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ACE2 Activator Diminazene Aceturate Reduces Adiposity but Preserves Lean Mass in Young and Old Rats

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Abstract

The obesity epidemic is multi-generational and is particularly debilitating in the aging population, necessitating the use of pharmaceutical interventions. Recent evidence suggests that increasing the activity of the angiotensin converting enzyme-2 [ACE2]/angiotensin-(1–7)[Ang-(1–7)]/Mas receptor (MasR) axis in obese animal models leads to significant reductions in body weight. It was hypothesized that activation of ACE2 via diminazene aceturate (DIZE) will significantly reduce body weight of rats fed a high fat diet. Young and old (4 and 23 months, respectively) male Fisher 344 x Brown Norway rats were fed 60% high fat diet for one week, and subsequently given either 15mg/kg/day DIZE s.c. or vehicle for three weeks. DIZE treatment resulted in a significant reduction of food intake and body weight in both young and old animals. However, that decrease was so dramatic in the older animals that they all nearly stopped eating. Interestingly, the TD-NMR assessments revealed that the weight-loss was primarily a result of decreased body fat percentage, with a relative preservation of lean mass. Tissue weights confirm the significant loss of white adipose tissue (WAT), with no change in muscle weights. Gene expression and serum ACE2 activity analyses implied that increased activation of the ACE2/Ang-(1–7)/MasR axis plays a role in reducing fat mass. Collectively, our results suggest that DIZE may be a useful tool in the study of obesity; however, caution is recommended when using this compound in older animals due to severe anorectic effects, although there is a mechanism by which muscle is preserved.

Keywords

Obesity; Pharmacology; Rats; Renin Angiotensin System

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Introduction

Obesity is a global epidemic affecting the health of both young and old generations. [1,2] Current recommendations for obesity treatment include adherence to a healthy diet and increased physical activity. Unfortunately, many patients, including the ever-growing aging-obese population, are physically unable or unwilling to adhere to these recommendations. [1] For such individuals, novel strategies must be implemented to induce weight-loss and reduce the risk of associated co-morbidities, such as type 2 diabetes and cardiovascular disease. To this end, recent evidence suggests treatment with Angiotensin-(1–7) [Ang-(1–7)], a component of the renin angiotensin system (RAS), improves metabolism, glucose homeostasis, and lipid profile in animal models of obesity and metabolic syndrome. [3,4] Furthermore, Ang-(1–7) treatment leads to reduced inflammation and oxidative stress, two key risk factors in the development of hypertension. These actions are in direct opposition to those of Ang II, the primary effector of the RAS, at the angiotensin type 1 receptor (AT₁R). [5–7] Ang-(1–7) is a derivative of Ang II, formed when ACE2 cleaves the amino acid, phenylalanine, from the C-terminus.[8]As a consequence, increasing ACE2 activity has a two-fold effect: 1) it elevates the levels of the beneficial Ang-(1–7), while 2) decreasing the detrimental effects of Ang II. This study tests the hypothesis that increasing ACE2 activity will exert beneficial metabolic actions that thwart high-fat diet-induced obesity in both young and aged rats.

To test this hypothesis, we utilized diminazene aceturate (DIZE), a compound that is commonly used in livestock to treat Trypanosomiasis, or African sleeping sickness, [9] and that has more recently, come to be recognized for its ability to increase ACE2 activity *in vitro* and *in vivo*. [10] Due to the ACE2 activating properties of this drug, recent studies have evaluated its potential as a pharmacological intervention in multiple rodent models of cardiovascular disease and each of these studies have revealed novel roles of increased ACE2 activity. [10–12] Of particular relevance for the present studies, de Macedo *et al.* demonstrated a decrease in lipogenic enzymes in adipose tissue of mice treated with DIZE. [13] Here, we assess the efficacy of this drug to prevent diet-induced obesity in both young and aged rats and also its impact on indices of ACE2/Ang-(1–7)/MasR axis activation in serum and tissue homogenates of these animals.

