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

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Rationale and Objectives: **Although many clinical physiology and epidemiology studies show an association between obstructive sleep apnea (OSA) and markers of insulin resistance, no causal pathway has been established. The purpose of the current study was to determine if the intermittent hypoxia (IH) stimulus that characterizes OSA causes insulin resistance in the absence of obesity. Furthermore, we assessed the impact of IH on specific metabolic function in liver and muscle. Finally, we examined the potential mechanistic role of the autonomic nervous system (ANS) in mediating insulin resistance in response to IH.**

Methods and Results: **Hyperinsulinemic euglycemic clamps were conducted and whole-body insulin sensitivity, hepatic glucose output, and muscle-specific glucose utilization assessed in conscious, chronically instrumented adult male C57BL/6J mice exposed to (*1*) IH (achieving a nadir of $F_{I_{O_2}}$ = 5–6% at 60 cycles/h for 9 h), (*2*) intermittent air as a control, (*3*) IH with ANS blockade (hexamethonium), or (*4*) IA with ANS blockade. IH decreased whole-body insulin sensitivity compared with intermittent air (38.8 ± 2.7 vs. 49.4 ± 1.5 mg/kg/min, $p < 0.005$) and reduced glucose utilization in oxidative muscle fibers, but did not cause a change in hepatic glucose output. Furthermore, the reduction in whole-body insulin sensitivity during IH was not restored by ANS blockade.**

Conclusion: **We conclude that IH can cause acute insulin resistance in otherwise lean, healthy animals, and that the response is associated with decreased glucose utilization of oxidative muscle fibers, but that it occurs independently of activation of the ANS.**

Keywords: blood glucose; hyperinsulinemic euglycemic clamp; muscle glucose utilization; obstructive sleep apnea

Obesity is associated with the development of the metabolic syndrome, and is also a major risk factor for obstructive sleep apnea (OSA) (1). A large body of evidence supports the concept that the physiologic disturbances of OSA can cause systemic hypertension, a component of the metabolic syndrome, independent of the effects of obesity (2–5). Recently, data from epidemiologic cohorts and clinical populations have indicated that OSA may also contribute to the development of insulin resistance (6–8), another component of the metabolic syndrome. However, technical demands associated with directly assessing insulin sensitivity using the hyperinsulinemic euglycemic clamp, and a lack of studies in animal models, have precluded demonstrating that a cause-and-effect relationship exists between OSA and insulin resistance independent of obesity.

The potential exists for OSA to independently contribute to the development of insulin resistance. One of the major physiologic perturbations of OSA is the development of repetitive, acute cycles of intermittent hypoxemia (IH) resulting from periodic collapse of the upper airway during sleep. Epidemiologic studies have shown an association between indices of insulin resistance and the degree of hypoxemia from OSA (6–8). Furthermore, a recent clinical intervention study has shown that treatment of OSA using continuous positive airway pressure eliminates the hypoxemia and reduces insulin resistance (9). However, there are currently no data from animal models of OSA showing that IH causes insulin resistance similar to prior demonstrations that IH causes hypertension in rodents (3, 10, 11).

Potential mechanisms that may account for IH causing insulin resistance have not been directly examined. However, given that nasal continuous positive airway pressure, the predominant treatment for OSA, can improve insulin sensitivity in patients within 2 days, and before any significant changes in body fat distribution occur, it has been suggested that the autonomic nervous system (ANS) may play a key role (9). The ANS has recently been proposed as a potential mediator of insulin resistance and type 2 diabetes (12). It is known that increased sympathetic nerve activity (SNA), release of catecholamines, and deactivation of vagal parasympathetic pathways can induce insulin resistance (12–19). Given that OSA can cause marked disturbances in autonomic function that are corrected by continuous positive airway pressure (20–22), the potential exists for changes in ANS function to account for the rapid 2-day improvement of insulin sensitivity reported in patients treated for OSA (9). It is currently unknown what contributions changes in autonomic activity make in the relationship between IH and insulin resistance.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Clinical studies suggest that sleep apnea can independently contribute to development of insulin resistance. However, there is no direct evidence of a cause-and-effect relationship or potential mechanisms linking sleep apnea and insulin resistance.

What This Study Adds to the Field

Intermittent hypoxia produces insulin resistance in lean mice. Insulin resistance during intermittent hypoxia was not dependent on the autonomic nervous system.

