Browning and Beiging of Adipose Tissue: Its Role in the Regulation of Energy Homeostasis and as a Potential Target for Alleviating Metabolic Diseases

ACE2 exerts anti-obesity effect via stimulating brown adipose tissue and induction of browning in white adipose tissue

Yasuhiro Kawabe,1 Jun Mori,1 Hidechika Morimoto,1 Mihoko Yamaguchi,1 Satoshi Miyagaki,1 Takeshi Ota,1 Yusuke Tsuma,1 Shota Fukuhara,1 Hisakazu Nakajima,1 Gavin Y. Oudit,2,3 and Hajime Hosoi1

1Department of Pediatrics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan; 2Department of Physiology, Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Canada; and 3Division of Cardiology, Department of Medicine, Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Canada

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INTRODUCTION

Obesity is increasing worldwide and associated with increased risk of diabetes mellitus, cardiovascular disease, and cancer (22). The combination of diet and exercise is a main treatment of obesity, but the therapeutic effects are limited. Thus, new therapeutic approaches for obesity are urgently needed.

Brown adipose tissue (BAT) increases energy expenditure by producing heat. Consequently, BAT is regarded as a new target for anti-obesity treatment.

The renin-angiotensin system (RAS) plays a pivotal role in the pathophysiology of obesity. In the setting of obesity, the increased angiotensin II (ANG II) exacerbates insulin resistance and metabolic syndrome through ANG II type 1 receptor (AT1R; see Refs. 5, 11, 24). On the other hand, in recent years, angiotensin 1–7 (Ang 1–7), which is converted from ANG II by angiotensin-converting enzyme 2 (ACE2; see Ref. 1), has been reported to improve glucose metabolism and exert an anti-obesity effect (2, 6, 14, 15). We previously reported that ACE2 and the Ang 1–7 axis represent a potential therapeutic approach to prevent the development of obesity.

ACE2; angiotensin 1–7; BAT; browning; obesity

MATERIALS AND METHODS

Experimental animals and protocol. Male C57BL/6 mice (4 wk old) were purchased from CLEA Japan (Tokyo, Japan). Mice were housed at 23°C and on a 12:12-h light-dark cycle. Mice were fed normal chow diet (NCD; CLEA Rodent Diet CE-2; CLEA Japan) or high-fat diet (HFD; 60% Clea High Fat Diet 32; CLEA Japan) for 8 wk. NCD-fed mice were injected normal saline intraperitoneally (NCD + saline mice). HFD-fed mice were randomly assigned to groups injected intraperitoneally with normal saline (HFD + saline mice) or rhACE2 (2 mg kg−1·day−1, HFD + rhACE2 mice) for 4 wk. rhACE2 was kindly provided by GlaxoSmithKline (Stevenage, UK), and its biochemical characterization and use in preclinical models have been well documented (19, 28). All animal experiments were approved by the Animal Care and Use Committee of the Kyoto Prefectural University of Medicine, Japan (e-mail: jun1113@koto.kpu-m.ac.jp).
Prefectural University of Medicine and conducted in accordance with the guideline.

**Biochemical analyses.** Plasma total cholesterol, triglyceride, low-density lipoprotein cholesterol, and free fatty acid levels were analyzed using reagents from Wako (Osaka, Japan).

We performed glucose tolerance tests to investigate systemic glucose metabolism as previously described (15). Blood glucose was measured using a compact glucose analyzer (Antsense II; Horiba, Kyoto, Japan). Plasma insulin levels were checked using an ELISA kit (catalog no. M1104; Morinaga Institute of Biological Science, Kanagawa, Japan).

**Oxygen consumption and locomotor activity.** Oxygen consumption and locomotor activity were performed after 4 wk of treatment with rhACE2 or saline. Oxygen consumption was analyzed by the O2/CO2
RESULTS

rhACE2 suppressed the body weight gain by activating BAT and increasing energy expenditure in HFD-induced obese mice. The body weight of HFD-fed mice significantly increased compared with that of NCD-fed mice. rhACE2 treatment significantly reduced the body weight gain in HFD-fed mice independent of food intake (Fig. 1, A–C), which was accompanied with the decreased fat mass (Table 1). Oxygen consumption of HFD-fed mice in both dark and light phases was significantly higher in response to rhACE2 treatment (Fig. 1, D and E), but locomotor activity was not significantly different (Fig. 1, F and G). These data suggest that rhACE2 treatment increased energy expenditure in HFD-fed mice. Thus, we next investigated BAT since it is known as a main organ of energy expenditure by inducing thermogenesis.