Materials and Methods

Experimental animals

Three month-old and 22 month-old male Fisher 334 X Brown Norway rats were obtained from National Institute on Aging. Upon arrival, rats were examined and remained in quarantine for one week. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals, and the University of Florida Institutional Animal Care and Use Committee approved all protocols. Rats were housed individually on a 12:12 h light-dark cycle and were fed standard chow for one month before the start of the experiment, whereupon they were fed 60% High Fat Diet (HF) (60% kcal from fat, 20% kcal from protein, 20% kcal from carbohydrates; Research Diets Inc., New Brunswick, NJ, USA).

Experimental design

Eight days after the start of HF, rats were **pseudo-randomized** into four groups (Young Control, n=6; Young DIZE, n=6; Old Control, n=8; Old DIZE, n=9) based on body weight **to ensure that rats of various weights were represented equally in each group**, and given either 15 mg/kg/day DIZE (LKT Laboratories Inc.; St. Paul, MN) or vehicle (water) s.c. Body weight and food intake were measured daily during the first week to document the hyperphagic response to the introduction of the HF diet and then subsequently measured twice weekly. Food and water were provided *ad libitum* in a food hopper that rested inside the cage above the animal. Daily food intake was measured by placing all food pellets remaining in the hopper on the scale. Body composition was measured at weeks 1 and 3 after treatment began via time-domain nuclear magnetic resonance (TD-NMR) in restrained but fully conscious rats (TD-NMR Minispec, Bruker Optics, The Woodlands, TX, USA). Treatment lasted for three weeks, and animals were sacrificed 24 hrs after final DIZE injection.

Tissue harvest

Rats were euthanized by thoracotomy under 5% isoflurane anesthetic. Whole blood was taken by cardiac puncture and serum collected following centrifugation in serum separator tubes. Subsequently, 15 ml of cold saline were perfused through the circulatory system. The perirenal, retroperitoneal, and epididymal white adipose depots (PWAT, RTWAT, and EWAT, respectively) along with interscapular brown adipose tissue (BAT), tibialis anterior (TA), and heart were excised, blotted dry, and weighed. The tibia was collected and used as a measurement of rat growth.

Serum ACE2 Activity and Leptin Levels

Serum ACE2 activity was determined using the protocol described by Bennion et.al. [12] Briefly, serum samples (6µl) were incubated in black flat-bottomed 96-well plates in 100µl of reaction mixture containing ACE2 buffer (1mol/L NaCl, 75mmol/L Tris HCl, pH 7.5, and 50µmol/L ZnCl₂), 10µmol/L captopril, and 25µmol/L fluorogenic Mca- YVADAPK(Dnp)-OH ACE2 substrate (R&D Systems, Inc., #ES007). Relative fluorescence (RFU) for all samples was measured for 120 minutes using a Synergy Mx Microplate Reader (BioTek Instruments, Inc.) with excitation at 320nm and emission at 405nm. The slope of the fluorescence curve from 30–60 minutes was used to calculate RFU per minute. Substrate concentrations were selected following determination of reaction K_m and V_{max} using control samples and recombinant human ACE2 (R&D Systems, Inc., #933-ZN-010) as a positive control, and all samples were run in duplicate. Serum leptin levels were determined by ELISA (Millipore, Billerica, MA).

mRNA Gene Expression via qPCR

RTWAT (100mg) and TA muscle (50mg) was placed in 1 ml TriReagent (Sigma-Aldrich, St. Louis, Mo) and briefly sonicated. mRNA was isolated following standard TriReagent protocol from Sigma. mRNA was converted into cDNA using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Isle, NY). Real-Time

PCR was used to determine gene expression of AT₁R, AT₂R, MasR, ACE, and ACE2 using TaqMan probes and the One-Step PCR machine (Life Technologies, Grand Isle, NY).

