

Intermittent hypoxia reverses the diurnal glucose rhythm and causes pancreatic β -cell replication in mice

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Obstructive sleep apnoea (OSA) and type 2 diabetes frequently co-exist and potentially interact haemodynamically and metabolically. However, the confounding effects of obesity have obscured the examination of any independent or interactive effects of the hypoxic stress of OSA and the hyperglycaemia of type 2 diabetes on haemodynamic and metabolic outcomes. We have developed a chronically catheterized, unhandled, lean murine model to examine the effects of intermittent hypoxic (IH) exposure and exogenous glucose infusion on the diurnal pattern of arterial blood pressure and blood glucose, as well as pancreatic β -cell growth and function. Four experimental groups of adult male C57BL/J mice were exposed to 80 h of (1) either IH (nadir of inspired oxygen 5–6% at 60 cycles h⁻¹ for 12 h during light period) or intermittent air (IA; control) and (2) continuous infusion of either 50% dextrose or saline (control). IH exposure during saline infusion caused a sustained increase in arterial blood pressure of 10 mmHg ($P < 0.0001$), reversed the normal diurnal rhythm of blood glucose ($P < 0.03$), doubled corticosterone levels ($P < 0.0001$), and increased replication of pancreatic β -cells from 1.5 ± 0.3 to $4.0 \pm 0.8\%$ bromodeoxyuridine (BrdU)-positive β -cells. The combined stimulus of IH exposure and glucose infusion attenuated the hypertension, exacerbated the reversed diurnal glucose rhythm, and produced the highest rates of apoptosis in β -cells, without any additive effects on β -cell replication. We conclude that, in contrast to the development of sustained hypertension, IH impaired glucose homeostasis only during periods of hypoxic exposure. IH acted as a stimulus to pancreatic β -cell replication, but the presence of hyperglycaemia may increase the hypoxic susceptibility of β -cells. This model will provide a basis for future mechanistic studies as well as assessing the metabolic impact of common comorbidities in OSA, including obesity, insulin resistance and type 2 diabetes.

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Obesity is the predominant risk factor for both obstructive sleep apnoea (OSA) and the development of insulin resistance, hyperglycaemia and type 2 diabetes. Several epidemiological studies have shown that OSA is associated with the presence of hyperglycaemia and indirect indices of insulin resistance (Ip *et al.* 2002; Punjabi *et al.* 2002, 2004). However, because of the confounding presence of obesity it has been difficult to interrogate pathways linking OSA to the development of insulin resistance and type 2 diabetes.

We recently examined insulin resistance in a murine model that simulates the hypoxic stress of OSA. During a period of exposure to intermittent hypoxia (IH), we determined that whole-body insulin resistance occurred, as measured by the hyperinsulinaemic euglycaemic clamp,

and was associated with impaired glucose uptake in oxidative muscle fibres (Iiyori *et al.* 2007). In contrast, earlier data from our group indicated that after a period of exposure to IH, when the animals were returned to breathing room air for several hours, there was actually a lowering of blood glucose and improved glucose tolerance, consistent with a decrease in insulin resistance (Polotsky *et al.* 2003). Taken together, these data suggest that there may in fact be a diurnal rhythm in glucose homeostasis that is dependent on the presence or absence of IH. Such a concept would be in contrast to the cardiovascular sequelae of OSA or IH, where the development of acute hypertension during episodes of airway obstruction or IH results in a sustained 24 h hypertensive response (Brooks *et al.* 1997; Fletcher *et al.* 1992; Nieto *et al.* 2000). To date,

there has been no systematic attempt in animal models to determine if there is a diurnal component to glucose homeostasis in response to IH exposure.

