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TITLE:

Experimental Approach to Examine Leptin Signaling in the Carotid Bodies and its Effects on Control of Breathing

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KEYWORDS:

leptin, carotid body, hypoxic ventilatory response, carotid sinus nerve, viral vector, obesity

SHORT ABSTRACT:

Our study focuses on the effects of leptin signaling in carotid body (CB) on the hypoxic ventilatory response (HVR). We performed 'loss of function' experiments measuring the effect of leptin on HVR after CB denervation and 'gain of function' experiments measuring HVR after overexpression of the leptin receptor in CB.

LONG ABSTRACT:

An adipocyte-produced hormone leptin is a potent respiratory stimulant, which may play an important role in defending respiratory function in obesity. The carotid bodies (CB), a key organ of peripheral hypoxic sensitivity, express the long functional isoform of leptin receptor ($LepR^b$) but the role of leptin signaling in control of breathing has not been fully elucidated. We examined the hypoxic ventilatory response (HVR) (1) in C57BL/6J mice before and after leptin infusion at baseline and after CB denervation; (2) in *$LepR^b$*-deficient obese *db/db* mice at baseline and after $LepR^b$ overexpression in CBs. In C57BL/6J mice, leptin increased HVR and effects of leptin on HVR were abolished by CB denervation. In *db/db* mice, $LepR^b$ expression in CB augmented the HVR. Therefore, we conclude that leptin acts in CB to augment responses to hypoxia.

INTRODUCTION:

An adipocyte produced hormone leptin acts in the hypothalamus to suppress food intake and increase metabolic rate. Studies performed in our laboratory[1,2] and by other investigators[3,4] showed that leptin increases the hypercapnic ventilatory response (HVR) preventing obesity hypoventilation in leptin deficient obesity. However, a majority of obese individuals have high plasma leptin levels and demonstrate resistance to the metabolic and respiratory effects of the hormone[5–8]. Resistance to leptin is multifactorial, but limited permeability of the blood-brain barrier (BBB) to leptin plays a major role. We propose that leptin acts below BBB in a key organ

of peripheral hypoxic sensitivity, the carotid bodies (CB), to defend breathing in obese individuals. CBs express the long functional isoform of leptin receptor, LepR^b, but the role of CB in respiratory effects of leptin has not been sufficiently elucidated[9,10].

The goal of our method was to examine the effect of leptin signaling in the CB on HVR. Our rationale was to perform (a) loss of function experiments infusing leptin in mice with intact carotid bodies and denervated carotid bodies followed by HVR measurements; (b) gain of function experiments in *db/db* mice lacking LepR^b, in which we measured the HVR at baseline and after expression of LepR^b exclusively in CB. The advantage of our techniques was that we performed all our experiments in unrestrained unanesthetized mice during sleep and wakefulness. Previous investigators either performed their experiments under anesthesia[9] or did not measure effects of leptin during sleep[10]. In addition, our study is the first to utilize a unique gain of function approach with selective LepR^b expression in CB described above.

In the broad context, our approach can be generalized to other receptors expressed in CB and their role in hypoxic sensitivity. Investigators can infuse a ligand to a receptor of interest and measure the HVR at baseline and after CB denervation. As a complementary approach, a receptor of interest can be overexpressed in CB and HVR measurements can be performed before and after overexpression using our technology described in this manuscript.

PROTOCOLS:

All experimental protocols have been approved by the Institutional Animal Care and Use Committee (MO18M211).

1. Leptin infusion

NOTE: In order to examine the effect of leptin on breathing, we infused leptin subcutaneously in lean C57BL/6J mice by an osmotic pump to raise circulating leptin levels to those observed in obese mice.

1.1. Osmotic pump preparation

1.1.1. Weigh the empty pump to check the net weight of the solution loaded.

1.1.2. Add Leptin (5 mg/mL) to the osmotic pump (1 µL/h for 3 days). Fill the pump with a small syringe (1 mL). After filling, close the pump with the provided blunt tipped 27 gauge filling tube.

NOTE: The syringe and the attached tube should be free of air bubbles.

