental rats. B) Western blots for SMAD2/3 phosphorylation in gastrocnemius muscle tissues. C) Quantitative phosphorylation rate of phosphorylated SMAD2/3/SMAD2/3. D) Relative mRNA expression levels of Tnfa, Il1b, and Il6 in the gastrocnemius muscle of experimental rats. The mRNA expression levels were measured by qRT-PCR, and Gapdh was used as internal control for qRT-PCR. E) Western blots for NF- $\kappa$ B p65 phosphorylation in the gastrocnemius muscle of experimental rats. F) Quantitative phosphorylation rate of phosphorylated NF- $\kappa$ B p65/NF- $\kappa$ B p65. GAPDH was used as the loading control for western blotting B and E). Quantitative values are indicated as fold changes to the values of CSAA group C and D) or CDAA+vehicle group F), respectively. Data are mean ± SD (A and D; n = 10, C and F; n = 4, respectively), a p < 0.05 and b p < 0.01 versus CSAA+vehicle group, c p < 0.05 and d p < 0.01 versus CDAA+vehicle group, e p < 0.05 and f p < 0.01 versus CDAA+Losartan group, g p < 0.05 and h p < 0.01 versus CDAA+BCAAs group.

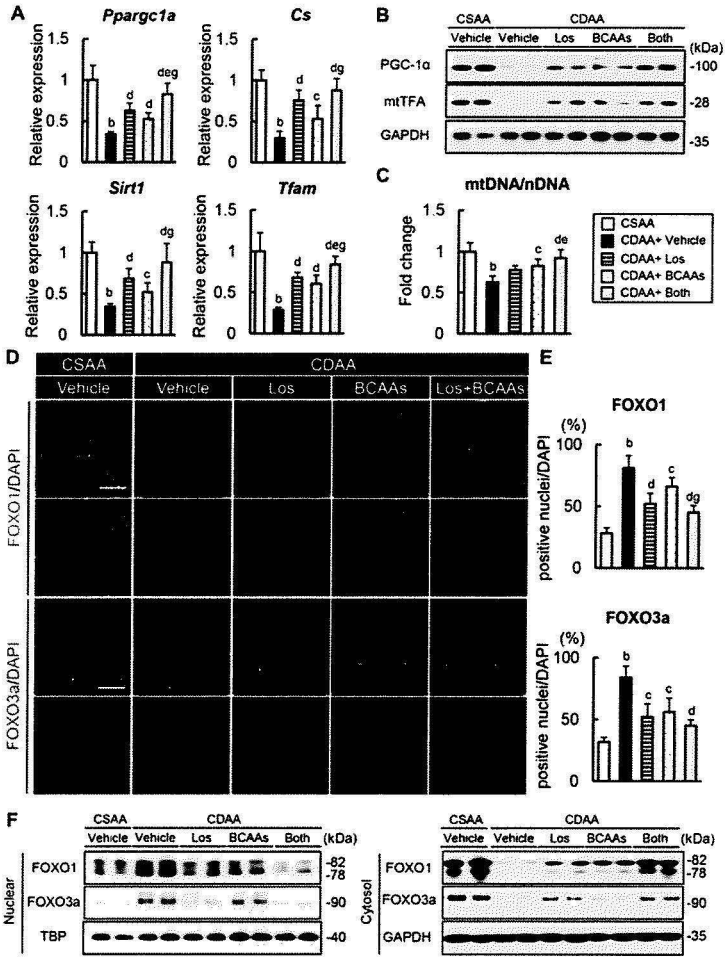


Figure 5

Losartan and BCAAs on mitochondrial biogenesis and FOXOs activation in CDAA-fed rats. A) Relative mRNA expression levels of Ppargc1a, Cs, Sirt1, and Tfam in the gastrocnemius muscle of experimental rats. The mRNA expression levels were measured by qRT-PCR, and Gapdh was used as internal control for qRT-PCR. B) Western blots for PGC-1 $\alpha$ and mtTFA proteins in the gastrocnemius muscle of experimental rats. GAPDH was used as the loading control. C) The mitochondrial DNA copy number was assessed using real-time PCR as described in above RNA extraction and RT-qPCR according to mtDNA (Rnr2)/nDNA (Gapdh). D) Representative microphotographs of immunofluorescence with FOXO1/DAPI (upper panels) and FOXO3a/DAPI (lower panels) staining in the gastrocnemius muscle sections of experimental groups. Scale bar; 50 μm. E) Semi-quantitation of FOXO1 (left panel) and FOXO3a (right panel) positive nuclei in high-power field (HPF) by ImageJ software in the experimental groups. The ratio of FOXO1/DAPI or FOXO3a/DAPI-positive

myocytes per 50 DAPI-positive myocytes in HPF were calculated. Quantitative analyses included five fields per section. F) Western blots for FOXO1 and FOXO3a proteins in the nuclear extract from the gastrocnemius muscle of experimental rats. TBP and GAPDH were used as the loading control. Quantitative values are indicated as fold changes to the values of CSAA group A and C). Data are mean ± SD (n = 10), a $p < 0.05$ and b $p < 0.01$ versus CSAA+vehicle group, c $p < 0.05$ and d $p < 0.01$ versus CDAA+vehicle group, e $p < 0.05$ and f $p < 0.01$ versus CDAA+Losartan group, g $p < 0.05$ and h $p < 0.01$ versus CDAA+BCAAs group.