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The primary goal of the current study was to determine if the pattern of IH that characterizes OSA causes insulin resistance in the absence of obesity. In lean, chronically instrumented C57BL/6J mice we used the hyperinsulinemic euglycemic clamp, which is the most sensitive measurement of whole-body insulin sensitivity. Furthermore, we assessed the impact of IH on hepatic glucose output and determined tissue-specific glucose utilization in oxidative, glycolytic, and mixed muscle fiber types. To determine a potential mechanism of IH-induced insulin resistance, we examined the combined impact of blockade of sympathetic, parasympathetic, and adrenal components of the ANS using the ganglionic blocking agent hexamethonium. We hypothesized that, during exposure to IH, lean mice exhibit insulin resistance and that this reduced insulin sensitivity is dependent on an intact ANS.

METHODS

Animals

Experiments were conducted in lean wild-type male C57BL/6J mice aged 8 to 12 weeks from Jackson Laboratories (Bar Harbor, ME). The University of Pittsburgh Institutional Animal Use and Care Committee approved the study. Anesthesia was induced and maintained using isoflurane administered through a facemask, and catheters were chronically implanted in the left femoral artery and vein for measurement of blood glucose and for infusion of solutions. All animals were allowed 48 to 72 hours to recover from the surgery before beginning data collection and maintained in a 12-hour light:12-hour dark environment beginning at 8:00 A.M.

Model of IH

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 (23). Control animals were exposed to alternating periods of room air (intermittent air [IA]) using an identical protocol of gas flows as the IH protocol except room air was used rather than nitrogen.

Experimental Design

In the first set of experiments beginning at 8:00–9:00 A.M., animals were exposed to either IH or IA (control) throughout a 9-hour protocol, and techniques similar to those described by Kim and colleagues (24) in mice were adapted for determination of insulin sensitivity, hepatic glucose output, and muscle glucose utilization. After 5.6 to 7 hours (baseline period) of IH or IA exposure, baseline hepatic glucose output was determined over 80 minutes, and after 7 to 9 hours, whole-body insulin sensitivity was determined by infusing a constant rate of human insulin (20 mU/kg/min) and a variable rate of D50 glucose through the femoral venous catheter, maintaining plasma glucose at 100 to 125 mg/dl. Blood glucose levels were sampled from the femoral artery catheter at 10-minute intervals and the average glucose infusion rate over the last 30 minutes of the hyperinsulinemic euglycemic clamp was used to determine insulin sensitivity (*see* the online supplement for further details).

In the second set of experiments, glucose utilization in soleus (oxidative), vastus (glycolytic), and gastrocnemius (mixed oxidative and glycolytic) muscle was determined in two groups of animals exposed to either IA or IH by administering [2-^{3}H]deoxyglucose (25) during a hyperinsulinemic euglycemic clamp identical to that described above (*see* the online supplement for further details).

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 intravenous infusion of the autonomic ganglionic blocker hexamethonium (10-mg/kg bolus + 20-mg/kg/h infusion), which was continued throughout the 9-hour protocol. Efficacy of the ganglionic blockade was assessed by administration of phenylephrine (*see* the online supplement for further details).

Plasma Insulin and Plasma Corticosterone

Plasma insulin levels (Linco Research, Inc., St. Charles, MO) and plasma corticosterone levels (Diagnostic Systems Laboratories, Inc., Webster, TX) were measured according to the manufacturers' specifications.

Statistical Analyses

Data are reported as mean ± SEM. Differences between means in animals exposed to IA and IH were determined by unpaired, two-tailed *t* tests. Comparisons of differences in means of glucose, insulin, hepatic glucose output, and blood pressure between intact and autonomically blocked animals under conditions of IA and IH exposure, before beginning the hyperinsulinemic euglycemic clamp, were determined by two-way analysis of variance (ANOVA). During the clamp, two-way ANOVA was not applied to compare differences between means because insulin levels differed between the intact and autonomically blocked animals (*see* DISCUSSION).