rhACE2 promotes differentiation and proliferation of BAT and improves insulin resistance, glucose intolerance, and lipolysis. BAT was enlarged by rhACE2 treatment in HFD-fed mice (Fig. 2A and Table 1). We measured the cold-induced alteration in body temperature. Rectal temperature of HFD-fed mice was significantly higher than that of NC-fed mice. rhACE2 treatment further increased rectal temperature in HFD-fed mice (Fig. 2B). In accordance with this result, UCP1 protein expression was significantly increased in the HFD-rhACE2 group (Fig. 2C). We previously reported that Ang I–7 activates via stimulating the sympathetic nervous system. rhACE2 treatment also upregulated β3-AR expression, PKA expression, and p38 MAPK expression (Fig. 2C). These results clearly indicate that rhACE2 activates BAT and increases energy expenditure via activation of the sympathetic nervous system (SNS), leading to the improvement of diet-induced obesity.

We analyzed the factors reported to control the differentiation and proliferation of BAT to elucidate the effect of rhACE2 on BAT enlargement. The treatment of rhACE2 increased protein levels of both PRDM16 (Fig. 2D) and PGC-1α (Fig. 2D), known as transcriptional factors inducing differentiation to BAT. AMPK, PRDM16, and mTOR are known to be essential for BAT proliferation (16). The phosphorylation of AMPK

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Values are shown as means ± SE. NCD, normal chow diet; HFD, high-fat diet; rhACE2, recombinant human angiotensin-converting enzyme 2; BAT, brown adipose tissue; vWAT, visceral white adipose tissue (WAT); sWAT, subcutaneous WAT. *P < 0.05 versus NCD + saline mice. **P < 0.05 versus HFD + saline mice.
Fig. 2. Effect of recombinant human angiotensin-converting enzyme 2 (rhACE2) on thermogenesis, differentiation, proliferation, lipolysis, and improvement in brown adipose tissue (BAT).

A: BAT weight (n = 5–10).

B: Cold-induced rectal temperature (n = 5–10).

C and D: Western blot analysis of phosphorylation and protein expression in BAT (n = 5). The arrangement is disclosed by white spaces.

E: Representative histological images of BAT.

F: Frequency distribution of lipid droplet in BAT (n = 9). NCD, normal chow diet; HFD, high-fat diet. Values are shown as means ± SE. *P < 0.05 vs. NCD + saline mice (a) and HFD + saline mice (b).
AMPKα, ERK1/2, and mTOR was increased in response to rhACE2 treatment in HFD-induced obese mice (Fig. 2D).

rhACE2 treatment decreased lipid droplet size in BAT of HFD-fed mice (Fig. 2, E and F) by increasing ATGL (Fig. 2D) and phosphorylation of HSL (Fig. 2D). These alterations in metabolism and BAT by rhACE2 treatment improved glucose tolerance (Fig. 3A), which is accompanied with lowered blood glucose levels (Fig. 3B) and decreased...
blood insulin levels (Fig. 3C), and the improvement in HOMA-IR (the indication of insulin resistance) (Fig. 3D). Blood glucose levels during intraperitoneal insulin tolerance test and area under the curve were lowered in response to rhACE2 treatment (Fig. 3, E and F). Also, rhACE2 treatment upregulates phosphorylation of both Akt (Fig. 3G) and PI3K (Fig. 3H). These data collectively demonstrate that rhACE2 stimulates the signaling pathway involved in BAT
differentiation with increased lipolysis and improved insulin signaling.

**rhACE2 promoted lipolysis and induced browning in sWAT.** We next assessed the effect of rhACE2 on browning in WAT. Another thermogenic organ characterized by UCP1 expression, rhACE2 treatment decreased both visceral WAT and sWAT (Table 1) and reduced lipid droplets in HFD-fed mice (Fig. 4, A–C), concomitant with increased phosphorylation of HSL (Fig. 4D) and ATGL (Fig. 4E) levels. More interestingly, rhACE2 treatment induced UCP1 expression in sWAT of HFD-fed mice (Fig. 4, F and G), suggesting that rhACE2 treatment induced browning in sWAT. The expression of β3-AR (Fig. 4H), PRDM16 (Fig. 4I), PGC-1α (Fig. 4J), and FGF21 (Fig. 4K), which are inducible factors of browning, increased. Consistent with these data, plasma total cholesterol (Fig. 4L), triglycerides (Fig. 4M), and free fatty acid (Fig. 4N) levels were decreased in response to rhACE2 treatment. Thermogenesis induced by rhACE2 was due to not only activation of BAT but also browning in WAT. Browning of WAT is associated with reduced adipose tissue inflammation and fibrosis (10). Therefore, we examined inflammation and profibrosis markers in sWAT. rhACE2 treatment decreased the expression of IL-6, TNFα, and monocyte chemotactic protein-1 of sWAT (Fig. 4O), but there were no significant changes in profibrosis markers (Fig. 4P).