In addition to the development of insulin resistance, the progression to type 2 diabetes is dependent on the compensatory responses of the pancreatic β -cells that produce insulin. Hyperglycaemia, itself, is known to significantly increase the rate of β -cell replication (Bonner-Weir *et al.* 1989; Steil *et al.* 2001), a response that can provide an enlarged source of insulin to combat the presence of insulin resistance. Potentially, the hypoxic stress of OSA could also modify the ability of the pancreas to replicate, both before and after the development of any associated hyperglycaemia. However, due to the limited opportunities to study morphometry and function in the clinical setting we currently have no knowledge of the impact of the hypoxic stress of OSA on pancreatic β -cells.

We have recently developed a model of 4 day continuous glucose infusion in chronically catheterized, unhandled mice that produced moderate levels of hyperglycaemia and hyperinsulinaemia and caused significant increases in pancreatic β -cell replication (Alonso *et al.* 2007). The purpose of the current study was to utilize this model to investigate the independent and interactive effects of hypoxic stress (IH exposure) and hyperglycaemia (glucose infusion) on the diurnal rhythms of blood pressure and blood glucose, as well as responses in pancreatic β -cell growth and function.

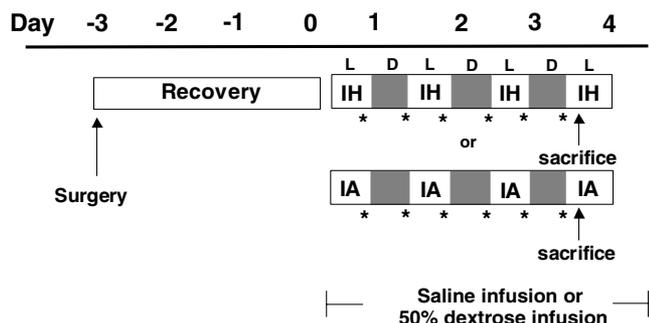


Figure 1. Schematic of experimental protocol and time line for the study

Exposure to either intermittent hypoxia (IH) or intermittent air (IA) occurred during the 12 h of the light (L) period (all animals kept in constant room air during dark (D) period) with a simultaneous infusion of either 0.9% sodium chloride (saline infusion) or 50% dextrose (glucose infusion) containing $500 \mu\text{g ml}^{-1}$ bromodeoxyuridine (BrdU) at a constant rate of $100 \mu\text{l h}^{-1}$. *Arterial blood was sampled twice daily: immediately prior to lights on at 8 am and 10 h into the light period at 6 pm. For each blood sample a total of $100 \mu\text{l}$ whole blood was removed and $2 \mu\text{l}$ used to measure blood glucose, insulin and corticosterone. Plasma was extracted from the remaining blood and the red blood cells re-suspended and re-infused into the mouse.

Methods

Ethical approval

Animal handling and experimentation was in accordance with approved Institutional Animal Care and Use Committee protocols at the University of Pittsburgh. Male C57bL/6J mice aged 10–12 weeks were kept on a 12 h light–dark cycle beginning at 8 am with free access to food and water. Femoral arterial and venous catheters were chronically implanted as previously described (Alonso *et al.* 2007). For catheterization, mice were anaesthetized with inhaled 2% isoflurane. In brief, microrenathane catheters (MRE-025; Braintree Scientific, Braintree, MA, USA) were prepared by heating, pulling, cutting to appropriate diameter, shaping into a J form in hot oil, and sterilizing (ethylene oxide). Catheters were inserted in the left femoral artery and vein, sutured in place, stabilized with superglue (Henkel Corp, Rocky Hill, CT, USA), tunneled subcutaneously to the upper back by threading through a blunt needle, taped to a wire attached to posterior cervical muscles for stiffness (792500; A-M-Systems, Sequim, WA, USA), and connected to a 360 deg dual channel swivel designed for mice (375/D/22QM; Instech, Plymouth Meeting, PA, USA). Prior to initiation of the experimental protocols detailed below, patency of the catheters was maintained by continuously flushing $7 \mu\text{l h}^{-1}$ saline containing 20 U ml^{-1} heparin (Baxter, Deerfield, IL, USA) using a syringe pump with multi-syringe adaptor (R99-EM; Razel Scientific Instruments, St Albans, VT, USA). Arterial catheters were monitored for patency daily and kept unclogged by manual flushes using a 1 cc syringe with 26G needle when necessary.