RESULTS

IH and Insulin and Glucose Regulation in Intact Animals

On the day of experimentation, animals were approximately 4% below their presurgical weight (Table 1) and were exhibiting a positive trend in food intake and weight, consistent with a complete recovery. Baseline insulin levels were not different between groups, but plasma blood glucose levels were higher in the intact animals exposed to IH compared with those exposed to IA (Table 1). At the completion of the clamp experiment, plasma insulin levels and hematocrit were comparable between the intact IA and IH groups, but plasma corticosterone levels were significantly elevated in the IH compared with the IA animals (Table 1).

During the last 30 minutes of the hyperinsulinemic euglycemic clamp, blood glucose levels and plasma insulin levels were identically matched between the IA and IH groups, but the glucose infusion rate was significantly reduced in IH (38.8 ± 2.7 mg/kg/min) compared with IA (49.4 ± 1.5 mg/kg/min) exposure, demonstrating a decrease in insulin sensitivity (Figure 1 and Table 1). During exposure to IH there was no difference in muscle glucose clearance (Kg) or muscle glucose utilization (Rg) in the vastus muscle (Figure 2, *left panels*). In contrast, there was a distinct divergence between experimental groups in Kg and Rg in soleus muscle, with IH causing reduced muscle glucose utilization in all animals (Figure 2, *right panels*). In the gastrocnemius muscle, all animals exposed to IH had lower Kg and Rg compared with those with IA exposure (Figure 2, *middle panels*), but the difference between the two groups was not as clearly delineated as the response seen in the soleus muscle. The steady-state glucose infusion rates during the muscle glucose utilization

TABLE 1. BASELINE CHARACTERISTICS AND GLUCOSE INFUSION RATES FOR THE HYPERINSULINEMIC EUGLYCEMIC CLAMP IN ANIMALS WITH AN INTACT AUTONOMIC NERVOUS SYSTEM

	Intermittent Air	Intermittent Hypoxia
Age, d	75 ± 2	66 ± 3
Presurgical weight, g	25.9 ± 0.8	24.2 ± 0.5
Experimental day weight, g	24.7 ± 0.8	23.4 ± 0.6
Baseline blood glucose, mg/dl	134 ± 5	168 ± 13*
Baseline plasma insulin, ng/ml	0.8 ± 0.1	0.9 ± 0.1
Baseline hepatic glucose output, mg/kg/min	12.9 ± 1.7	17.0 ± 2.3
Clamped blood glucose, last 30 min, mg/dl	109 ± 6	110 ± 5
Glucose infusion rate, mg/kg/min	49.4 ± 1.5	38.8 ± 2.7†
Hyperinsulinemic plasma insulin, ng/ml	2.7 ± 0.3	3.4 ± 0.3
Plasma corticosterone at end of clamp, ng/ml	79 ± 29	191 ± 38*
Hematocrit at end of clamp, %	36.0 ± 1.4	38.5 ± 1.9
Number of subjects	8	8

* $p < 0.05$ between intermittent air and intermittent hypoxia by unpaired *t* test.

† $p < 0.005$ between intermittent air and intermittent hypoxia by unpaired *t* test.

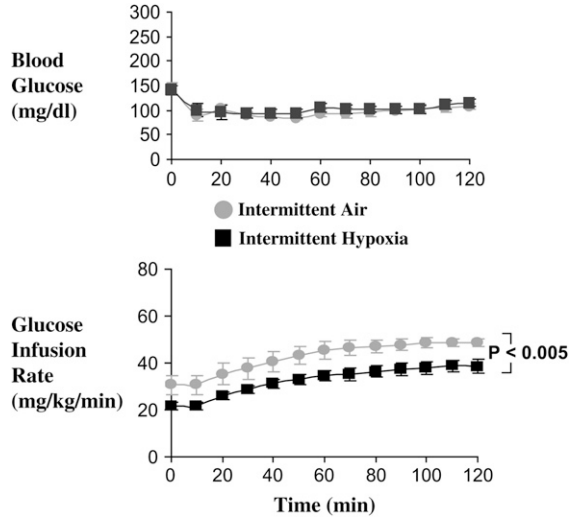

Figure 1. In neurally intact animals, insulin sensitivity was assessed by the steady-state level of exogenous glucose infusion during the 90–120-minute time period under conditions of hyperinsulinemia (20 mU/kg/min) in mice exposed to either intermittent hypoxia or intermittent air (control). Statistical differences between the means of the last 30 minutes of glucose infusion in the intermittent air and intermittent hypoxic groups were determined by unpaired two-tailed *t* test.

hyperinsulinemic euglycemic clamp protocol were 54.5 ± 5.2 mg/kg/minute for the IA exposure and 33.0 ± 4.7 mg/kg/minute for the IH exposure (Table 2), again consistent with the decrease in insulin sensitivity in the intact IH group shown previously in Figure 1 and Table 1.