Western Analysis

Brown adipose tissue (BAT; 30mg) was briefly sonicated in 300µl 10mM Tris, pH 6.8, 2% SDS for Western analysis. BAT homogenate was diluted 1:30 (10µl in 290 µl buffer) and filtered through 45µm syringe filter before protein assay. Protein was determined by DC Bradford assay (Bio-Rad, Hercules, CA). Protein homogenate was separated on a SDS-PAGE gel and electro-transferred to nitrocellulose membranes. Immunoreactivity was assessed with antibodies specific to UCP-1 (Abcam, Cambridge, MA), and was detected with ECL prime (GE Healthcare, Piscataway NJ), scanned with a ChemiDoc XRS+ (BioRad, Hercules, CA), and quantified using ImageQuant software (Molecular Dynamics, GE Healthcare Bio-Sciences, Pittsburgh, PA).

Statistical analysis

Data were analyzed by two-way ANOVA with repeated measures where appropriate, with Bonferroni post-hoc tests, using GraphPad Prism software (La Jolla, CA). Variables assessed in the analysis include age (Young vs. Old), Treatment (Control vs. DIZE), and Time. Values were considered statistically significant when $P < 0.05$.

Results

Effect of DIZE on Food Intake and Body Weight

DIZE treatment resulted in a significant reduction of caloric intake followed closely by a change in body weight in both young and old animals (Figure 1). Results from repeated measures two-way ANOVA on kcal intake showed a significant main effect of treatment and age ($p < 0.0001$; $F(3,24)=55.79$), and time ($p < 0.0001$; $F(15,360)=294.6$) with a significant interaction ($p < 0.0001$; $F(45,360)=24.9$). Analysis of delta body weight also demonstrated a significant effect of group ($p < 0.0001$; $F(3,24)=48.82$), time ($p < 0.0001$; $F(16,384)=142.8$), and an interaction ($p < 0.0001$; $F(48,384)=110.8$). Similarly, analysis of body weight showed a significant effect of group ($p < 0.0001$; $F(3,24)=79.85$), time ($p < 0.0001$; $F(16,384)=142.8$), and an interaction ($p < 0.0001$; $F(48,384)=110.8$). Further Bonferroni post-hoc analysis showed on day 11, Old DIZE animals demonstrated a significant decrease in food intake compared to Old Control animals, whereas Young DIZE animals did not demonstrate a significant decrease in food intake until day 18 (Figure 1A). In young rats, the decrease in food intake stabilized after 10 days of treatment; however, the old rats continued to decrease kcal intake to 13.01 ± 1.66 kcal/day compared to 66.66 ± 4.133 kcal/day in the control condition (Figure 1A). A significant decrease in delta body weight was observed in Old DIZE animals on Day 15, and Day 18 in Young DIZE animals when compared to age matched controls (Figure 1B). Delta body weight continued to decrease in both Young and Old DIZE animals, leading to a significant decrease in absolute body weight on day 21 in Old DIZE animals, and Day 32 in Young DIZE animals (Figure 1C). Despite the dramatic decrease in food intake and body weight, all rats remained bright, alert, and responsive, with a body condition score of 2.5 or higher (data not shown). Additionally, there was no evidence of diarrhea, and bowel movements were normal.