1.1.3. After filling, weigh the pump again to check the net weight of the solution.

1.1.4. Insert the leptin pump subcutaneously in the interscapular area.

NOTE: If you want to start the infusion immediately, incubate the pre-filled pump in the sterile saline at 37 °C for at least 4 to 6 h (preferably overnight).

2. Hypoxic ventilatory response (HVR)

NOTE: Thermoneutral conditions eliminate stressful factors imposed by cool ambient temperature and significantly modify metabolism in mice. Therefore, all respiratory measurements should be performed at the thermoneutral conditions (t = 30 °C) using a neonatal incubator[12] (**Figure 1A**). The whole body plethysmography chamber (WBP) has been used for all measurements. All animals should be acclimated to the barometric plethysmography chamber and to a sham neck collar for subsequent pulse oximetry recording for 3-5 days prior to the HVR measurements. HVR is recorded between 10 AM and 5 PM. HVR is suppressed during sleep, therefore it can be measured separately during sleep and quiet wakefulness[13]. To ensure the specific sleep-wake stage of the animal during the HVR measurement, EEG/EMG electrodes should be implanted as an EEG/EMG headmount as previously described[14]. Animals should be allowed to recover for at least 72 h after the headmount. Sleep staging should be performed in 5 s epochs. NREM sleep is recognized by the slow wave EEG activity occupying greater than 50% of the epoch. Quiet wakefulness is manifested by the alpha EEG activity in the absence of movements. REM sleep is identified by the predominant alpha and theta EEG activity in the presence of reduced muscle tone on EMG. Typically, REM sleep is not observed during the HVR gas challenges.

2.1. The HVR recording protocol

2.1.1. Use the WBP chamber of the following size: internal diameter of 80 mm, height of 50.5 mm, and volume of 0.4 L. The WBP chamber consists of two chambers, sealed and reference chambers, and a circular platform to place the mouse[14] (**Figure 1B**). Inflow and outflow inside the chamber are controlled by positive and negative pressure sources maintaining the atmospheric pressure. This control creates a steady bias flow in the chamber and prevents CO_2 retention. For more details about the WBP set up, see Hernandez and colleagues[15].

2.1.2. Measure the temperature inside the chamber and the relative humidity in the room prior to placing the mouse in the WBP.

2.1.3. Measure the body weight and rectal temperature.

2.1.4. Put the oximeter collar around the neck of the mouse (the area should be previously shaved).

2.1.5. Place the mouse inside the WBP and ensure that the chamber is completely sealed in order to avoid air leakages.

2.1.6. Wait for approximately 30 min until the mouse is quiet and the chamber is at a constant

volume.

2.1.7. Normoxia: after 30 min, if the mouse is quiet, start recording the respiratory signals and peripheral oxygen saturation (SpO_2) at normoxia phase (21% FiO_2) for 20 min, using LabChart 7 Pro (version 7.2).

2.1.8. Hypoxia: after 20 min of quiet normoxia, start recording the first cycle of acute hypoxia and SpO_2. For hypoxia phase, expose the animals to a constant mixed gas flow composed by 10% O_2 and 3% CO_2 for 5 min.

2.1.8.1. During the first 30 s of hypoxia, open the 2 small side ports at the base of the chamber allowing the influx of the mixed gas (FiO_2 drops from 21% to 10%).

2.1.8.2. After the initial 30 s, close one of the small side ports of the WBP chamber and keep recording at the constant hypoxia at 10% FiO_2 for 5 min.

NOTE: The mixture of 10% O_2 and 3% CO_2 at hypoxia is used in order to maintain eucapnea[11].

2.1.9. After 5 min of hypoxia, expose the mouse to room air again (21% FiO_2) by switching the inflow source.

2.1.10. Wait for at least 30 min until the next normoxia recording in order to recover from the previous hypoxic exposure to avoid the ventilatory roll-off phenomenon (i.e., central suppression of breathing during hypoxia[16]).

NOTE: Repeat normoxia/hypoxia cycles three times in each mouse to assure reproducibility of the measurements. In our experience, additional (greater than 3 times on a given day) hypoxic exposures should be avoided, because of the ventilatory adaptation (the roll-off phenomenon).