The baseline hepatic glucose output of 17.0 ± 2.3 mg/kg/minute during exposure to IH was not significantly greater ($p = 0.18$) than that seen during the IA exposure (12.9 ± 1.7 mg/kg/min) in intact animals (Table 1). During the hyperinsulinemic euglycemic clamp, hepatic glucose output was reduced to zero in both the IA and IH intact groups.

TABLE 2. HYPERINSULINEMIC EUGLYCEMIC CLAMP DURING MUSCLE GLUCOSE UTILIZATION EXPERIMENTS IN ANIMALS WITH AN INTACT AUTONOMIC NERVOUS SYSTEM

	Intermittent Air	Intermittent Hypoxia
Clamped blood glucose, last 30 min, mg/dl	102 ± 7	107 ± 8
Glucose infusion rate, mg/kg/min	54.5 ± 5.2	33.0 ± 4.7
Hyperinsulinemic plasma insulin, ng/ml	6.4 ± 2.3	7.6 ± 1.1
Number of subjects	4	3

IH and Insulin and Glucose Regulation in Autonomically Blocked Animals

Hexamethonium administration caused a significant decrease in arterial blood pressure ($p < 0.01$, two-way ANOVA), but did not completely block the transient bradycardic response to acute phenylephrine infusion in the animals exposed to IH ($p < 0.05$, paired *t* test; Table 3). There was no difference in baseline blood glucose levels between the IA and IH animals after autonomic blockade (Table 4), but two-way ANOVA of all four intact and autonomically blocked groups revealed a significant independent effect of IH to raise baseline blood glucose levels ($p < 0.025$) and of hexamethonium administration to lower baseline blood glucose levels ($p < 0.0001$). Baseline insulin levels were not different between IH and IA groups or with and without autonomic blockade. Baseline hepatic glucose output was comparable between IA and IH groups after autonomic blockade, but analyses of all four groups by two-way ANOVA revealed a significant independent effect ($p = 0.030$) for autonomic blockade to lower hepatic glucose output and a strong trend ($p = 0.071$) for an interaction between autonomic blockade and IH exposure. As

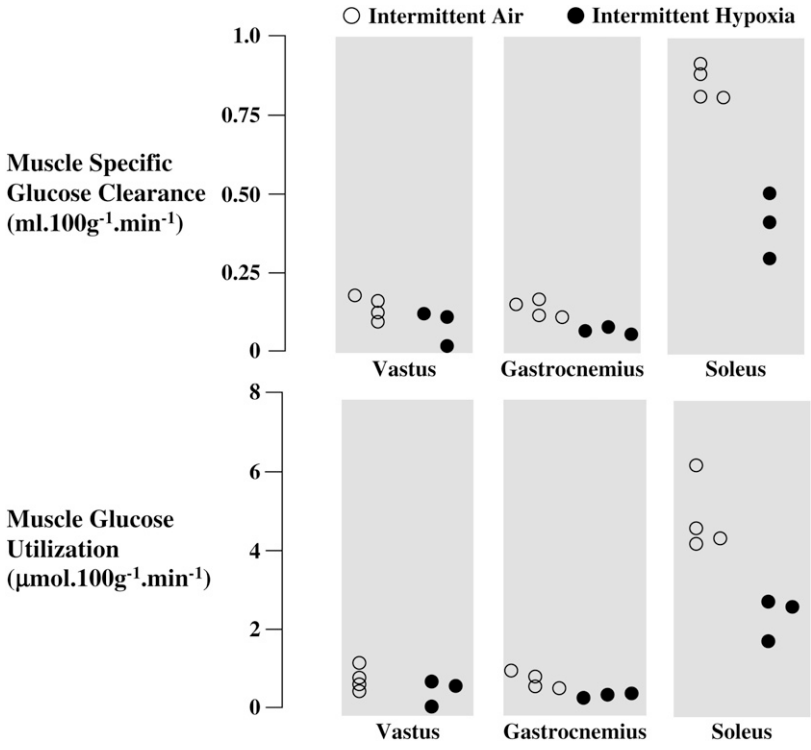

Figure 2. Muscle-specific glucose clearance and muscle glucose utilization in the vastus, gastrocnemius, and soleus muscle were determined in seven mice with intact autonomic nervous systems under intermittent hypoxia and intermittent air conditions.