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 (Iiyori *et al.* 2007). A series of programmable solenoids and flow regulators altered the inspired oxygen over a defined and repeatable profile. During each period of IH the inspired oxygen was reduced from 20.9% to 5.0–6.0% over a 30 s period and rapidly reoxygenated to room air levels in the succeeding 30 s period (i.e. 60 cycles h^{-1}). 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.

Protocol

A schematic timeline of the experimental protocol is shown in Fig. 1. A total of 58 animals were given 3 days post-operative recovery from surgery before beginning

one of four exposure and infusion regimens (IA + saline; IH + saline; IA + glucose; IH + glucose) on day 0. At the beginning of lights on (8 am) on day 0, animals were (1) exposed to either IH or IA for 12 h during the light period and maintained under stable room air conditions during the dark period, and (2) continuously infused with either 0.9% sodium chloride (saline infusion) or 50% dextrose (glucose infusion) containing 500 $\mu\text{g ml}^{-1}$ bromodeoxyuridine (BrdU; Sigma) at a constant rate of 100 $\mu\text{l h}^{-1}$. Arterial blood was sampled twice daily: immediately prior to lights on at 8 am and 10 h into the light period at 6 pm. For each blood sample a total of 100 μl whole blood was removed and 2 μl used to measure blood glucose (Ascencia elite XL glucometer; Bayer, Mishawaka, IN, USA). The remaining blood was centrifuged and red blood cells resuspended with 20 μl saline with 100 U ml^{-1} heparin and re-infused into the mouse. Plasma was stored at -80°C for measurement of insulin and corticosterone. Food intake was recorded every 24 h during the 3 days recovery and twice daily (8 am and 6 pm) during the exposure and infusion protocols. Continuous haemodynamic measurements were recorded using a pressure transducer (Argon, Athens, TX, USA) connected to the arterial catheter starting at day -1. The animals were killed by overdose of pentobarbital (60 mg I.P.) after 80 h exposure/infusion at 4 pm on day 4 and the pancreas removed for histological analyses.

In a second protocol, a total of eight uncatheterized animals were exposed to 28 days of either IH or IA, using the same daily regimen as detailed in Fig. 1, for assessment of pancreatic β -cell mass.

Immunostaining

The pancreas was fixed in Bouin's (Sigma) for 4 h at room temperature, dehydrated, embedded in paraffin, and sectioned (5 μm). For BrdU/insulin staining, sections were treated with 1 N HCl at 37°C for 60 min, blocked in PBS-5% rabbit serum-1% BSA, incubated with rat anti-BrdU (1 : 250, Abcam, Cambridge, MA, USA) and guinea-pig anti-insulin (1 : 50, Invitrogen, Carlsbad, CA, USA) overnight at 4°C , followed by anti-rat (1 : 200, Alexa 594; Invitrogen) and anti-guinea-pig (1 : 200, Alexa 488; Invitrogen) secondary antibodies for 30 min at room temperature. For TUNEL, the Promega (Madison, WI, USA) fluorescence Dead End kit was used following manufacturer's instructions; sections were then stained for insulin incubating with guinea-pig anti-insulin antibody overnight at 4°C , then anti-guinea-pig secondary antibody (1 : 200, TRITC, Sigma) and Hoescht (1 : 1000, Invitrogen) for 30 min at room temperature.