Effect of DIZE on Body Composition as Measured by TD-NMR

TD-NMR was measured 1 week and 3 weeks after DIZE treatment began. Repeated measures two-way ANOVA analysis of percent body fat showed a significant main effect of both group (DIZE and Age) ($p<0.0001$; $F(3,24)=63.44$) and time ($p<0.0001$; $F(1,24)=55.46$) with a significant interaction ($p<0.0001$; $F(3,24)=19.6$). Percent body fat was significantly decreased in both young ($p<0.001$; $2.32\pm0.636\%$) and old ($p<0.0001$; $2.99\pm0.449\%$) rats treated with DIZE for three weeks (Figure 2A). While there was no significant difference between young control and young DIZE rats, both groups decreased percent lean mass after three weeks (young control, $3.09\pm0.48\%$; young DIZE, $2.53\pm0.42\%$) (main effect of time ($F(1,24)=6.33$; $p=0.019$). Strikingly, old rats treated with DIZE demonstrated a significant increase in percent lean mass over time ($p<0.0001$; $3.26\pm0.71\%$) (Figure 2B). There was no change in percent lean mass in the old control animals (main effect of group $p<0.0001$; $F(3,24)=30.97$; significant interaction $p<0.0001$; $F(3,24)=45.62$). These data can be explained by the absolute values of fat and lean mass. Young DIZE rats lost 16.79 ± 4.15 g of fat mass, a significant decrease ($p<0.0001$); while their age matched controls gained 1.63 ± 2.59 g, a non-significant change. Old rats treated with DIZE display an even greater loss of fat mass ($p<0.0001$; 42.59 ± 6.60 g) over time; whereas their age-matched controls gained a non-significant amount (5.67 ± 8.91 g; Figure 2C) ($p<0.0001$; main effect of time $F(1,24)=91.18$ and group $p<0.0001$; $F(3,24)=128.8$; significant interaction $p<0.0001$; $F(3,24)=75.78$). Additionally, there was a significant decrease in absolute lean mass in both young (28.93 ± 4.27 g) and old rats (30.62 ± 6.65 g) treated with DIZE (Figure 2D) (main effect of time $p<0.0001$; $F(1,24)=97.32$ and group $p<0.0001$; $F(3,24)=98.88$; significant interaction $p<0.0001$; $F(3,24)=40.58$). In young rats treated with DIZE, the loss of lean mass and fat mass resulted in no change in Lean/Fat mass ratio over time (Figure 2E). However, when compared to age matched controls, there was a significant increase in the Lean/Fat mass ratio. Furthermore, in old rats treated with DIZE, the comparatively small loss of lean mass to fat mass resulted in a significantly greater Lean/Fat mass ratio over time, compared to age matched controls (Figure 2E) (main effect of time $p=0.0004$; $F(1,24)=16.62$ and group $p<0.0001$; $F(3,24)=19.88$; significant interaction $p=0.0003$; $F(3,24)=9.112$).

Effect of DIZE on Tissue weights

At sacrifice, tissues were excised and weighed. All weights are expressed relative to tibia length (TL) (cm) as a measure of natural growth. Old rats had significantly higher TL than young rats (data not shown). Assessment of tissue weights with two-way ANOVA revealed that age significantly increased all WAT weights (Figure 3A–D). Furthermore, there was an overall effect of DIZE to lower all WAT weights. Specifically, there was an interaction found in RTWAT weights ($p<0.0001$; $F(1,24)=32.05$) (Figure 3A) and the overall sum of WAT weights ($p=0.0011$; $F(1,24)=13.84$) (Figure 3D), in which DIZE-treated animals had significantly lower weight than age matched controls, as indicated by the Bonferroni post-hoc analysis. Additionally, DIZE treatment significantly lowered BAT weights in both young and old rats (main effect of DIZE $F(1,24)=117$). The loss of BAT mass in old DIZE animals is particularly striking as old control animals had significantly higher BAT mass than young control animals (0.017 ± 0.0008 g/cm vs. 0.012 ± 0.0007 g, respectively) (main effect of age $F(1,24)=15.33$). However, DIZE treatment in old rats significantly decreased BAT mass to

match those of young DIZE treated animals ($0.008 \pm 0.0004\text{g}$ vs. $0.007 \pm 0.0005\text{g}$, respectively) (significant interaction $p < 0.0001$; $F(1,24) = 29.13$).

In addition to adipose tissue mass, the TA muscle was collected as an indicator of DIZE's effect on lean mass. Age significantly lowered TA muscle weights (main effect of Age $p = 0.04$; $F(1,24) = 4.72$); however, DIZE had no effect on TA mass ($F(1,24) = 2.761$) (Figure 3F). Furthermore, there was a significant effect of age to increase heart weights (main effect of age $p < 0.0001$; $F(1,24) = 23.58$); however, DIZE significantly attenuated this increase (main effect of DIZE $p = 0.0073$; $F(1,24) = 8.61$).