TABLE 3. EFFECT OF HEXAMETHONIUM ADMINISTRATION ON BLOOD PRESSURE AND REFLEX BRADYCARDIA IN RESPONSE TO ACUTE PHENYLEPHRINE ADMINISTRATION BEFORE AND AFTER BLOCKADE OF THE AUTONOMIC NERVOUS SYSTEM

	Intermittent Air		Intermittent Hypoxia	
	Preblockade	Post-blockade	Preblockade	Post-blockade
Resting arterial blood pressure, mm Hg	111 ± 5	98 ± 7	126 ± 8	102 ± 7
Peak arterial blood pressure during phenylephrine, mm Hg	137 ± 4	147 ± 5	160 ± 15	157 ± 13
Resting heart rate, beats/min	718 ± 53	692 ± 24	691 ± 15	688 ± 51
Nadir heart rate during phenylephrine, beats/min	448 ± 42*	601 ± 65	469 ± 23*	601 ± 53†

Mean arterial blood pressures were measured over a 5-minute period before phenylephrine administration and peak arterial pressure and nadir heart rate were determined over a period of five cardiac cycles after phenylephrine administration.

* $p < 0.005$ between resting heart rate and nadir heart rate by paired *t* test.

† $p < 0.05$ between resting heart rate and nadir heart rate by paired *t* test.

described above, during the hyperinsulinemic euglycemic clamp, hepatic glucose output was reduced to zero in both the IA and IH autonomically blocked groups.

During the last 30 minutes of the hyperinsulinemic euglycemic clamp, blood glucose levels and plasma insulin levels were matched between the IA and IH groups (Table 4), but the glucose infusion rate was significantly reduced in IH (51.3 ± 2.7 mg/kg/min) compared with IA (72.0 ± 1.8 mg/kg/min) exposure (Figure 3 and Table 4). These data demonstrate that the effect of IH in reducing insulin sensitivity still occurs in the presence of autonomic blockade.

The hematocrit at the end of the clamp was not different between groups (Table 4), but plasma corticosterone levels were significantly elevated in the IH compared with the IA animals (Table 4).

DISCUSSION

The data from the current study show that, under conditions of acute exposure to IH, there is a decrease in whole-body insulin sensitivity and muscle-specific glucose utilization in C57Bl/6J mice. The decrease in insulin sensitivity during IH exposure occurred in the absence of obesity, the most common risk factor for development of OSA. The reduced insulin sensitivity was most pronounced in the predominantly oxidative, insulin-sensitive soleus muscle (26). Furthermore, we show that reduced insulin sensitivity during IH persists after blockade of the ANS, suggesting that, at least under the conditions of the current experimental paradigm, an increase in SNA is not a mechanistic link between hypoxic stress and the development of insulin resistance. If these findings extrapolate to the clinical setting, it would indicate that the central role ascribed to elevated SNA in OSA-induced nighttime and sustained hypertension is not the dominant factor causing insulin resistance in patients with OSA.

Our methodologic approach used a previously validated system of exposure to IH (23) in mice and techniques to simultaneously measure insulin sensitivity using the hyperinsulinemic clamp. Insulin sensitivity was determined in conscious, unrestrained animals in which the femoral, and not the carotid, artery was chronically catheterized to preserve bilateral carotid artery blood flow and function of the carotid body, the main acute sensor of hypoxia. We also chose to make the measurement of insulin sensitivity during the period of hypoxic exposure based on the results of two previous studies from our laboratory suggesting that insulin sensitivity may be reduced during the 12-hour exposure to IH in the light period (27) and follow with a rebound increase in insulin sensitivity in the 12-hour exposure to room

TABLE 4. BASELINE CHARACTERISTICS AND GLUCOSE INFUSION RATES FOR THE HYPERINSULINEMIC EUGLYCEMIC CLAMP IN ANIMALS AFTER AUTONOMIC BLOCKADE