2.1.11. At the end of the experiment, calibrate the WBP chamber (with the mouse is still inside) by injecting 1 mL of room air 3 times with a syringe plugged in one of the small side ports at the base of the chamber.

2.1.12. Measure the temperature in the chamber again with the animal inside it.

2.1.13. Open the chamber and measure the rectal temperature of the mouse before placing it back to its home cage.

2.2. HVR calculation

2.2.1. Digitize all signals for HVR calculation at 1,000 Hz (sampling frequency per channel).

NOTE: WBP tidal volume analysis is performed in Lab Chart 7 based on Drorbaugh and Fenn equation[17]. Required variables comprise mouse rectal temperature and the chamber

temperature (before and after HVR measurement), relative humidity, and the chamber gas constant. This constant is resultant from WBP pressure deflection after the 1 mL injections of air in the calibration phase. For more details, see Hernandez and colleagues[15].

2.2.2. After calculating the Drorbaugh and Fenn equation, multiply the tidal volume (Vt) chamber channel by the constant.

2.2.3. **Selection of recordings for the analysis**

2.2.3.1. Normoxia: select only sections of steady breathing with constant tidal volume. Avoid sections in close proximity to mouse's movements.

2.2.3.2. Hypoxia: discard first 30 s when O_2 levels are declining from 21% to 10%, select sections from 30 s to 2 min of 10% O_2 exposure (a 90 s interval). Within this interval, select only sections with steady breathing with constant tidal volume. Avoid sections in close proximity to mouse's movements. The analysis is reliable if at least 10 s of hypoxia is selected in each cycle.

NOTE: The peripheral component of chemoreflex governed by CB predominates during the first 1-2 min of exposure[16,18,19]. During the second phase, between 2 min and 5 min of hypoxic exposure, both peripheral and central component play a role. Finally, the third phase, > 5 min, is characterized by hypoventilation (the roll-off phenomenon) governed predominantly by central chemoreceptors. Our experience shows that mice are often awake during hypoxic exposition because of the manual switches in the airflow source.

2.2.4. After the selection, calculate the mean minute ventilation (V_E) at normoxic and hypoxic conditions using the formula V_E = respiratory rate X tidal volume.

2.2.5. Calculate the mean SpO_2 at normoxia and hypoxia conditions.

2.2.6. Calculate the HVR manually using the formula HVR = (V_E (10% O_2) - V_E (21%O_2)) / (SpO_2 (10% O_2) – SpO_2 (21% O_2)).

NOTE: In C57BL/6J mice, the osmotic pump for leptin infusion may impair the accurate SpO_2 signal detection by the neck collar. In this case, HVR is calculated based on the FiO_2 values at normoxic and hypoxic conditions as HVR = ΔV_E / ΔFiO_2[11].

3. Carotid body denervation or carotid sinus nerve dissection (CSND)

NOTE: We performed combined surgical and chemical denervation one week apart, because surgical denervation alone does not abolish the hypoxic chemoreflex.

3.1. Surgical preparation

3.1.1. Perform all the procedures on adult male C57BL/6J mice. Sterilize all surgical

instruments. Use sterile surgical gloves, syringes and cotton tipped applicators.

3.1.2. Anesthetize male C57BL/6J mice with 1-2% isoflurane using a nose cone and place a warm blanket to prevent hypothermia. Isoflurane is carefully titrated to maintain respiratory rate at 1 Hz (60 breaths/min). The adequacy of anesthesia prior to starting surgeries will be assessed by the breathing frequency, the absence of movements and audible noises, the absence of response to tactile stimuli and the forelimb or hindlimb pedal withdrawal reflex.

3.1.3. Administer Buprenorphine (0.05 mg/kg) intraperitoneally to prevent pain discomfort. Remove the hair at ventral region of the neck and disinfect the area with betadine and alcohol.

3.1.4. To prevent corneal desiccation, lubricate the mice's eyes with sterile ophthalmic ointment during anesthesia.

3.2. CSND procedures

Stage 1: Surgical denervation

3.2.1. After midline incision, expose the bifurcation of the common carotid arteries by removing connective tissues and adipose tissue.