Effect of DIZE on Serum ACE2 Activity and RAS components in WAT and Muscle

In order to verify that DIZE increased ACE2 activity as expected based on previous literature, serum samples were assessed in an ACE2 activity assay described previously by Bennion *et al.* [12] While there was no effect of age on serum ACE2 activity ($F(1,20) = 1.548$), DIZE did increase serum ACE2 activity in both young and old animals (main effect of DIZE $p = 0.0035$; $F(1,20) = 10.94$) (Figure 4). Additionally, we assessed the effect of age and DIZE treatment on gene expression of RAS components in both the muscle and RTWAT as measured by qPCR (Table 1). DIZE significantly decreased ACE mRNA expression in TA muscle. There was no effect of either DIZE or age on ACE2 or AT1R mRNA expression in muscle. Both AT2R and MasR expression were increased within the TA muscle of old rats; however, there was no effect of DIZE treatment. In RTWAT, ACE ($p = 0.002$), ACE2 ($p < 0.0001$), and AT1R ($p < 0.0001$) expression were significantly lower with age AT2R ($p < 0.0001$) and MasR ($p < 0.0001$) expression was significantly higher in aged animals, with DIZE treatment causing a greater increase in expression (AT2R $p < 0.0001$; MasR $p = 0.0326$).

Effect of DIZE on Leptin and BAT UCP1

Serum leptin levels were significantly elevated in old animals (40.08ng/mL) compared to young (12.49ng/mL) (main effect of age $p > 0.0001$; $F(1,24) = 67.95$) (Figure 5A). Furthermore, DIZE significantly lowered serum leptin levels in both young (3.72ng/mL) and old animals (17.61ng/mL) when compared to age-matched controls (main effect of DIZE $p > 0.0001$; $F(1,24) = 42$) (Figure 5A).

Interestingly, there was a striking difference in UCP1 expression in young and old animals treated with DIZE, as measured by western blot. Two-way ANOVA showed a main effect of DIZE ($p > 0.0001$; $F(1,21) = 41.82$) and a significant interaction ($p > 0.0001$; $F(1,21) = 90.76$). Bonferroni's Posthoc analysis demonstrated old control animals had a significant increase in UCP1 expression when compared to young controls. Although there was a slight increase (Young HF = $100 \pm 8.86\%$, Young HF+DIZE = $126.67 \pm 9.2\%$), DIZE did not have a significant effect on young animals fed HF diet. However, there was a significant decrease in UCP1 in old animals treated with DIZE (Old HF = $175.67 \pm 11.62\%$, Old HF+DIZE = $36.29 \pm 4.81\%$; $p > 0.0001$; Figure 5B).

Discussion

With obesity becoming an epidemic, novel pharmacological interventions are necessary to promote overall health and weight loss. Our data suggest that increasing endogenous ACE2 activity, as with DIZE administration, may be a viable intervention; however this may be true in only younger populations. Young and old rats fed 60% HF diet continuously gained a significant amount of weight over a 4-week period; however, DIZE treatment significantly reduced body mass, which can be explained, in part, by a decrease in food intake. Furthermore, TD-NMR measurements of body composition indicate that the primary source of weight loss is due to loss of fat mass, as evidenced by the increase in lean/fat mass ratio in both young and old rats. Furthermore, tissue weights at the time of sacrifice confirm DIZE treatment resulted in a significant loss of WAT mass, with no significant difference in tibialis anterior (TA) muscle weights, indicating no muscle wasting. Importantly, when assessing heart weights, we observed a significant reduction in aged rats treated with DIZE when compared to age-matched controls, suggesting a prevention of cardiac hypertrophy associated with obesity. [14–16] The differences observed in gene expression of RAS components in fat and muscle mass between young and old rats on HF diet, and their subsequent reactions to DIZE treatment are novel, and raise intriguing new questions regarding the RAS and its involvement with aging and obesity.

The loss of body weight in these animals closely followed a decrease in food intake. It is likely that the decrease in food intake contributes to the rapid loss of body weight. However, the primary loss of fat mass and lack of muscle loss suggests an alternative or additional mechanism of weight loss specific to adipose tissue. To confirm this hypothesis, a pair-feeding study is necessary.