Histological analyses

For BrdU, islets were photographed at $400\times$, assigned blinded filenames, and the number of β -cells and

BrdU-positive β -cells were manually counted. At least 1500 β -cells were counted per mouse, from two sections separated by at least 50 μm . To measure β -cell size, $400\times$ images of insulin-stained islets were converted to grey scale, thresholded, and insulin-positive area electronically measured using image J software (NIH, Bethesda, MD, USA); insulin area was divided by the number of β -cells (manually counted) to obtain an average β -cell area. To measure β -cell mass, two insulin-stained pancreas sections from each mouse, separated by at least 50 μm , were scanned in entirety (Nikon CoolScan), red and blue hues separated, converted to grey scale and thresholded using Adobe Photoshop (Adobe, San Jose, CA, USA). Pancreatic and islet areas were quantified using Image J (NIH, Bethesda, MD, USA). To measure apoptosis, TUNEL positive cells were quantified using an identical protocol to BrdU quantification. To measure pyknosis, Hoescht-stained pancreas sections were photographed as for BrdU and TUNEL. Nuclear morphology of β -cells was scored for pyknotic features, including reduced nuclear diameter and increased Hoescht density.

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, USA). Plasma corticosterone levels were measured using a kit from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA).

Arterial blood pressure measurement

Arterial blood pressure measurements were made with pressure transducers (Cobe Inc.; Lakewood, CO, USA) zeroed at midthoracic level. Calibrations were checked at the beginning and end of each experiment. A pen recorder (Grass Instruments; Quincy, MA, USA) was used to amplify the signal which was digitized at 300 Hz (DI-200 data acquisition board; Dataq Instruments; Akron, OH, USA) and stored on disk with Windaq/200 acquisition software (Dataq Instruments). Mean arterial blood pressure was averaged over 12 h periods.

Statistical analyses

The overall effects of IH exposure and glucose infusion, and their interaction, were tested by two-way ANOVA across exposure (IH and IA) and infusion (glucose and saline) factors. Differences between means in animals exposed to IA and IH during saline or glucose infusion, as well as differences between means during light and dark periods within each of the four experimental groups, were determined by one-way ANOVA. Differences between the

Table 1. Daily food intake of standard dry chow in four experimental groups starting in the immediate post-operative period (day -2) and continuing through the 80 h exposure and infusion protocol starting day 0

	Days relative to start of exposure and infusion protocols									
	-2	-1	0	1		2		3		4
				light	dark	light	dark	light	dark	
IA + saline food intake (gm)	1.0 ± 0.1	2.8 ± 0.3	3.4 ± 0.2	0.7 ± 0.1	3.4 ± 0.4	0.4 ± 0.1	2.3 ± 0.3	0.4 ± 0.1	2.6 ± 0.4	0.4 ± 0.1
IH + saline food intake (gm)	1.0 ± 0.2	2.4 ± 0.2	3.8 ± 0.3	0.8 ± 0.1	3.3 ± 0.2	0.7 ± 0.1	3.1 ± 0.2	0.4 ± 0.1	2.6 ± 0.2	0.4 ± 0.1
IA + glucose food intake (gm)	0.9 ± 0.1	2.6 ± 0.2	3.7 ± 0.2	0.2 ± 0.1	1.5 ± 0.2	0.2 ± 0.1	1.3 ± 0.2	0.1 ± 0.1	1.3 ± 0.2	0.1 ± 0.1
IH + glucose food intake (gm)	0.8 ± 0.2	2.9 ± 0.3	4.0 ± 0.2	1.0 ± 0.1	1.4 ± 0.2	0.2 ± 0.1	1.1 ± 0.1	0.2 ± 0.1	1.1 ± 0.1	0.1 ± 0.1

Note: food intake was recorded every 24 h during the 3 day post-operative recovery period and twice daily (light period: 8 am to 6 pm; dark period: 6 pm to 8 am) during the exposure and infusion protocol starting on day 0. IH, intermittent hypoxia; IA, intermittent air.

