Online Data Supplement

Intermittent Hypoxia Causes Insulin Resistance in Lean Mice Independent of Autonomic Activity

Nao Iiyori, Laura C. Alonso, Jianguo Li, Mark H. Sanders, Adolfo Garcia-Ocana, Robert M. O'Doherty, Vsevolod Y. Polotsky, Christopher P. O'Donnell

Animals

Experiments were conducted in lean wild-type male C57BL/6J mice aged 8-12 weeks from Jackson Laboratory (Bar Harbor, ME). The study was approved by the University of Pittsburgh Institutional Animal Use and Care Committee and complied with the American Physiological Society Guidelines for Animal Studies.

Surgical Procedures

Anesthesia was induced and maintained using isoflurane administered through a facemask. Catheters (MRE025 Braintree Scientific, Inc., Ma) were chronically implanted in the left femoral artery and vein for measurement of blood glucose and for infusion of solutions. The catheters were attached to a double channel fluid swivel (375/25 Instech Laboratories Inc., PA) and perfused throughout the recovery period by an infusion pump (7.7 μl/hr) with a sterile saline solution containing heparin (20 U/ml). The success rate for femoral artery and vein catheterization was above 90%. All animals were allowed 48-72 hrs to recover from the surgery before beginning data collection and maintained in a 12hr light:12 hr dark environment beginning at 0800 hr. Behaviorally the animals recovered to a normal food intake (3.5-4.0 gm of dry chow per day), groomed themselves, moved freely, and were able to construct nests from nestlets (Ancare, Bellmore, NY) in their home cages. Most animals displayed some loss of function of the catheterized left hind limb immediately after surgery that typically recovered to allow weight bearing by 48-72 hrs.

Model of Intermittent Hypoxia

A gas control delivery system was designed to regulate the flow of nitrogen and room air into a customized cage housing individual mice during the experimental period, as previously described[1]. A series of programmable solenoids and flow regulators altered the FIO_2 level over a defined and repeatable profile. During each period of IH the FIO_2 was reduced from 20.9% to 5.0-6.0% over a 30 sec period and rapidly reoxygenated to room air levels in the succeeding 30 sec period (i.e. 60 cycles per hour). Control animals were exposed to intermittent air (IA) using an identical protocol of gas flows as the IH protocol except room air was used rather than nitrogen.

Experimental Design

On the day of experimentation, mice were moved from their holding cage to the customized IH cage at 0800-0900 hr for the duration of the nine-hour experiment. During the experimental period mice were given free access to water, but food was removed. In the first set of experiments, animals were exposed to either IH or IA (control) throughout the nine-hour protocol and similar protocols to those described by Kim et al. [2] in mice were adapted for determination of insulin sensitivity, hepatic glucose output, and muscle glucose utilization. Between 5.6 and 7-hrs (baseline period) of IH or IA exposure, baseline hepatic glucose output was determined over an 80 min period by infusing [3-^{3}H] glucose (10 μCi bolus + 0.1 μCi/min; NEN Life Science Products Inc.) through the femoral vein and acquiring a 100 μl sample of arterial blood for determination of [3-^{3}H] glucose levels at the 80 min time point. The blood was centrifuged at 10,000 g, the supernatant collected and the red blood cells resuspended in heparinized saline and reinfused in the mouse. During the clamp procedure (7-9 hr), [3-

^{3}H] glucose (0.1 μCi/min; NEN Life Science Products Inc.) was administered in combination with insulin to allow assessment of hepatic glucose output under hyperinsulinemic euglycemic conditions. Whole-body insulin sensitivity was determined by infusing a constant rate of human insulin (20 mU/kg/min; Novalin R, Novo Nordisk, Princeton, NJ) and a variable rate of D50 glucose (Hospira, Inc, Lake Forest, IL) through the femoral venous catheter and maintaining plasma glucose at 100-125 mg/dl. Blood glucose levels were sampled from the femoral artery catheter at 10 min intervals using an Ascencia elite XL glucometer (~2μl samples; Bayer, Mishawaka, IN). The average glucose infusion rate over the last 30 minutes of the hyperinsulinemic euglycemic clamp was used to determine insulin sensitivity. Pilot studies were undertaken to determine the appropriate starting rates of D50 glucose infusion required to maintain euglycemia under each experimental condition. Once the starting rates for D50 glucose infusion were established the steady-state glucose infusion rate could be achieved with minimal fluctuations in the level of euglycemia in individual animals for a given experimental condition.

In the second set of experiments, muscle glucose utilization was determined in two groups of animals exposed to either IA or IH. A hyperinsulinemic euglycemic clamp identical to that described above was performed and a bolus of 12 mCi [2-^{3}H] deoxyglucose was administered and 20 μl blood samples obtained at 1, 2.5, 5, 10, 15, and 25 min during the steady-state phase of the glucose infusion. At the end of the experiment the animals were euthanized with pentobarbital (200 mg/kg) and the soleus, vastus, and gastrocnemius muscle from the contralateral side to the catheters was dissected and frozen in liquid nitrogen and stored at –80^{C}.

In the third set of experiments, mice were exposed to IH or IA and hepatic glucose output and the hyperinsulinemic euglycemic clamp performed as detailed above, during a continuous i.v. infusion of the autonomic ganglionic blocker hexamethonium (10 mg/kg bolus + 20 mg/kg/hr) that was continued throughout the 9-hr protocol. Efficacy of the ganglionic blockade was determined by assessing acute baroreflex reductions in heart rate during an i.v. bolus administration of phenylephrine (50 μg/ml) to acutely raise blood pressure.

Plasma Insulin and Plasma Corticosterone

Plasma insulin levels were measured with a rat insulin ultrasensitive radioimmunoassay kit (cross reactivity with mouse insulin 100%) from Linco Research, Inc. (St. Charles, MO). Plasma corticosterone levels were measured using a kit from Diagnostic Systems Laboratories, Inc. (Webster, Texas).

Assessment of Hepatic Glucose Output and Muscle Glucose Utilization

The basal hepatic glucose output was determined as the ratio of rate of infusion of [3-^{3}H]glucose to [3-^{3}H]glucose/mg glucose in the sampled plasma, as previously described[2]. For assessment of hepatic glucose output during the hyperinsulinemic euglycemic clamp, the steady-state rate of glucose infusion required to maintain euglycemia was subtracted from the ratio of rate of infusion of [3-^{3}H]glucose to [3-^{3}H]glucose/mg glucose in the sampled plasma.

The protocol of Shearer et al.[3] was used to determine muscle specific clearance (Kg) of [2-^{3}H] deoxyglucose from the ratio of [2-^{3}H] deoxyglucose-6-phosphate in

muscle to the area under the curve of plasma [2-^{3}H] deoxyglucose. Muscle glucose utilization (Rg) was determined as $Rg = Kg * Glucose_{plama}$.

Statistical Analyses

Data are reported as mean ± s.e.m. Differences between means in animals exposed to IA and IH were determined by unpaired, two-tailed, t-tests. Comparison of differences in means between intact and autonomically blocked animals under conditions of IA and IH exposure were determined by two-way ANOVA.

References

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