DIZE treatment has been shown to be beneficial in models of cardiovascular diseases such as pulmonary hypertension, myocardial infarction, and stroke. [10,12,17] These studies revealed that the beneficial effects of this drug were via the observed increase in ACE2 activity, as effects were blocked by inhibition of either ACE2 with C16, [17] or MasR with A779.[10]With this in mind, we measured indirect blood pressure, via tail cuff (Kent Scientific) in these animals and observed no significant differences (data not shown). While this does not reveal the effect of DIZE on age and diet-induced end organ damage, it **does suggest that the observed anorectic effects observed in the older animals did not have an adverse impact on BP, another indicator of good health.**

Multiple studies have found that the RAS plays an important role in metabolic regulation. For example, ACE inhibitors and ARBs reduce adiposity, attenuate metabolic syndrome, and promote longevity, [3,18,19] and Ang-(1–7) actions at the MasR improve insulin sensitivity and reduce adiposity. [4,20–23] The present study found increased serum ACE2 activity occurred in young and old animals on HF diet treated with DIZE. Additionally, these animals demonstrated a significant decrease in ACE mRNA expression in the TA muscle. One interpretation of these results is that the balance of the RAS has been altered towards increased ACE2 activity and decreased ACE expression, which may suppress the production of Ang II production but increase the synthesis of Ang-(1–7), and may be responsible for the decrease in adiposity as demonstrated in other studies. [4,19,22,24] However, this hypothesis

cannot be confirmed by this study, and should be addressed in future studies comparing ACE inhibition with ACE2 activation in young and old obese animals. Aged rats treated with DIZE demonstrated an increase in both AT₂ and Mas receptor expression in the RTWAT. This significant difference may explain, at least partially, the greater effects seen in old animals treated with DIZE compared to young animals. Notably, there was a significant loss of ACE2 expression in RTWAT in aged animals. The increase in MasR seen in the TA muscle of old rats, along with the DIZE-induced increase of MasR in RTWAT of the same animals, suggests that increased ACE2 activity will have a greater impact in these animals with more receptor available for Ang-(1–7) to activate. Moreover, Ang-(1–7) also binds to the AT₂R to provide beneficial effects. [25–27] As AT₂R was increased in both muscle and RTWAT of old rats fed HF diet, increased ACE2 activity in these animals may have a greater impact at the level of the AT₂R as well. Increased ACE2 activity in aged obese rats may counteract the loss of ACE2 expression in the RTWAT, and thus promote anti-obesogenic effects of HF diet. While further studies are needed to identify specific mechanisms, our results suggest that rousing the counter-regulatory limb of the RAS with DIZE down-regulates ANGII production but augments the synthesis of Ang1–7, which suppresses adiposity, possibly by altering AT₂ and MasR signaling. Future studies using AT₂R and MasR antagonists, and ACE2 inhibitors are necessary to confirm our hypothesis.

Identifying differences between young animals and aged animals on HF diet could lead to further development of therapies targeted to the unique populations. This study in particular highlights the differences that may arise when treating an aging animal in a similar fashion to young animals. As noted previously, the dramatic loss of ACE2 expression in the RTWAT of aged animals may play a role in the pathophysiology of the disease, as well as the dramatic differences seen with DIZE treatment. Additionally, aged rats treated with DIZE demonstrated a significant decrease in UCP-1 expression in BAT tissue, consistent with a decrease in SNS activation. This was a noticeable difference from young rats, which showed no significant change in UCP-1 expression when treated with DIZE. This striking difference may be attributed to central actions of DIZE that are specific to aged rats, as the blood brain barrier of these animals is more permeable. [28] Therefore, it is possible that, in aged rats, DIZE gains greater access to brain nuclei that are sensitive to angiotensin receptor signaling and have been shown to suppress SNS activation. [9,29] Consistent with this speculation, multiple studies have demonstrated differences between the brain RAS and peripheral RAS activation on metabolic outcomes. Animals fed high fat diet with the AT_{1a}R knock-down in the PVN demonstrated increased food intake and body weight gain. [30] Furthermore leptin-induced SNS activation of thermogenesis in the BAT is reduced in mice with AT_{1a}R knock-down in the PVN[31] or in the SFO. [32] These studies demonstrate that central suppression of AT_{1a}R in obesity will exacerbate the phenotype. Therefore, if ACE2 activity is increased centrally we would expect a similar phenotype as increased Ang-(1–7) would act at MasR and AT₂R to oppose AT₁R activation.