3.2.2. Identify the hypoglossal nerve, which is usually very prominent, and then lift it up to expose the glossopharyngeal nerve immediately underneath. Dissect carotid sinus nerves bilaterally using micro spring scissors.

3.2.3. Close the incision with 6-0 silk suture.

3.2.4. Administer 1 mL of normal saline subcutaneously to prevent dehydration.

3.2.5. House the mice in a recovery chamber and monitor their behavior every 15 min for initial 1 h until the mice regain consciousness to maintain sternal recumbency. Return the mice to their home cages after they are fully recovered. Keep monitoring the mice twice per day for next 3 days. Give additional buprenorphine if mice show any signs of pain (e.g., reduced appetite, restlessness).

Stage 2: Chemical denervation

3.2.6. One week after the surgery, paint the carotid artery with a solution of 2% phenol diluted in ethanol at the points of branching from the glossopharyngeal nerve to the cranial pole of the CB. The same post-operative care should be provided after the chemical denervation as described above in the surgical denervation section.

NOTE: For a sham surgery group, the same procedures are performed except the carotid sinus nerve dissection.

3.2.7. Let the animals recover for 5-7 days before HVR measurements.

4. Expression of LepR^b in the CB using an adenoviral vector (*Ad-LepR^b*) *vs* control (*Ad-LacZ*)

4.1. *Ad-LepR^b* and *Ad-LacZ* suspension

4.1.1. Transfect the mice with adenovirus (*Ad-LepR^b-GFP*, 2-5x10^{10} pfu/mL for overexpression, *Ad-Lacz*, 1x10^{10} pfu/mL for control) to the CB area.

4.1.2. Thaw the Matrigel matrix by submerging the vial in ice in a 4 °C refrigerator overnight.

4.1.3. Gently suspend adenovirus in liquid Matrigel matrix at 1:5 (1 µL of Matrigel matrix and 4 µL of adenovirus applied bilaterally).

4.1.4. Always keep viral suspension on ice until it is applied in the CB.

4.2. Surgical preparation

4.2.1. Use the same surgical instruments as the CSND protocol.

4.2.2. Anesthetize *LepR^b*-deficient *db/db* mice with 2-2.5% isoflurane using a nose cone and place the animals on a warm blanket to prevent hypothermia. Isoflurane is carefully titrated to maintain respiratory rate at 1 Hz (60 breaths/min). The adequacy of anesthesia will be assessed by the breathing frequency, the absence of movements and audible noises, the absence of response to tactile stimuli and the forelimb or hindlimb pedal withdrawal reflex.

4.2.3. Administer buprenorphine (0.05 mg/kg) intraperitoneally.

4.2.4. Remove the hair at ventral region of the neck and disinfect the area with betadine and alcohol as in the CSND protocol.

4.2.5. To prevent corneal desiccation, lubricate the mice's eyes with sterile ophthalmic ointment during anesthesia.

4.3. Adenovirus treatment of the CB

4.3.1. Expose CBs as described in the CSND protocol and apply 5 µL of the viral suspension to the CB area bilaterally.

4.3.2. Wait 2-3 min until the liquid Matrigel matrix becomes a gel. After confirming that the viral suspension was congealed, close the incision with 6.0 silk suture.

4.4. After surgery

4.4.1. House the mice in a recovery chamber and monitor their behavior every 15 min for initial 1 h until the mice regain consciousness to maintain sternal recumbency. Return the mice to their home cages after they are fully recovered. Keep monitoring the mice twice per day for next 3 days. Give additional buprenorphine if mice show any signs of pain (e.g., reduced appetite, restlessness).

4.4.2. Administer Buprenorphine (0.05 mg/kg) intraperitoneally as needed to prevent pain discomfort during the post-operative period.

