EXPERIMENTAL DESIGN AND SAMPLE HANDLING

Animal handling

Animals

Adult male and female Fischer 344 (F344) inbred rats were obtained from the National Institute on Aging (NIA) rodent colony in three cohorts of 20-30 rats. Rats arrived on site at least 4 weeks prior to initiation of exercise training and were handled daily to reduce stress. Upon arrival at the University of Iowa, rats were adapted to a reverse dark-light cycle with lights off at 9:00 AM and lights on at 9:00 PM so that the treadmill training occurred during the normal active part of the day for rats. Rats were housed two per cage (146.4 square inches of floor space) in ventilated racks (Thoren Maxi-Miser IVC Caging System) on Tekland 7093 Shredded Aspen bedding. Animals were fed ad libitum (not measured) with the pelleted Charles River Rat and Mouse 18% (Auto) 5L79 LabDiet (Gateway Lab Supply, St. Louis, Missouri), which has the following calorie composition: 21.196% protein, 14.774% fat (ether extract), 64.030% carbohydrates. These are the standard bedding and diet used at the NIA rodent colony. The animal housing room was monitored daily and maintained at a temperature of 68-77°F and relative humidity of 25-55%. Red lights were used during the rat's dark cycle to provide adequate lighting for the staff to perform routine housing tasks and rodent handling and training. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Treadmill familiarization and training

Treadmill exercise was performed on a Panlab 5-lane rat treadmill (Harvard Instruments, Model LE8710RTS). All animal handling and exercise was performed in the active, dark phase of the rats. Upon arrival, rats were acclimated to the reverse light cycle for a minimum of 10 days. Following the initial acclimation period, rats went through a 12-day treadmill familiarization protocol to expose the rats to the treadmill and to identify potential non-compliant rats. Those rats that were unable to run on the treadmill for 5 minutes at a speed of 10 m/min and grade of 0° were classified as non-compliant and removed from the study. Rats that successfully completed the 12-day familiarization protocol were entered in the rat database and randomized into a control or training group so that mean body weight of the groups were equal. The 8-week rats were randomly assigned to control or training within sex and tertile of weight. 4-week rats were assigned to control without randomization. 1- and 2- week rats were randomly assigned to 1- or 2-week training within sex and tertile of weight.

Exercise training began at 6 months of age in male and female rats and lasted for a duration of 1, 2, 4 or 8 weeks. Rats were exercised on the treadmill 5 days per week using a progressive training protocol designed to exercise the rats at approximately 70% of VO_2max. The starting

treadmill speed was based on VO_2max measurements obtained following familiarization and prior to training in the compliant rats. Training occurred during the dark cycle of the rat, started no earlier than 10:00 AM, and ended no later than 5:00 PM over 5 consecutive days per week. Training was initiated with the treadmill set at a grade of 5°, speeds of 13m/min for males and 16m/min for females, and a duration of 20 minutes. As outlined in Table 1, the duration of exercise was increased by one minute each day until day 31 of training (start of week 7), when a duration of 50 min was reached. The treadmill grade was increased from 5° to 10° at the start of week 3 and stayed at 10° for the remainder of the training. The treadmill speed increased at the start of week 2, 4, 5, 6 and 7. At the start of week 7, speed, grade and duration were fixed and maintained for the final 10 days of the protocol to ensure steady-state had been achieved. If a rat was unable to perform at least 4 days of training per week, it was removed from the study and euthanized. Rats assigned to the control group were placed on the treadmill (0 m/min) for 15 min/day, 5 days per week, and followed a schedule similar to the 8-week training group. The control animals were age-matched to the 8-week training group. Importantly, 6-9 month old rats are mature and stable with limited physiological differences until 12 months of age[1].

Table 1. Progressive training protocol for male and female 6-month-old rats.

Week	Day	Speed (meters/min) Male/Female	Grade (degrees)	Duration (mins)
1	1	13/16	5	20
	2	13/16	5	21
	3	13/16	5	22
	4	13/16	5	23
	5	13/16	5	24
2	6	15/18	5	25
	7	15/18	5	26
	8	15/18	5	27
	9	15/18	5	28
	10	15/18	5	29
3	11	15/18	10	30
	12	15/18	10	31
	13	15/18	10	32
	14	15/18	10	33
	15	15/18	10	34
4	16	18/21	10	35
	17	18/21	10	36
	18	18/21	10	37
	19	18/21	10	38
	20	18/21	10	39
5	21	20/23	10	40
	22	20/23	10	41
	23	20/23	10	42
	24	20/23	10	43
	25	20/23	10	44

6	26	23/26	10	45
	27	23/26	10	46
	28	23/26	10	47
	29	23/26	10	48
	30	23/26	10	49
7	31	25/28	10	50
	32	25/28	10	50
	33	25/28	10	50
	34	25/28	10	50
	35	25/28	10	50
8	36	25/28	10	50
	37	25/28	10	50
	38	25/28	10	50
	39	25/28	10	50
	40	25/28	10	50