Xiao *et al.* demonstrated this phenomenon with transgenic mice in which ACE2 expression is specifically increased centrally; there is a concomitant suppression of SNS function. [33] Our data demonstrating a reduction in UCP-1 expression in aged animals, coupled with the literature supporting a suppression of SNS activity when ACE2 activity is increased in the brain, are consistent with the notion that DIZE crosses a leaky BBB and suppresses the SNS

in aged animals. Furthermore, there was a significant decrease in serum leptin in animals treated with DIZE. This loss of serum leptin, likely further contributes to the decrease in UCP-1 expression from BAT tissue in aged animals. However, these central effects are not enough to counteract the anti-obesity effects of activating the ACE2/Ang-(1–7)/MasR axis peripherally. Therefore, aged animals have a significant loss of weight, despite a significant suppression of thermogenesis-related protein expression. These data pose interesting questions regarding the dichotomy between brain RAS activation and peripheral RAS activation, as well as the effects of aging on RAS and the pathophysiology of obesity. Comparing an animal model which specifically over-expresses ACE2 in the brain to one in which ACE2 is specifically over-expressed in the adipose tissue could further elucidate the metabolic differences of activating the ACE2/Ang-(1–7)/Mas axis centrally versus peripherally.

There are a few limitations to the current study. Not all components of the RAS were examined in circulation and/or in specific tissues. Future studies will address whether levels of AngII and Ang(1–7) are indeed increased. This will further validate our working hypothesis that it is DIZE's action in converting AngII to Ang(1–7) is responsible for the beneficial effects observed in this study.

An intriguing alternative hypothesis to the beneficial effects observed with DIZE in this study is that the decrease in HF food intake and weight loss is actually the result of DIZE-induced toxicity. Converging literature from other animal models seems to confirm this. For example, Baldissera et al reported an increase in liver and renal oxidative stress and a reduction in anti-oxidant enzymes (catalase, SOD) following a 21-day treatment with 3.5 mg/kg of DIZE. [34] The study also documented histological damage to the kidney and liver by day 21 of treatment. At 10 mg/kg, it was reported to cause GI and respiratory issues in dogs. [9]

However, several other studies have shown benefits of DIZE in a variety of conditions including myocardial infarction, stroke, and pulmonary hypertension; although there was no report of food intake or body weight and were generally conducted in young animals. [10,12,17] Therefore, our paper is the first to demonstrate that this intervention may be ill-recommended for use in older animals in the treatment of obesity. A caveat to this cautionary recommendation is that even with this dramatic weight loss in older animals, the loss of mass was more attributable to fat loss rather than muscle mass loss. Thus there may still be protective effects rendered by DIZE with regards to muscle loss. We would still not recommend this treatment in older animals, at this dose, based on the dramatic anorectic impact although this does argue for more targeted ACE2 interventions.

Collectively, the present study indicates that DIZE may serve as a novel pharmacological agent to treat obesity, **but must be pursued with caution**, particularly in the ever-increasing aged population. Importantly, the therapeutic utility of this drug is augmented by its ability to also prevent or reverse many of the co-morbidities often impacting obese patients. Further studies, using the ACE2 inhibitor, MLN-4760, or the MasR antagonist, A779, **and more comprehensively measuring multiple RAS components** are needed to ascertain the precise mechanisms of these effects.

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Figure 1:

Food intake and body weight. A) DIZE treatment significantly decreased kcal intake in both young and old rats fed 60% high fat diet. DIZE treatment significantly decreased B) delta body weight followed by a significant decrease in C) absolute body weight. Data presented as mean and SEM. Two-Way ANOVA with repeated measures. $P < 0.05$; *, significantly different from age-matched controls.