4.4.3. Measure HVR 9 days after the adenovirus transfection.

REPRESENTATIVE RESULTS:

Continuous infusion of leptin significantly increased HVR in lean C57BL/6J mice from 0.23 to 0.31 mL/min/g/ΔFiO_2 ($P<0.001$, **Figure 2**)[11]. CSND abolished the leptin-induced increase in HVR (**Figure 2**), while no attenuating effects of CSND on HVR were observed in sham surgery group after leptin infusion.

$LepR^b$ expression in the CB of *$LepR^b$*-deficient obese *db/db* mice induced a significant increase in HVR from 0.05 to 0.06 mL/min/g/ΔSpO_2 (**Figure 3**). In animals transfected with control *Ad-LacZ* in CB, HVR did not change.

FIGURE LEGENDS:

Figure 1. HVR measurements. Experiments should be performed at thermoneutral conditions using **(A)** a neonatal incubator at 30 °C and are recorded in a **(B)** whole body plethysmography chamber.

Figure 2. **Leptin augmented the hypoxic ventilatory response (HVR) and the effects were abolished by carotid sinus nerve dissection (CSND) in C57BL/6J mice.** This figure has been modified from Caballero-Eraso et al[11].

Figure 3. $LepR^b$ expression in the carotid bodies (CB) of *$LepR^b$*-deficient *db/db* mice increased the hypoxic ventilatory response (HVR). This figure has been modified from Caballero-Eraso et al[11].

DISCUSSION:

The main focus of our study was to examine respiratory effects of leptin signaling in the CB. Several protocols have been developed to assess the role of leptin in a mechanistic fashion. First, specific contribution of CB to the HVR was analyzed by careful quantification of the HVR during the first 2 min of hypoxic exposure. Second, the relevance of CB in leptin-mediated up-regulation of control of breathing was examined by two complementary approaches. In lean wild-type mice with low leptin levels, the HVR was measured at baseline and after continuous infusion of leptin; the experiment was repeated after CB denervation. In *$LepR^b$*-deficient *db/db* mice, the HVR was measured at baseline and after $LepR^b$ expression in the CB.

Several protocols have been used to measure HVR in rodents, exposing the animals to hypoxic gases. We have developed an HVR protocol to examine the role of CB in the peripheral chemoreflex and the effects of leptin in CB on the control of breathing. CB chemoreflex has a relatively short time domain. The first 1-2 min of hypoxia are characterized by an acute augmentation of ventilatory response followed by a slow return to baseline ventilation after 2 min of the carotid sinus nerve stimulation[16,18,19]. Thus, in our HVR protocol, the analyses are conducted within a 90 s interval between the first 30 s of hypoxia and 2 min of 10% O_2 exposure, corresponding to the domination of peripheral chemoreflex governed by CB. This short-time analysis avoids the hypoxic ventilatory depression in the animals. In our HVR protocol, we also used a fixed 3% CO_2 tension in hypoxia in order to circumvent the hypocapnia induced by hyperventilation during acute hypoxic exposure. Finally, we have developed the HVR protocol under constant thermoneutral conditions, keeping the mice at a temperature of around 30 °C. Our experience shows that short exposures to hypoxia can decrease rectal temperature in mice if exposure occurs at room temperature[11], while hypoxia at thermoneutrality does not induce significant metabolic changes[12].

CSND in mice is technically challenging, because of the small size of the animals and their CBs. We have developed a consistently successful approach with nearly 100% survival rate by strict adherence to our protocol. Controlled conditions in our protocol includes thermoneutral environment, carefully controlled anesthesia and standard sterile microsurgical techniques with visualization of the glossopharyngeal nerve as vigilant post-operative management with pain control. Our experience shows that surgical denervation alone does not abolish the hypoxic chemoreflex. The second step, chemical denervation, is also followed by careful post-operative management to improve survival.

Our most innovative technique is selective gene over-expression in the CB area. This approach has not been implemented previously, because of the small size of the CBs and the lack of specific drivers allowing to express a gene of interest in a particular cell type. In fact, type I CB cells are very similar to sympathetic neurons or cells of the adrenal medulla, whereas type II cells are similar to astrocytes[20, 21]. We took advantage of *db/db* mice, which lack the *LepR*^{b} gene, our ability to apply adenoviral suspension almost exclusively to the CB area, and properties of the Matrigel matrix, which rapidly solidifies at 37 °C. Our novel approach can be used in the future to study a role of any gene expressed in CB using mice with the whole body tyrosine hydroxylase specific (type I cells) or GFAP specific (type II cells) knockouts.