Tissue collection

Tissues were collected from all rats 48 hours following the last exercise bout. On the day of collection, food was removed at 8:30 AM, three hours prior to the start of dissections, which occurred between 11:30 AM and 2:30 PM. Rats were sedated with inhaled isoflurane (1-2%). Under isoflurane anesthesia, blood was drawn via cardiac puncture. Under continued isoflurane anesthesia, the right triceps surae muscles (soleus, gastrocnemius, and plantaris), subcutaneous white fat on the right side, right lobe of the liver, heart, and lungs were removed in that specific order. Removal of the heart resulted in death. Immediately following removal of the heart, a guillotine was used for decapitation. The brain was removed from the skull, and the following regions were dissected out in the specified order: hypothalamus, right and left hippocampus, right and left cerebral cortex. Following decapitation, specific organs were removed in the following order: right kidney, right & left adrenal glands, spleen, brown adipose tissue, small intestine (jejunum), colon (transverse and descending) and feces, right testes or ovaries, right vastus lateralis, and tibia. All tissues were flash-frozen in liquid nitrogen immediately upon removal, placed in cryovials, and stored at -80°C. All tissues were subsequently shipped on dry ice to the Biospecimens Repository at the University of Vermont.

Sample distribution

Archiving

Frozen tissue samples received by the Biospecimens Repository at the University of Vermont were logged in to Freezerworks (Dataworks Development Inc., Mountlake Terrace, Washington) and stored in -80ºC freezers. All samples were labeled with a unique 11-digit barcode.

Selection of animals for molecular assays

Tissue samples were collected for between 12 and 20 animals per sex, per training group (i.e., sedentary controls or animals trained for 1, 2, 4, or 8 weeks). Six animals per sex per training group were randomly selected for molecular profiling. A subset of five replicates were used for metabolomic assays. Additional rats were selected at random if there were insufficient aliquots for specific molecular assays.

Cryopulverization

Tissues samples were stored at -80ºC until time of processing. Frozen tissue samples were transferred from storage vials to Covaris tissueTUBEs (Covaris, Inc, Woburn, Massachusetts) flash-chilled in liquid nitrogen. Larger tissues were first broken into smaller pieces on a chilled, foil-covered stainless steel block using a foil-covered hammer or pestle. The tissue piece(s) were centered in the tissueTUBE pouch (primary impact zone), and a pre-chilled glass transfer tube was attached. Filled tissueTUBEs were placed on dry ice while the Covaris CryoPREP CP02 (Covaris Inc, Woburn, Massachusetts) was set to the appropriate setting for the tissue type. TissueTUBEs were dipped in liquid nitrogen, and the glass transfer tube was loosened ¼ turn to prevent pressure build-up inside the tissueTUBE before being inserted in the Covaris CP02 to pulverize. Tissues requiring an additional round of pulverization were first inspected to ensure tissueTUBE integrity and dipped in liquid nitrogen before being put back into the Covaris CP02. After pulverization, tissueTUBEs were dipped in liquid nitrogen and then inverted to move the pulverized sample into the glass transfer tube. Pulverized samples were transferred to cryogenic storage vials for long term storage at -80ºC.

Aliquoting

Frozen tissue storage vials were removed from the -80ºC freezer and placed on dry ice. Aliquot vials were set up in a prechilled CoolRack XT CFT24 (Corning, Corning, New York) sitting in dry ice inside an AirClean 600 Dead Air Workstation (AirClean Systems, Creedmoor, North Carolina). Working with one storage vial at a time, disposable plastic transfer scoops of predetermined volumes were chilled in liquid nitrogen and then used to measure and transfer tissue to aliquot vials. A chilled metal spatula was used to break up any tissue clumps in the storage tubes before aliquoting. Aliquot vials were capped and stored at -80ºC before shipping to chemical analysis labs. By experimentation we determined that the target weights could be aliquoted with a reproducibility of +/- 15% of the target. Different sized scoops were needed for different tissues depending on fat content.

Reference standards

Aliquots of assay- and tissue-specific reference standards were included in molecular assays in order to evaluate technical differences across batches. Samples from the same tissue and sex were pulverized together to create a homogenous pool. One aliquot each of the male and female reference standards was included on each plate of study samples. Tissue-matched reference standards were used when possible. Metabolomics and lipidomics reference

standards were from pilot samples collected at the University of Florida, Joslin Diabetes Center, and the University of Iowa for a separate MoTrPAC study. Samples were split to create pools of sedentary and immediate-post-exercise reference standards. One aliquot each of the sedentary and exercised reference standards was included with each batch of study samples. All samples were from Fischer 344 (F344) inbred rats obtained from the NIA rodent colony.