Figure 2:

TD-NMR measurements. A) DIZE treatment significantly decreased percent fat mass in both young and old rats fed 60% high fat diet compared to age-matched controls. B) DIZE treatment significantly increased percent lean mass in old animals compared to age-matched controls. DIZE treatment significantly reduced both C) Total Fat Mass and D) Total Lean Mass compared to age-matched controls. E) DIZE treatment lead to a significant increase in Lean/Fat ratio in both young and old rats compared to age-matched controls. Data presented as mean and SEM. Two-Way ANOVA with repeated measures. $P < 0.05$; *, significantly different from age-matched controls.

Figure 3:

Tissue Weights. A) Old age significantly increased RTWAT compared to young animals, and DIZE treatment significantly decreased RTWAT weight in both young and old rats fed 60% high fat diet compared to age-matched controls. There was a significant effect of both Age and DIZE treatment on PWAT (B) and EWAT (C) weights. D) Old age significantly increased the Sum of WAT weights compared to young animals, and DIZE treatment significantly decreased WAT weight in both young and old rats. E) BAT weight is significantly increased in old controls compared to young controls. DIZE treatment lead to a significant decrease in BAT weight in both young and old rats compared to age-matched controls. F) Age significantly decreased TA weight; however, there was no significant effect of DIZE treatment. G) There was a significant effect of DIZE treatment and Age on heart weights. Data presented as mean and SEM. Two-Way ANOVA with repeated measures. P 0.05; *, significantly different from age matched controls; #, significant effect of DIZE; ^, significant effect of Age.

Serum ACE2 Activity

Figure 4:

DIZE treatment significantly increased serum ACE2 activity. Data presented as mean and SEM. Two-Way ANOVA. $P < 0.05$; #, significant effect of DIZE.

Figure 5:

A) Serum leptin levels were significantly elevated in old animals compared to young, and DIZE significantly lowered serum leptin levels in both young and old animals when compared to age-matched controls. B) Old age lead to a significant increase in UCP-1 expression. DIZE treatment had a significant effect on UCP-1 expression. The interaction between Old Age and DIZE treatment lead to a significant decrease in UCP-1 expression compared to age-matched controls. Data presented as mean and SEM. Two-Way ANOVA

with repeated measures. $P < 0.05$; *, significantly different from age-matched controls; #, significant effect of DIZE; ^, significant effect of Age.

Table 1:

mRNA expression of RAS components in TA Muscle and RTWAT.

Tissue	Gene	Young Control	Young DIZE	Old Control	Old DIZE
TA Muscle					
	<i>ACE</i>	1.02±0.09	0.69±0.06 [#]	1.27±0.22	0.73±0.07 [#]
	<i>AT1R</i>	1.09±0.20	1.01±0.14	1.46±0.20	1.39±0.24
	<i>ACE2</i>	1.04±0.13	1.28±0.18	1.20±0.07	0.80±0.08
	<i>AT2R</i>	1.05±0.17	0.55±0.09	1.48±0.24 [^]	1.78±0.39 [^]
	<i>MasR</i>	1.10±0.21	0.69±0.10	1.38±0.21 [^]	1.58±0.25 [^]
RTWAT					
	<i>ACE</i>	1.00±0.04	0.94±0.11	0.42±0.07 [^]	0.63±0.10 [^]
	<i>AT1R</i>	1.05±0.13	1.20±0.09	0.62±0.04 [^]	0.69±0.04 [^]
	<i>ACE2</i>	1.04±0.12	1.07±0.10	0.08±0.01 [^]	0.07±0.01 [^]
	<i>AT2R</i>	1.08±0.20	0.91±0.20	2.41±0.45 [^]	4.03±0.62 ^{^*}
	<i>MasR</i>	1.08±0.17	1.23±0.11 [#]	1.45±0.19 [^]	2.42±0.34 ^{^#}

Data presented as mean and SEM. Two-Way ANOVA. P = 0.05;

*, significant interaction with Post Hoc analysis indicating significant difference from age matched controls;

#, significant main effect of DIZE;

^, significant main effect of Age.