**rhACE2 treatment promotes H3K9 dimethylation, H3K9 and H3K27 acetylation.** Epigenetic regulation, such as histone methylation and acetylation, is involved in BAT development. Thus, we next examined epigenetic alterations induced by rhACE2 treatment. rhACE2 treatment upregulated EHMT1 in both BAT (Fig. 5A) and sWAT (Fig. 5B) in HFD-fed mice. H3K9me2 was increased in sWAT (Fig. 5C) by rhACE2 treatment. However, rhACE2 treatment did not increase H3K9me2 in BAT, even though there was a trend of increase (Fig. 5D). Histone acetylation is regulated by histone acetyltransferase and HDAC. Histone acetyltransferase, Gcn5, and its homolog PCAF have been reported to be important for BAT differentiation by promoting H3K9 acetylation in the PRDM16 promoter region (12). In HFD-fed mice, rhACE2 treatment increased H3K9 acetylation (Fig. 5E) with upregulation of GCN5 (Fig. 5F) and PCAF (Fig. 5G) and increased H3K27 acetylation (Fig. 5H) with the downregulation of HDAC3 in sWAT (Fig. 5I).

**DISCUSSION**

BAT can dissipate energy by nonshivering thermogenesis. Browning of sWAT also plays an important role in thermogenesis. To control the activation and increase of BAT and browning is drawing attention as a new target for obesity treatment. In this study, we demonstrated that rhACE2 activated BAT and increased energy expenditure in HFD-fed mice. The activation of UCP1 through lipolysis is needed for thermogenesis. Especially, SNS stimulation via β3-AR is essential for the upregulation of UCP1 expression and lipolysis in BAT. The increased lipolysis supplies fatty acids as oxidative substrate in BAT, leading to the heat generation (3). rhACE2 increases UCP1 expression, accompanied with the activation of SNS (β3-AR/PKA/p38 MAPK). Importantly, rhACE2 treatment elevates the rectal temperature in HFD-fed mice under the cold environment, as well as at 22°C. However, Ang 1–7 treatment does not elevate the rectal temperature in HFD-fed mice at 22°C (16). These results might show that rhACE2 has a stronger thermogenesis compared with Ang 1–7. BAT is characterized with the UCP1 expression, and the amount of UCP1 expression represents the volume of BAT. BAT differentiation was established by the upregulation of PRDM16 and PGC-1α, critical transcriptional factors of BAT. In fact, BAT was enlarged, and cold-induced thermogenesis was higher in response to rhACE2 treatment. rhACE2 increases Ang 1–7 levels via converting ANG II to Ang 1–7. We previously reported that Ang 1–7 activates BAT and improves obesity through AMPK and mTOR in HFD-fed mice (16). In line with the previous report, rhACE2 stimulated BAT and reduced obesity in HFD-fed mice, accompanied with upregulation of AMPK and mTOR. MasR, a specific receptor of Ang 1–7, is highly expressed in BAT (16). Thus, the ACE2-Ang 1–7–MasR axis might directly activate BAT in addition to norepinephrine-induced activation. We did not examine the effect of rhACE2 in NCD-fed mice, even though it leads to a better understanding of the rhACE2 effect on energy metabolism. RAS, especially ANG II, is activated in the setting of obesity. The Ang 1–7–MasR axis counteracts the ANG II-AT,R axis. In fact, Ang 1–7 treatment shows effects only in HFD-fed obese mice, not in NCD-fed mice (16). Based on this, we think rhACE2 treatment might have an effect only in HFD-fed obese mice as well as Ang 1–7. Further investigations are needed to clarify if ACE2 directly or indirectly activates BAT.

