Intermittent hypoxia-induced glucose intolerance is abolished by α-adrenergic blockade or adrenal medullectomy

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INTRODUCTION

Obstructive sleep apnea (OSA) is a common condition that causes upper airway collapse during sleep. There is emerging evidence that OSA may be linked to the development of diabetes mellitus. For example, OSA is associated with diabetest in cross-sectional studies (64), and OSA increases risks of developing diabetes in longitudinal studies (12, 16, 53). Furthermore, core features of diabetes, such as insulin resistance (29, 32, 76) and hyperglycemia (4, 17, 18, 59), are improved by treatment of OSA with continuous positive airway pressure (57). Mechanisms by which OSA might induce metabolic impairment are poorly understood. Repetitive airway collapse causes many stressful consequences such as sleep fragmentations and intermittent hypoxia (IH), and hypercapnia, which increase sympathetic nervous system (SNS) activity. In turn, catecholamines oppose the regulatory actions of insulin by causing skeletal muscle insulin resistance (19), suppressing pancreatic insulin secretion (24), and stimulating hepatic glucose mobilization (5). Thus, excessive SNS activity might contribute to changes in glucose homeostasis observed in OSA patients.

Hypoxia, in particular, may be an important stimulus for metabolic dysfunction in OSA. Healthy humans exposed to acute intermittent (52) or sustained hypoxia (58) develop glucose intolerance. IH has also been found to induce features of the metabolic syndrome in rodents (21, 36, 37, 49). However, rodent experiments often induce hypoxia that is considerably more severe than that typically encountered in OSA. For example, a commonly used IH protocol lowers the ambient oxygen to ~5%, 60 times/h. This stimulus can lower the PaO2 to <30 Torr (75) with a SaO2 nadir ranging from <50% (65) to 67% (38). By contrast, relatively mild degrees of hypoxia in human OSA are associated with glucose dysregulation (73). Second, rodent studies have been performed without warming the experimental environment to maintain thermonutrality (~30°C in mice). In small mammals, cool ambient temperatures significantly raise the metabolic rate (15, 50), and hypoxia suppresses this reflex (31). Hence, interactions of cold and hypoxia can obscure the effects of hypoxia itself (41). Third, most studies involve IH exposures lasting days to weeks in an effort to simulate the chronic IH of OSA. However, brief and reversible changes in metabolism occur during IH (39, 47, 52, 77), high-altitude hypoxia (45, 78), and OSA (18, 59). To place chronic effects of IH in their proper context, these acute changes and their mechanisms must first be understood.

Based on the above considerations, we performed this study to examine the effects of acute hypoxia on glucose homeostasis and the role of the SNS in mediating effects of IH. We used a novel, thermonutral model of IH that simulates a clinically realistic burden of hypoxia in OSA. To capture the full spectrum of OSA severity, we exposed mice to IH at different frequencies, simulating an oxygen desaturation index (ODI) of 0, 15, 30, or 60 with a fixed SaO2 nadir of 80%. This approach was used to probe dose-response relationships between simulated OSA severity and metabolism that have been observed in clinical studies (3).

MATERIALS AND METHODS

Ethical approval. The study was approved by the Johns Hopkins University Animal Care and Use Committee and complied with the American Physiological Society Guidelines for Animal Studies.

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Experimental animals. Forty-eight 6- to 8-wk-old male C57BL6/J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and acclimated to the laboratory for 2 wk before commencing experiments. All mice were maintained on an ad libitum chow diet. They were fasted for 5 h before the initiation of any experimental procedures involving blood tests. For surgical procedures, anesthesia was maintained with 2% isoflurane. The room lights were programmed to maintain a 12:12-h light-dark cycle, with lights on from 0900 to 2100. To maintain thermoneutrality, cages were placed in a Draeger IC 8000 incubator set to 30°C. To prevent accumulation of carbon dioxide and to ensure maintenance of cage temperature by the incubator, cage lids were left partially open to allow mixing of gas with the surrounding environment. Tubes for delivery of air, nitrogen, or oxygen were passed through ports in the incubator to deliver gas mixtures.