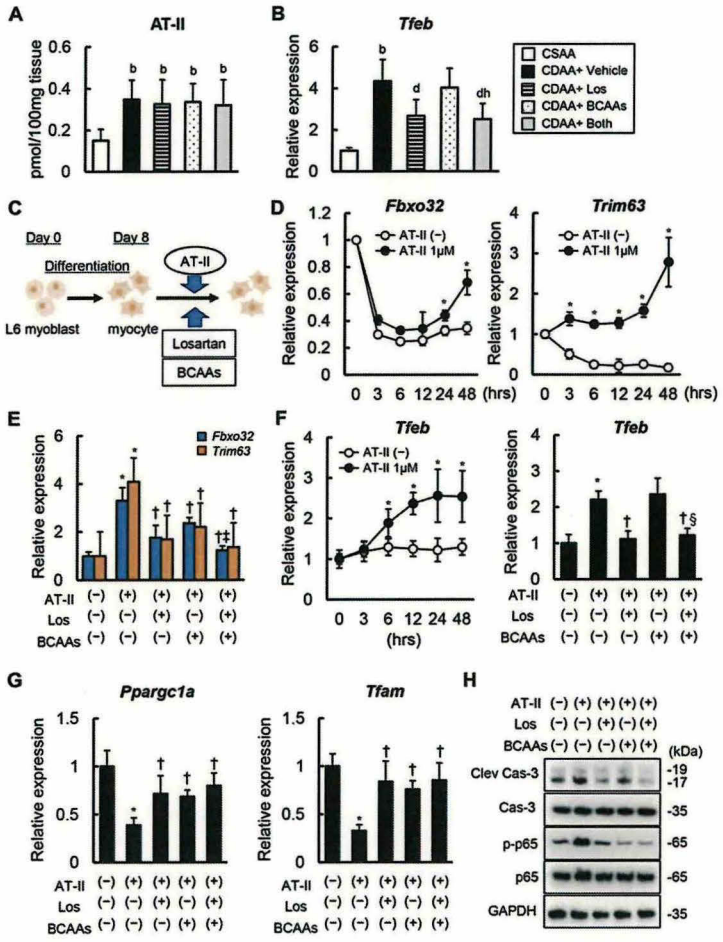


Figure 6

Losartan and BCAAs on AT-II-stimulated skeletal muscle cells. A) AT-II concentrations in gastrocnemius muscle tissues (100 mg) of experimental rats. B) Relative mRNA expression levels of Tfeb in the gastrocnemius muscle of experimental rats. Quantitative values are indicated as fold changes to the values of CSAA group. C) In vitro experimental protocol. D) Time-dependent effects of AT-II (1 μM) on the mRNA expression levels of Fbxo32 and

Trim63 in rat L6 myocytes. E) The effects of losartan and/or BCAAs on the mRNA expressions of Fbxo32 and Trim63 in the AT-II-stimulated rat L6 myocytes. F) (Left panel) Time-dependent effects of AT-II (1 μM) on the mRNA expression levels of Tfeb in rat L6 myocytes. (Right panel) The effects of losartan and/or BCAAs on the mRNA expressions of Tfeb in the AT-II-stimulated rat L6 myocytes. G) The effects of losartan and/or BCAAs on the mRNA expressions of Ppargc1a and Tfam in the AT-II-stimulated rat L6 myocytes. H) Western blots for cleavage of caspase-3 and phosphorylation of NF-κB p65 in the AT-II-stimulated rat L6 myocytes. GAPDH was used as the loading control for western blotting. The mRNA expression levels were measured by qRT-PCR, and Gapdh was used as internal control for qRT-PCR B, D-G). The cell lines were cultured with AT-II (1 μM), losartan (1 μM) and/or BCAAs (2 mM) for 48 h E–H). Quantitative values are indicated as fold changes to the values of 0 h D and F; left) or AT-II(-)/Los(-)/BCAAs(-)-group E, F; right and G). A and B) Data are mean ± SD ($n = 10$), a $p < 0.05$ and b $p < 0.01$ versus CSAA+vehicle group, c $p < 0.05$ and d $p < 0.01$ versus CDAA+vehicle group, e $p < 0.05$ and f $p < 0.01$ versus CDAA+Losartan group, g $p < 0.05$ and h $p < 0.01$ versus CDAA+BCAAs group. D–G) Data are mean ± SD ($n = 8$), * $p < 0.01$, versus AT-II (−) at each time point D–G). † $p < 0.05$ versus AT-II(+)/Los(-)/BCAAs(-)-group, ‡ $p < 0.05$ versus AT-II(+)/Los(+)/BCAAs(−)-group, § $p < 0.05$ versus AT-II(+)/Los(−)/BCAAs(+)-group E–G).

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