	Intermittent Air	Intermittent Hypoxia
Age, d	73 ± 4	76 ± 1
Presurgical weight, g	24.9 ± 0.9	25.2 ± 0.7
Experimental day weight, g	23.5 ± 0.6	23.9 ± 0.9
Baseline blood glucose, mg/dl	81 ± 5	89 ± 7
Baseline plasma insulin, ng/ml	0.7 ± 0.1	0.7 ± 0.2
Baseline hepatic glucose output, mg/kg/min	12.2 ± 1.2	10.1 ± 1.2
Clamped blood glucose, last 30 min, mg/dl	103 ± 6	102 ± 3
Glucose infusion rate, mg/kg/min	72.0 ± 1.8	51.3 ± 2.7*
Hyperinsulinemic plasma insulin, ng/ml	8.3 ± 1.8	9.1 ± 2.0
Plasma corticosterone at end of clamp, ng/ml	242 ± 52	399 ± 59†
Hematocrit at end of clamp, %	36.5 ± 1.4	38.5 ± 1.0
Number of subjects	9	8

* $p < 0.00005$ between intermittent air and intermittent hypoxia by unpaired *t* test.

† $p < 0.05$ between intermittent air and intermittent hypoxia by unpaired *t* test.

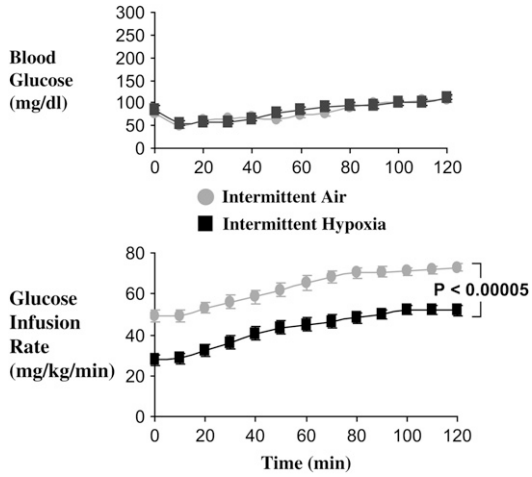

Figure 3. In autonomically blocked animals (hexamethonium 10 mg/kg bolus + 20 mg/kg/h), insulin sensitivity was assessed by the steady-state level of exogenous glucose infusion during the 90–120-minute time period under conditions of hyperinsulinemia (20 mU/kg/min) in mice exposed to either intermittent hypoxia or intermittent air (control). Statistical differences between the means of the last 30 minutes of glucose infusion in the intermittent air and intermittent hypoxic groups were determined by unpaired two-tailed *t* test.

air in the dark period (28). Therefore, we decided the most likely time to observe a decrease in insulin sensitivity was during the period of IH exposure, a hypothesis our data clearly support.

We examined the putative role of activation of the sympathetic nervous system in mediating the decreased insulin sensitivity during IH exposure by continuously infusing hexamethonium to pharmacologically denervate both the sympathetic and parasympathetic arms of the ANS. Hexamethonium produces blockade of the autonomic ganglia by occupying cholinergic receptor sites on the postsynaptic membrane (29) and was originally tested for clinical use as an antihypertensive in the 1950s (30, 31). However, numerous side effects, including an exacerbated hypoglycemic response to insulin administration, limited ganglionic blockers from a broader role in blood pressure control (32–34). Furthermore, an early animal study reported that administration of a 2-mg/kg bolus of hexamethonium could lower baseline blood glucose levels in dogs (32). Similarly, we report that the higher dose of hexamethonium used in our study (10-mg/kg bolus + 20-mg/kg/h infusion) also resulted in lower baseline blood glucose levels (Table 4). Although we chose a relatively high dose of hexamethonium based on our previous experience in acutely blocking autonomic cardiovascular reflex responses in dogs (35), the data in Table 3 show that a very small bradycardic response to acute phenylephrine-mediated hypertension still persisted (only significant for IH, $p < 0.05$). However, the relatively high corticosterone levels in the IA control group suggests that side effects to an even higher dose of hexamethonium may have been poorly tolerated.