Hypoxia. A gas control system was designed to regulate the flow of air, nitrogen, and oxygen into cages as described previously (38), with software modifications to induce IH of varying frequency (ODI of 0, 15, 30, or 60/h). A separate gas regulator system was used to clamp ambient oxygen levels for sustained hypoxia experiments. In a preliminary set of experiments, we simultaneously measured mouse oximetry (neck cuff; Starr Life Sciences, Oakmont, PA) and cage oxygen level (ProOx 110 analyzer; Biospherix, Redfield, NY) during IH. We found that the desired $S_{\text{aO}_2}$ nadir of 80% could be achieved targeting a cage oxygen nadir of 10–11%. Thereafter, mice were exposed to IH with continuous monitoring of cage $O_2$ level. Interim tent air (IA) control was delivered at identical flow rates of 21% $O_2$ and is denoted ODI 0 in IH experiments. Following IA or IH exposure for 30 min (or for 5 h in separate time course experiments), animals underwent rapid induction with isoflurane, and blood was obtained by retro-orbital puncture. For experiments where glucose was the outcome of interest, blood glucose was obtained from tail bleeding without anesthesia; tails were cut >4 h before procurement of samples to minimize stress during sampling.

Telemetry. Mice were anesthetized with isoflurane and implanted with transmitters from Data Sciences International (St. Paul, MN). PA-C10 telemeters (pressure transducer) were placed in the left
femoral arteries of mice. Signals were captured using PowerLabs 16/35 interfaced with LabChart Pro software from ADInstruments (Colorado Springs, CO).

Pharmacological inhibitors. Mice were injected with either 0.9% phosphate-buffered saline as placebo, or with active drug 5 min before IH exposure. We used the α-adrenergic blocking agent phentolamine at 5 mg/kg and the β-blocking agent propranolol at 5 mg/kg. These doses were selected based on prior studies where these drugs effectively inhibited lipolysis (2, 23, 28) and hyperglycemia (30, 70) under various types of stressful stimuli. In addition, in a separate set of pilot experiments, phentolamine at this dose affected neither systemic blood pressure nor heart rate, whereas propranolol lowered blood pressure by ~10% and heart rate by ~15%.

Adrenal medullectomy. Mice were anesthetized with 1–2% isoflurane. The lumbar area was shaved and prepped with chlorhexidine and alcohol. A 1-cm dorsal midline incision was performed between the first and third lumbar vertebra. The muscle wall was entered with mosquito forceps 1.5 cm lateral to the spine on each side. The left adrenal gland was located lateral and cranial to the spleen, and the right adrenal gland was located cranial to the right kidney. The adrenal glands were exteriorized. Small incisions were made on the adrenal capsule bilaterally, and medulla was gently squeezed out with atraumatic forceps. The adrenal capsule and attached fat pads were returned to the abdominal cavity, and the skin incision was closed. Burprenorphine (0.01 mg/kg) was administered subcutaneously at the end of surgery to minimize discomfort. Sham surgery was performed in an identical fashion, except that medulla was not removed. At least 1 wk of recovery time was allotted to mice before exposures to hypoxia for metabolic studies.

Intraperitoneal glucose tolerance test, insulin tolerance test, and biochemical assays. For the glucose tolerance test (GTT) mice (n = 10/group) were injected with 1 g/kg glucose in the peritoneal cavity. Blood was collected from the tail in unanesthetized mice 0, 10, 20, 30, 60, 90, and 120 min after glucose injection. IH was begun immediately following the time 0 blood sample and continued for the 2 h of the assay. Blood glucose was tested with Accu-Chek Comfort Curve kits from Roche Diagnostics (Indianapolis, IN). For the insulin tolerance test (ITT), a similar procedure was followed except mice were injected with 0.5 U/kg insulin immediately at time 0 followed by exposure to acute IH. Free fatty acids (FFA) were measured by colorimetric assays (Wako Diagnostics, Richmond, VA). For GTT and ITT, IH was begun immediately following the time 0 blood sample and continued for the 2 h of the assay.

### Table 1. Mouse oximetry

<table>
<thead>
<tr>
<th>Hypoxia Condition</th>
<th>Mean $\text{SaO}_2%$</th>
<th>Average Low $\text{SaO}_2%$</th>
<th>High $\text{SaO}_2%$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODI 0 (control)</td>
<td>97 ± 1</td>
<td>81 ± 4</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>ODI 15</td>
<td>96 ± 3</td>
<td>80 ± 7</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>ODI 30</td>
<td>93 ± 5</td>
<td>77 ± 4</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>ODI 60</td>
<td>88 ± 4</td>
<td>78 ± 7</td>
<td>97 ± 1</td>
</tr>
</tbody>
</table>

ODI, oxygen desaturation index.