Our protocols have several limitations. First, we used 3% CO_2 to determine the HVR and the question remains open if the fraction of the HVR can actually be attributed to the hypercapnic response. In order to address this limitation, investigators can simultaneously measure responses to 3% CO_2 balanced in hyperoxic gas, which would turn the CB off. Second, HVR may not be completely eliminated by CSND[22]. This phenomenon can be attributed to neuroplasticity, which is particularly prominent in mice. Therefore, it is important to study HVR as soon as possible after CSND and always use sham surgery control. Third, our CB gene expression approach lacked cell type and organ specificity. Molecular techniques with the

future use of more selective promoters may help to counter this limitation.

In conclusion, despite described above limitations, our protocols in combination allow to study the role of specific CB genes in physiological responses to hypoxia.

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DISCLOSURES:
The authors have no conflicting interests or disclosures.

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Figure 1

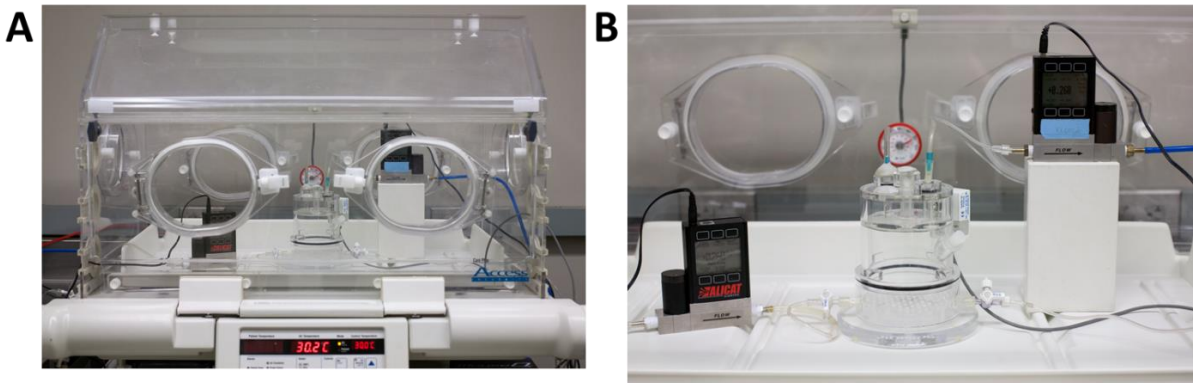


Figure 2

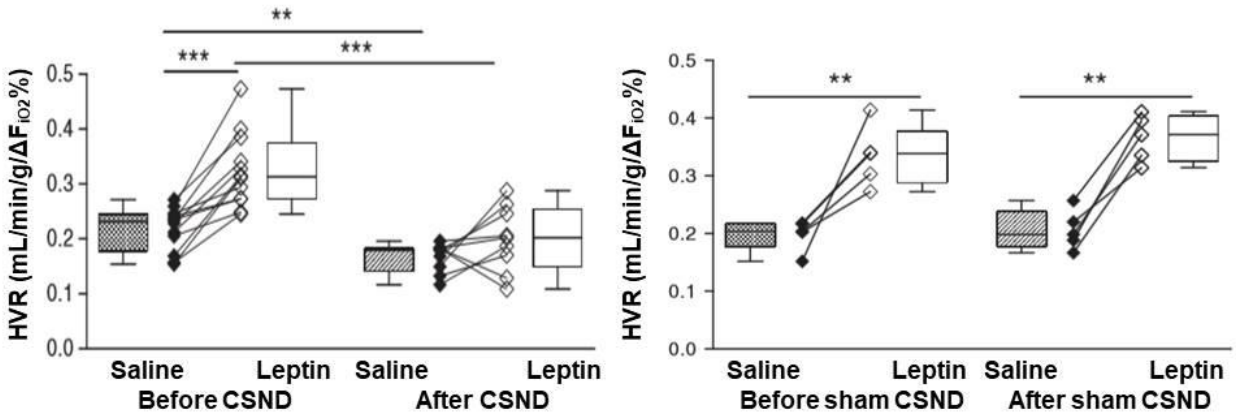
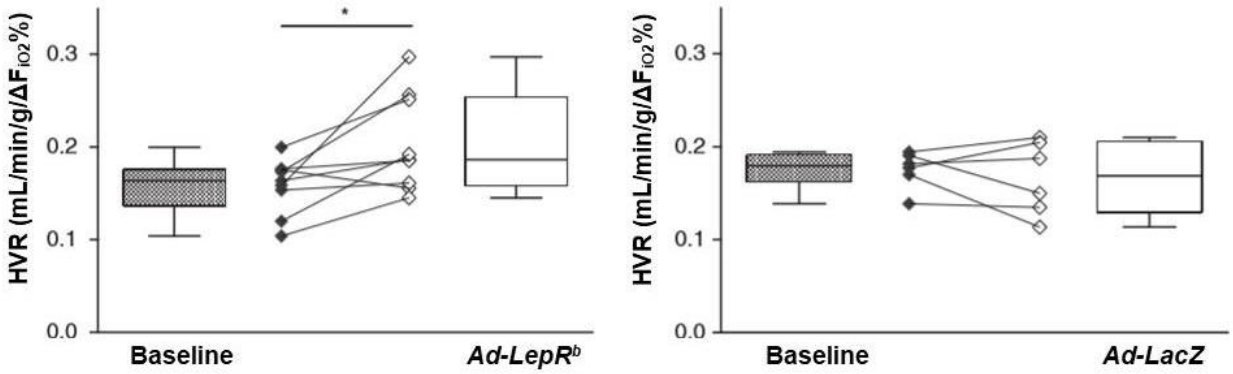


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1ml Insulin Syringes	BD Biosciences	309311	
1x PBS (pH 7.4)	Gibco	10010-023	500 ml
Ad-Lacz	Dr. Christopher Rhodes (University		$1x10^{10}$ pfu/ml