Recently, in addition to classical BAT, beige adipocytes in WAT have attracted much attention as a target for anti-obesity treatment. WAT mainly consists of white adipocyte. Beige adipocyte is induced in especially sWAT in response to some stimulating factors, such as SNS signaling and cold challenge. Beige adipocyte is also characterized with the UCP1 expression. FGF21 promotes browning of WAT (UCP1 expression) via PGC-1α in association with the increment of glucose uptake and oxidation (8). In the present study, rhACE2 treatment increases the UCP1 expression in sWAT of HFD-fed mice, accompanied with the increased FGF21 and PGC-1α. These results clearly show that rhACE2 induces browning in sWAT. Furthermore, browning of WAT induced by rhACE2 treatment was accompanied with decreased inflammation markers. This result is compatible with the previous report showing rhACE2 represses inflammation in adipose tissue (20). We previously reported that Ang 1–7 upregulates thermogenic capacity via activation of BAT, independent of browning in WAT (16). In this study, rhACE2 treatment exerts a stronger

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**Fig. 5. Effect of recombinant human angiotensin-converting enzyme 2 (rhACE2) on epigenetic change in subcutaneous white adipose tissue (sWAT).** A: Eue-HMTase 1 (EHMT1) protein expression in sWAT (n = 5). B: EHMT1 protein expression in brown adipose tissue (BAT, n = 5). C: dimethylation of H3K9 in sWAT (n = 5). D: dimethylation of H3K9 in sWAT (n = 5). E: H3K9 acetylation level in sWAT (n = 5). F: mRNA expression of GCN5 in sWAT (n = 5). G: mRNA expression of P300/CBP-associated factor (PCAF) in sWAT (n = 5). H: H3K27 acetylation level in sWAT (n = 5). I: mRNA expression of histone deacetylase (HDAC) 3 in sWAT (n = 5). J: schematic drawing of rhACE2 on epigenetic change in sWAT (n = 5). NCD, normal chow diet; HFD, high-fat diet. Values are shown as means ± SD. P < 0.05 vs. NCD + saline mice (a) and HFD + saline mice (b).
anti-obesity effect compared with Ang 1–7 by inducing browning in sWAT in addition to promoting BAT differentiation and proliferation. In the setting of obesity, ANG II levels are high in the various tissues and involved with the pathological process. Furthermore, AT1R predominates over ANG II type 2 receptor, and the signal via AT1R suppresses browning of WAT. AT1R antagonist increases browning of WAT and body temperature by the increasing UCP1 expression and oxygen consumption, resulting in the reduction of WAT mass and serum lipid levels (25). rhACE2 decreases ANG II levels by converting to Ang 1–7. Therefore, rhACE2 treatment not only increases the Ang 1–7 level but also decreases ANG II/AT1R signaling, thereby inducing WAT browning and exerting stronger anti-obesity effect compared with Ang 1–7 treatment. ACE2 treatment can also elicit anti-inflammatory effects in adipose tissue, which can also promote BAT function (20).

Identification of the molecular mechanism involved in the differentiation of browning and induction of the thermogenic program are promising fields in the treatment of obesity. Several transcription factors have been reported as determining factors of browning in WAT. In particular, PRDM16 and EHMT1, which construct the transcriptional complex, are important molecular switches that determine WAT browning differentiation (4, 18). EHMT1 has enzymatic activity on H3K9 di- or tri-methylation. Deletion of EHMT1 in brown adipocytes decreases the H3K9me2 levels on the promoter regions of muscle-specific genes and causes a loss of brown adipocyte differentiation (17). We established that rhACE2 promotes WAT browning by upregulating PRDM16 and EHMT1, and further increased H3K9me2 in WAT. More interestingly, rhACE2 also increased acetylation of H3K9 by upregulating GCN5/PCAF expression and increased acetylation of H3K27 by downregulating HDAC3 expression. GCN5 and PCAF were found to be critical for brown adipocyte differentiation by promoting peroxisome proliferator-activated receptor (PPAR)-γ transcription and by facilitating recruitment of RNA polymerase II to PRDM16 promoter region (12). Selective ablation of HDAC3 in WAT promotes browning of WAT via enhancers in Pparγ and Ucp1 genes, and increased acetylation of H3K27 in the putative regulatory region of Ppara gene (7, 9). Furthermore, Ucp1 expression in WAT increases H3K27 acetylation in the Ucp1 promoter lesion through β3-AR by both cold and isoproterenol stimulation (27). These results suggest that browning of WAT is related to the downregulation of HDAC3 via the β3-AR. Thus, it is likely that rhACE2 treatment induces browning of WAT by upregulating H3K9 2me, H3K9ac, and H3K27ac via the β3-AR (Fig. 5).

In conclusion, rhACE2 treatment reduces HFD-induced obesity in association with not only the activation of BAT but also the browning of WAT. The browning of WAT is controlled by epigenetic change, such as histone methylation and acetylation. ACE2 and its downstream epigenetic effects represent a potential therapeutic approach to prevent the development of obesity.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES
ACE2 ACTIVATES BAT AND WAT BROWNING

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