For the glucose tolerance test, only ODI 60 increased FFA levels. Severe SH increased lactate. *P < 0.001 vs. 21% O2; **P < 0.01 vs. 21% O2; #P < 0.01 vs. ODI 0; ¥P < 0.05 vs. ODI 0.

Fig. 2. Effect of sustained hypoxia (SH) or IH on plasma glucose, free fatty acid (FFA), glycerol, insulin, and lactate. Both forms of hypoxia caused dose-dependent increases in glucose, whereas only ODI 60 increased FFA levels. Severe SH increased lactate. *P < 0.001 vs. 21% O2; **P < 0.01 vs. 21% O2; #P < 0.01 vs. ODI 0; ¥P < 0.05 vs. ODI 0.
Fig. 3. Time course of glucose, FFA, and lactate levels during 5 h of 10% O₂ SH or IH (ODI 60). Glucose was elevated by both forms of hypoxia, with the greatest increase observed in the first 2 h (P < 0.05 for ODI 0 vs. ODI 60 and SH for the 1.5-h time point). A similar early rise in FFA was observed during IH (P < 0.05 for ODI 0 vs. ODI 60 at the 1.5- and 3-h time points). SH decreased FFA relative to the other conditions at 5 h (P < 0.01). Lactate was increased only by SH (P < 0.05 for SH vs. other groups at 1.5-, 3-, and 5-h time points).

AUC Glucose mg/dl *min
0 50 100 150 200 250 300 350 400

%O₂
21% 15% 10% 7%

GTT-AUC

Fig. 4. Effect of SH on glucose tolerance (glucose tolerance test (GTT)) and insulin sensitivity (insulin tolerance test (ITT)). The glucose area under the curve (AUC) was used for statistical analysis. The degree of glucose intolerance and insulin resistance correlated with severity of hypoxia. GTT AUC: ANOVA P < 0.0001; *P < 0.05 vs. 15 or 10% O₂. ITT AUC: ANOVA P < 0.0001; **P < 0.01 vs. other groups; ¥P < 0.01 vs. ODI 30 or ODI 60.
Glucose-stimulated insulin secretion. Mice were fasted 5 h and injected with 2 mg/kg glucose in the peritoneal cavity. With the use of heparinized capillary tubes, 30 μl blood were collected from the tail before glucose injection and 5 min following injection. Blood was centrifuged to obtain plasma and insulin levels measured using an ultrasensitive ELISA assay from Alpco (Manchester, NH). Given the brief time available for obtaining samples, we exposed mice to IH for 10 min before the test and continued IH after glucose injection. Phentolamine injections were performed before the start of IH.

Statistical analysis. For GTT and ITT data, we calculated the area under the curve (AUC) using a trapezoidal rule. The initial 40 min of the ITT curve were used in the AUC calculations, corresponding to the primary period of insulin action. For dose-response effects of sustained hypoxia or IH, a one-way ANOVA was used followed by Bonferroni posttests. For intervention experiments (phentolamine, propranolol, medullectomy) a two-way ANOVA was performed to examine the independent effects of the intervention and IH, followed by Bonferroni posttests. Statistics were calculated using GraphPad Prism (GraphPad Software, La Jolla, CA). All values are reported as means ± SE. A P value of < 0.05 was considered significant.

RESULTS

IH model. We developed an IH system capable of inducing IH at variable frequency while maintaining a constant oxygen nadir. Figure 1 shows cage oxygen saturation, blood pressure, and heart rate in a mouse during exposures to 30 min of IH at varying ODI. Table 1 shows average mouse SaO₂ during these exposures. As expected, increasing ODI lowered the average 30-min oxygen level, without altering the amplitude of fluctuations in SaO₂. Telemetry monitoring also revealed that each oxygen desaturation was accompanied by a brief acceleration of heart rate, without altering blood pressure in a significant manner.

