Chronic AT1 blockade improves glucose homeostasis in obese OLETF rats

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Short title: ARB improves hyperglycemia
Supplemental methods

Blood pressure and heart rate. Once assigned, a subset of animals from each group (LETO n=3, OLETF n=4, and ARB n=4) were surgically implanted with radiotelemeters (PA-C40; DAI, St. Paul, MN) as previously described (Ortiz, et al. 2007a; Ortiz, et al. 2007b). Animals were allowed to recover from the surgery for seven days before the initiation of systolic blood pressure (SBP) recordings at -4 weeks of age. For each animal, SBP values represented the weekly average of measurements taken every other day for 15 weeks. SBP and heart rate were measured at -4, 0, 2, 7 and 11 weeks of age by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan). Treatment with ARB was started at 13 weeks of age (T0). At this age, OLETF rats were characterized by obesity (360 ± 10 vs. 465 ± 8 g), fasting hyperglycemia (4.3 ± 0.2 vs. 6.5 ± 0.3 mmol/L) and elevated mean SBP (125 ± 2 vs. 143 ± 4 mmHg) as compared to LETO.

Pancreatic oxidative damage and antioxidant enzyme activities. A piece of frozen pancreas was homogenized on ice in 50 mM potassium phosphate buffer containing EDTA, PIC (Thermo, Waltham, MA) and PMSF (EMD Millipore, Billerica, MA). Supernatants were used to measure pancreatic 4-hydroxy-2-nonenal (4-HNE) and nitrotyrosine (NT) levels as markers of oxidative damage using antibodies as previously described (Minas, et al. 2015). Uniform protein loading was confirmed by Ponceau S staining. Pancreatic SOD, catalase, and GPx activities were measured using commercially available kits (Cayman Chemical) as previously described for heart (Vázquez-Medina, et al. 2013) and liver (Montez, et al. 2012) in our lab. Total protein content was measured with the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) and used to normalize enzyme activities (Montez et al. 2012; Vázquez-Medina et al. 2013).

Body mass and food intake. BM was measured every other day to calculate the appropriate ARB dose. Food intake was measured at -4, 2 and 11 weeks.
**Tissue Collection.** At 2 and 11 weeks, animals were fasted for 12 hours (21:00 – 09:00), and tissues collected the following morning between 09:00 – 12:00. After BM was measured, animals were decapitated, and trunk blood was collected into chilled vials containing 50 mM EDTA and protease inhibitor cocktail (PIC; Sigma-Aldrich, St. Louis, MO). Immediately after, the pancreas, liver, and epidydimal fat were rapidly removed and snap-frozen in liquid nitrogen. Frozen samples were kept at −80°C until analyzed. Additionally, retroperitoneal fat was removed, its mass was recorded, and a piece fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Blood samples were centrifuged (3000 g x 15 min at 4°C) and plasma was transferred to cryovials, frozen by immersion in liquid nitrogen, and immediately stored at -80°C.

**Histological analysis of adipose tissue**

Paraffin-embedded retroperitoneal fat was sectioned (10 µM thick), deparaffinized, rehydrated and stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO). Representative photos were taken using an EVOS XL Core microscope (Thermo, Waltham, MA) fitted with a 20X objective. Adipocyte diameter and area were measured using Adiposoft software (Galarraga, et al. 2012). Adipocytes were separated by size as follows: 25-50 µm, 51-100 µm, and 101-200 µm as previously described (Muñoz, et al. 2006). For this study, adipocytes between 25-50 µm, 51-100 µm, and 101-200 µm were classified as small, medium, and large adipocytes, respectively.

**Western blot.** Total protein content was measured by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated on 6% to 15% Bis-Tris gels. Proteins were electroblotted using the Bio-Rad Trans-Blot SD semi-dry cell onto 0.45 µm PVDF membranes (EMD Millipore, Billerica, MA). Membranes were blocked with Odyssey blocking solution (LI-COR Biosciences, Lincoln, NE). After blocking, membranes were incubated overnight with one or two of the following primary antibodies: phosphorylated (p)-insulin receptor (IR)-β (Tyr1150/1151), IR-β (Assay Designs, Ann Arbor, MI), glucose transporter 2 (EMD Millipore, Billerica, MA), phosphoenolpyruvate carboxykinase, glucose 6-phosphatase, tumor necrosis factor-alpha and β-actin (Santa Cruz
Biotechnology, Santa Cruz, CA) in Odyssey blocking solution + 0.2% Tween 20 at 4°C. Membranes were washed, incubated for 1 hour with specific secondary antibodies (IRDye; Li-COR Biosciences, Lincoln, NE) in TBS-T + 5% nonfat milk + 0.01% SDS, re-washed, and scanned in an Odyssey infrared imager (LI-COR Biosciences, Lincoln, NE). In addition to consistently load the same amount of total protein per well, densitometry values were further normalized by correcting for the densitometry values of β-actin or Ponceau S staining as previously described (Thorwald, et al. 2018; Vazquez-Anaya, et al. 2016).

**Biochemical analyses.** Fasting plasma glucose and triglycerides were measured using an Analox GM7 analyzer (Analox Instruments, London, UK). Plasma non-esterified fatty acids were measured using a commercially available kit (WAKO, Osaka, Japan). Plasma insulin (EMD Millipore, Billerica, MA), adiponectin and TNF-α (R&D Systems, Minneapolis, MN) were measured using a commercially available rat-specific RIA and ELISA kits. All samples were analyzed in duplicate and run in a single assay with intra-assay, percent coefficients of variability of < 10% for all assays.

References