Ad-LepR ^{b} *-GFP*	Vector Biolabs	ADV-263380	$2\text{-}5x10^{10}$ pfu/ml
Anesthetic cart	Atlantic Biomedical		
Betadine	Purdue Products Ltd.	12496-0757-5	
Buprenorphine (Buprenex)	Reckitt Benckiser Healthcare Ltd.	12496-0757-5	0.3mg/ml
C57Bl/6J	Jackson laboratory	000664	Mice Strain
Cotton Gauze Sponges	Fisherbrand	22-362-178	
db/db	Jackson laboratory	000697	Mice Strain
Ethanol	Pharmco-AAPER	111000200	
Isoflurane	Vetone	502017	
Lab Chart	Data Science International (DSI)		Software
Matrigel Matrix	BD Biosciences	356234	
Micro Spring Scissors	World Precision Instruments (WPI)	14124	
Mouse Ox Plus	STARR Life Sciences Corp.		Software
Mouse Ox Plus Collar Sensor	STARR Life Sciences Corp.	015022-2	Medium Collar Clip Special 7”
Mouse Whole Body Plethysmography Chamber	Data Science International (DSI)	PLY3211	
Ohio Care Plus Incubator	Ohmeda	HCHD000173	
Operating Scissors	World Precision Instruments (WPI)	501753-G	Straight
Osmotic Pump	Alzet	1003D	1ul per hour, 3 days
Phenol	Sigma-Aldrich	P4557	

Recombinant Mouse Leptin protein	R&D systems	498-OB-05M	5mg
Saline	RICCA Chemical	7210-16	0.9% Sodium Chloride
Sterile Surgical Suture	DemeTech	DT-639-1	Silk, size 6-0
Thermometer	Innovative Calibration Solutions (INNOCAL)	EW 20250-91	

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Title of Article: Experimental Approach to Examine Leptin Signaling in the Carotid Bodies and its Effects on Control of Breathing

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