Effects of acute IH and sustained hypoxia. Next, we examined the effect of acute sustained or IH (Fig. 2) on plasma glucose, FFA, glycerol, insulin, and lactate. Sustained hypoxia and IH both caused a dose-dependent increase in plasma glucose. Sustained hypoxia did not affect glycerol or insulin levels. During the most severe hypoxic condition (7%), FFA trended lower (P = 0.072) and lactate levels significantly increased. IH, by contrast, increased FFA at ODI 60, and had no effect on insulin or lactate at any frequency of ODI. To further examine kinetics of substrate elevations, we performed 5-h exposures to ODI 60 or 10% sustained hypoxia and measured glucose, FFA, and lactate at 1- to 1.5-h intervals (Fig. 3). Both IH and sustained hypoxia increased glucose to the greatest extent in the first 2 h of exposure, followed by a decrease toward baseline by the end of the exposure. A similar pattern was observed for IH-induced FFA elevations. Sustained hypoxia decreased FFA after 5 h (P <
Sustained 10% hypoxia increased lactate throughout the 5-h exposure.

Sustained hypoxia caused glucose intolerance and insulin resistance (Fig. 4); the greater the severity of hypoxia, the more pronounced the effect on both parameters. A qualitatively similar pattern was observed during IH (Fig. 5) in which the extent of glucose intolerance and insulin resistance was dependent upon the ODI.

**Metabolic effects of acute IH during SNS blockade.** Sustained hypoxia has been shown to mediate hyperglycemia and impaired insulin secretion and signaling by SNS activation (8, 9, 34, 35). We therefore investigated the SNS in mediating IH-induced metabolic changes by administering the α-adrenergic antagonist phentolamine or the β-antagonist propranolol 10 min before IH exposure. We also performed adrenal medullectomy to examine the role of circulating catecholamines, having previously demonstrated increased epinephrine and norepinephrine following acute sustained hypoxia (40) or IH (68).

Figure 6 shows that phentolamine abolished IH-induced hyperglycemia and glucose intolerance without affecting FFA levels. Based on GTT, phentolamine independently improved glucose tolerance \( (P < 0.001) \) and abolished IH-induced glucose intolerance \( (IH \times \text{drug interaction}, P < 0.05) \). Phentolamine improved insulin sensitivity in both ODI 60 and ODI 0 groups \( (P < 0.001) \). However, there was no interaction between effects of phentolamine and IH.

Collectively, these experiments demonstrate that IH induces hyperglycemia and glucose intolerance in a manner dependent upon intact α-adrenergic signaling. Metabolic effects of β-blockade with propranolol are shown in Fig. 7. Propranolol prevented IH-induced FFA elevation but did not affect glucose levels, glucose tolerance, or insulin sensitivity. These results suggest that β-adrenergic signaling mediates lipolysis during IH but does not play a significant role in glucose homeostasis. Adrenal medullectomy independently lowered fasting glucose \( (P < 0.0001) \) and improved glucose tolerance (Fig. 8).

**DISCUSSION**

In this study we examined metabolic consequences of acute IH and the role of the SNS in mediating these effects. Comparing sustained hypoxia with IH, we noted many similarities.
Both stimuli caused hyperglycemia, glucose intolerance, and insulin resistance. During sustained hypoxia, the magnitude of effect was proportional to the depth of hypoxia; during IH, effects were proportional to the frequency of hypoxia (ODI). In addition, effects of IH on glucose intolerance and hyperglycemia were abolished by α-adrenergic blockade or adrenal medullectomy and unaffected by β-blockade. These findings offer a physiological explanation for the metabolic consequences of IH, and perhaps for the metabolic consequences of OSA. Figure 10 is a schematic that summarizes our findings. In the discussion that follows, we will elaborate upon these findings and their significance.

Within minutes, we observed that hypoxia caused a significant increase in plasma glucose, glucose intolerance, and insulin resistance. Glucose levels remained elevated for ~3 h and later normalized during ongoing exposure to IH or sustained hypoxia (Fig. 3). Similarly, humans exposed to 30 min of sustained hypoxia (58) or 5 h of IH exhibited impaired insulin sensitivity in association with markers of heightened sympathetic activity (52). These results agree with other studies of acute hypoxia in animals (54, 74, 77). Lee et al. (47) demonstrated nearly equivalent effects of acute IH or sustained hypoxia on insulin sensitivity. After chronic IH, insulin sensitivity continued to resemble that of acute hypoxia, whereas, after chronic sustained hypoxia, insulin sensitivity improved (47). Thus, our results underscore similarities between IH and sustained hypoxia in the acute setting. We also revealed a strong relationship between ODI and plasma glucose. In OSA, disordered breathing severity correlates with HbA1c (60, 69) and glucose levels during sleep (10, 17, 18, 22, 59). Taken together, these results suggest that OSA has dynamic effects on glucose metabolism that may correlate with the burden of hypoxia during sleep. Even transient changes in glucose homeostasis could have chronic implications for the development of diabetes mellitus (14, 22, 26).