Although the degree of autonomic blockade we achieved with hexamethonium lowered baseline blood glucose, caused significant hypotension, and mostly eliminated acute hypertension–induced reflex bradycardia, pronounced insulin resistance still occurred during exposure to IH. This finding, that insulin sensitivity was significantly reduced by IH after blockade of the ANS, was unexpected. Several studies indicate that hypoxic stress and subsequent activation of the sympathetic nervous system in patients with OSA, and in animals exposed to IH, represents the predominant causal pathway to the development of sustained daytime hypertension (21, 22, 36–40). Similarly, several epidemiologic studies have shown that abnormal glucose regulation in patients with OSA is positively associated with hypoxic stress (6–8), and by implication increased SNA. Furthermore, one study showed that in lean (body mass index < 30 kg/m^2) patients with OSA, the insulin sensitivity, as assessed directly by the hyperinsulinemic euglycemic clamp, could significantly improve after just 2 days of removal of the hypoxic stimulus by treatment with nasal continuous positive airway pressure (9). The authors speculated that the rapidity of this improvement in insulin sensitivity with treatment is potentially consistent with a reduction in sympathetic drive. Thus, there is considerable correlative evidence linking IH, increased SNA, and reduced insulin sensitivity, but the existence of a causal pathway has not been tested previously.

Our data demonstrate that, under conditions of acute exposure to IH, a reduction in insulin sensitivity is not dependent on activation of the ANS. Another potential mechanism that may account for such a rapid reduction in insulin sensitivity during IH is an increase in counterregulatory hormones. In the present study, we did measure corticosterone, the dominant glucocorticoid in mice, and, under both ANS intact and ANS blocked conditions, the corticosterone levels were higher in mice exposed to IH compared with mice exposed to IA. However, these data should be interpreted cautiously because they were single measurements taken at the termination of the experiment and there was a clear independent effect of hexamethonium to elevate corticosterone levels. Very few studies in the clinical literature have examined the effect of OSA or its treatment with nasal continuous positive airway pressure on cortisol levels, although one study suggested that OSA could elevate cortisol levels (41). Another study in neonatal rats has shown that administration of the glucocorticoid dexamethasone can produce a state of relative insulin resistance and mimic the metabolic responses seen with hypoxic exposure (42). Clearly, there is a need for future studies to investigate the relationship between glucocorticoids and potentially other counterregulatory hormones in mediating decreased insulin sensitivity in response to OSA or IH.

Alternatively, the fiber-specific response of muscle glucose uptake to IH may argue against a role for glucocorticoids, or other circulating counterregulatory hormones, in mediating insulin resistance. The data shown in Figure 2 suggest that some intrinsic characteristic of the different muscle fiber types is contributing to the development of insulin resistance. In other words, if the IH-induced reductions in insulin sensitivity were due to a circulating counterregulatory hormone, then muscle glucose uptake should be reduced in all muscle fiber types, with the magnitude of the decrease in insulin sensitivity being greater in the more insulin sensitive soleus muscle compared with the less insulin sensitive vastus muscle. However, our data show significant reductions in muscle glucose uptake in soleus, but not vastus, muscle during exposure to IH. One possible explanation for this finding is that reduced oxygen delivery to muscle during IH preferentially slows the rate of oxidative metabolism, decreases the rate of glycolysis, and consequently makes insulin less effective at disposing glucose in the oxidative soleus muscle. In contrast, the glycolytic vastus muscle has little dependence on oxidative metabolism, and glycolysis and insulin disposal of glucose may therefore be unaffected by reduced oxygen delivery during IH. Thus, the potential exists for reduced oxygen delivery to contribute to the development of insulin resistance in OSA by directly impairing the ability of tissues to oxidatively metabolize glucose. Although reduced oxidative capacity of muscle during IH under hyperinsulinemic conditions would fit the broadest definition of "insulin resistance," such a mechanism would occur independently of any disruption in classical insulin signaling pathways. Potentially decreased oxidative capacity in respiratory muscles during IH may impair muscle function, contribute to fatigue, and increase vulnerability to breathing disorders during sleep.