The impact of IH on fasting hyperglycemia and impaired glucose tolerance was abolished by phentolamine, a finding that can be understood considering the metabolic consequences of α- and β-adrenoreceptor (AR) stimulation (1, 24, 27, 44, 51). Systemic α-adrenergic stimulation raises plasma glucose via hepatic glycogenolysis and gluconeogenesis and suppresses pancreatic insulin secretion. Hyperglycemia during IH, without changes in plasma insulin (Fig. 2), suggests mobilization of glucose by α-AR stimulation of gluconeogenesis. Glucose intolerance during IH is at least partially attributable to impaired pancreatic GSIS (Fig. 9). Interestingly, phentolamine interacted with IH to increase insulin secretion, and this effect was likely caused by an “unmasking” of the high insulin demand...
during systemic hyperglycemia and insulin resistance. To our knowledge, this is the first study to demonstrate that IH causes hyperglycemia and lowered insulin secretion by α-adrenergic pathways. In this regard, IH is analogous to exercise (13, 42), surgical stress (56), epinephrine infusion (30, 62), and acute sustained hypoxia (9, 35, 61). Our results may shed light on the finding that IH caused insulin resistance without a compensatory increase in insulin secretion in humans (52).

A crucial implication of this work is that clinically realistic IH does not cause glucose intolerance or hyperglycemia by inducing cellular oxygen insufficiency. Rather, metabolic effects of IH can be ascribed to SNS activation. This implicates the arterial chemoreflexes in the metabolic consequences of IH (72). Our findings do not refute literature demonstrating that hypoxia can stimulate or suppress lipolysis depending on experimental conditions (6, 7, 43, 66). Perhaps ODI 60 induces a lipolytic SNS response without elevating lactate, which can inhibit lipolysis (25). Propranolol or medullectomy prevented IH-induced lipolysis, which is consistent with the role of β-AR in the regulation of hormone-stimulated lipolysis. Excessive FFA inhibit insulin-stimulated glucose uptake in skeletal muscle (11). However, lowering FFA during IH did not alleviate insulin resistance, suggesting that FFA were not responsible under these experimental conditions.

This study is subject to certain limitations. First, we examined only acute forms of hypoxia. OSA is a chronic condition, and the physiological consequences of chronic IH may differ. In particular, IH can lead to long-lasting SNS activation that persists beyond the period of hypoxia (20, 48). We plan subsequent studies in this model examining the effects of chronic IH. Second, we induced IH without other aspects of OSA such as sleep arousals and thoracic pressure changes. However, this approach also provides unique insights into the isolated significance of IH in OSA. Third, we did not directly measure catecholamine levels or their peripheral interactions at
the tissue level. We have previously shown acute elevations of serum epinephrine and norepinephrine during sustained hypoxia or IH (40, 68) and the effectiveness of adrenal medullary stimulation (68). Classic studies have defined the metabolic effects of catecholamine stimulation in liver (71), fat (44), and pancreas (63). Thus, substantial evidence supports the central role of circulating catecholamines in mediating glucose mobilization and intolerance during IH.

In summary, IH or sustained hypoxia both acutely cause a similar pattern of hyperglycemia, glucose intolerance, and insulin resistance. During IH, circulating catecholamines, through their action on α-ARs, promote glycogenolysis and inhibit pancreatic insulin secretion. Insulin resistance during IH may occur independently of SNS activation.

**REFERENCES**

Fig. 10. Effects of acute IH on glucose homeostasis. IH stimulates arterial chemoreceptors that in turn activate the sympathetic nervous system (SNS). Downstream activation of α-AR in the pancreas and liver, respectively, inhibit insulin secretion and stimulate glycogenolysis. Activation of β-AR stimulates white adipose tissue lipolysis. The net effect of IH is glucose intolerance, hyperglycemia, and FFA elevation.

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