In addition to IH, OSA is characterized by a significant disruption and fragmentation of sleep (41, 43, 44). Similarly, in our mouse model of IH, we know that sleep is severely disrupted in the first 12 hours of exposure (23). The total amount of non-REM sleep is significantly reduced, REM sleep is effectively abolished, and what sleep does occur is fragmented by arousal at the 5% nadir of each hypoxic exposure (23). It is possible that fragmented sleep, restricted sleep, or arousals *per se*, in addition to IH, may have contributed to the decreases in insulin sensitivity that we report. Data from normal young human volunteers demonstrate that sleep restriction can lead to impaired glucose tolerance and is associated with alterations in the circadian cortisol rhythm (45). Thus, both IH and sleep disruption may potentially contribute to a stress response that can impair glucocorticoid rhythm and reduce insulin sensitivity in patients with OSA and in animal models of IH.

In addition to studying whole-body insulin sensitivity under hyperinsulinemic conditions, we also examined under basal fasting conditions whether exposure to IH resulted in an increase in hepatic glucose output. Although there was no independent effect of IH, two-way ANOVA demonstrated that autonomic blockade resulted in a significantly reduced hepatic glucose output, and a strong trend existed for an interaction between autonomic blockade and IH exposure on hepatic glucose output.

The latter finding suggests that the ability of IH to increase hepatic glucose output is more pronounced in intact rather than in ANS blocked animals. Thus, the ANS may potentially contribute to elevating the hepatic glucose output in response to IH and create a susceptible environment for the development of elevated glucose levels and progression to type 2 diabetes.

A caveat of the current study was that the plasma insulin levels measured at the end of the clamp period were lower in the experiments in the ANS intact compared with experiments in the ANS blocked animals (*see* Tables 1 and 4). The exact reason for the difference in insulin levels is unclear, although we did change the insulin stock between the earlier intact ANS and later blocked ANS experiments. Despite the lower levels of insulin measured during the clamp in the intact ANS experiment, there are two reasons to suggest that this level of hyperinsulinemia was effectively maximal with respect to its action on peripheral tissues. First, for the intact ANS experiments, as well as the ANS blocked experiments, glucose production from the liver was completely absent during the clamp period of hyperinsulinemia. Second, the latter insulin stock solution was used in the muscle glucose utilization experiments and the insulin levels during this clamp procedure were more than double (6.4 ± 2.3 ng/ml IA and 7.6 ± 1.1 ng/ml IH; Table 2) that of the initial intact ANS experiment (Table 1), yet the steady-state glucose infusion rates were almost identical to the earlier intact ANS experiments. Taken together, these data indicate that, in ANS intact animals, the ability of insulin to stimulate peripheral glucose utilization and inhibit glucose production from the liver was effectively maximal in all experiments. Nevertheless, we have adopted a conservative approach to our data analyses and refrained from conducting any direct statistical comparison between the ANS intact and ANS blocked groups during the clamp procedure. Most important, however, for both the intact ANS and blocked ANS experiments, the insulin levels were comparable under IH and IA conditions, allowing direct comparison between the hypoxic and control state. A second caveat of the study involves the use of hexamethonium to block the sympathetic, parasympathetic, and adrenal arms of the ANS. Although our approach represented a first attempt at evaluating a role for the ANS in IH-induced insulin resistance, it is possible that opposing actions of different components of the ANS masked important contributions to the development of insulin resistance. Future studies with more specific blockers of the ANS will be necessary to discriminate between the different arms of the ANS. A final caveat of the study relates to the severity of the 5 to 6% oxygen nadir that the animals were exposed to during IH. In mice, exposure to a 5 to 6% IH nadir is severe, but nonetheless physiologic. We have previously reported in a model of spontaneous sleep-induced hypoxia in C57BL/6J mice that, on average, 63 events per 24-hour period were terminated by arousal from sleep when inspired oxygen levels had reached 6% or less (46). However, it is unclear how a nadir level of less than 6% oxygen in mice translates to the severity of hypoxic stress in the clinical setting.

In summary, we have shown that, during exposure to a regimen of IH that simulates severe hypoxic stress in OSA, there is an acute decrease in both whole-body insulin sensitivity and muscle glucose utilization in oxidative, insulin-sensitive soleus muscle. These data demonstrate, at least under conditions of the present study, that a cause-and-effect relationship exists between exposure to IH and the development of insulin resistance in lean, otherwise healthy animals. Unexpectedly, this rapid reduction in insulin sensitivity of peripheral tissues was not dependent on activation of the ANS, suggesting that other mechanisms, such as an increase in counterregulatory hormones or the hypoxic stress *per se*, should be a focus of future studies.

***Conflict of Interest Statement*:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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