Table 2. Baseline metabolic and haemodynamic assessments in all four experimental groups in the 24 h (day -1) prior to initiation of intermittent hypoxia (IH) or intermittent air (IA) exposure and infusion protocols

	IA + saline	IH + saline	IA + glucose	IH + glucose
Sample size (<i>n</i>)	13	17	14	14
Age (days)	76 ± 4	75 ± 2	76 ± 3	74 ± 1
Body weight (gm)	23.6 ± 0.6	23.2 ± 0.4	23.2 ± 0.6	23.2 ± 0.5
Blood pressure (mmHg)	103 ± 2	104 ± 1	102 ± 1	104 ± 2
Blood glucose (mg dl ⁻¹)	117 ± 2	113 ± 2	110 ± 3	107 ± 4
Plasma insulin (ng ml ⁻¹)	1.4 ± 0.2	1.4 ± 0.2	1.7 ± 0.3	1.2 ± 0.2
Plasma corticosterone (ng ml ⁻¹)	52 ± 14	52 ± 7	53 ± 13	51 ± 14

means of the four experimental groups for replication and apoptosis were determined by two-way ANOVA as above, as well as by one-way ANOVA with Dunnett's *post hoc* analysis relative to the IA + saline group. Data are reported as mean ± S.E.M.

Results

Food intake occurs predominantly in the dark period and is reduced during glucose infusion

Daily food intake recovered back to normal levels by the third post-operative day in all four experimental groups (Table 1). In both the IA and IH saline-infused groups daily food intake remained constant over the 4 day protocol with the major proportion of the food being consumed in the dark or active period. In contrast, in both the IA and IH glucose-infused groups, daily food intake was reduced and total calorie intake was maintained in the normal range through the addition of approximately 40% of the daily calorie requirements via glucose infusion. Despite

daily food intake being reduced by glucose infusion, the consumption of dry chow still predominantly occurred during the dark period.

Table 2 verifies that animals in all four experimental groups had mean arterial blood pressure, blood glucose, plasma insulin, and plasma corticosterone in the normal range for chronically instrumented mice during the 24 h prior to the initiation of the exposure and infusion protocols.

Intermittent hypoxia causes sustained hypertension

In all four experimental groups the mean arterial blood pressure drifted slightly downwards over the course of the 4 day exposure and infusion protocol, presumably as a result of the animals' continued adaptation to their chronic instrumentation and environmental surroundings (Fig. 2A and B). Regardless of these time-related drifts in pressure, in the saline-infused animals exposure to IH resulted in an increase in mean arterial blood pressure of approximately 10 mmHg compared to the IA control

group (Fig. 2A and C) and was maintained throughout both the light and dark periods over the 4 day protocol (Fig. 2A and C).

During glucose infusion, IH also resulted in an elevated mean arterial blood pressure of approximately 5 mmHg that was maintained throughout the 4 day protocol (Fig. 2A and C). Analysis of all four experimental groups by two-way ANOVA showed an overall effect of IH to raise mean arterial pressure ($P < 0.0001$) and glucose infusion to lower mean arterial pressure ($P < 0.0001$), and also a significant interaction indicating that the hypertensive effects of IH were reduced in the presence of hyperglycaemia ($P < 0.05$).

Normal diurnal glucose rhythm is reversed by intermittent hypoxia

The saline-infused IA control group exhibited a diurnal glucose rhythm with blood glucose being approximately 13 mg dl^{-1} higher at the end of the dark period compared to the light period (Fig. 3A and C; $P < 0.005$). This higher

blood glucose in the dark period was associated with the dominant period of food intake (Table 1). In contrast, exposure to IH in saline-infused animals abolished the diurnal rhythm seen in the IA group and there was a trend for blood glucose to be approximately 7 mg dl^{-1} higher in the light period of hypoxic exposure compared to the dark period (Fig. 3A and C; $P = 0.070$). This effective reversal in the diurnal rhythm of blood glucose with IH exposure could not be explained by altered patterns of calorie consumption, as food intake remained comparable during the dark and light periods between the IH + saline and IA + saline groups (Table 1). Furthermore, the opposing patterns of diurnal glucose rhythm in the IH and IA groups were unrelated to plasma insulin levels that remained basal throughout the 4 day protocol and did not exhibit any detectable diurnal rhythm (Fig. 4A and C).

During glucose infusion in the IA exposure control group there was a moderate hyperglycaemia ($139 \pm 3 \text{ mg dl}^{-1}$; Fig. 3B) relative to the overall blood glucose level in the saline-infused IA group

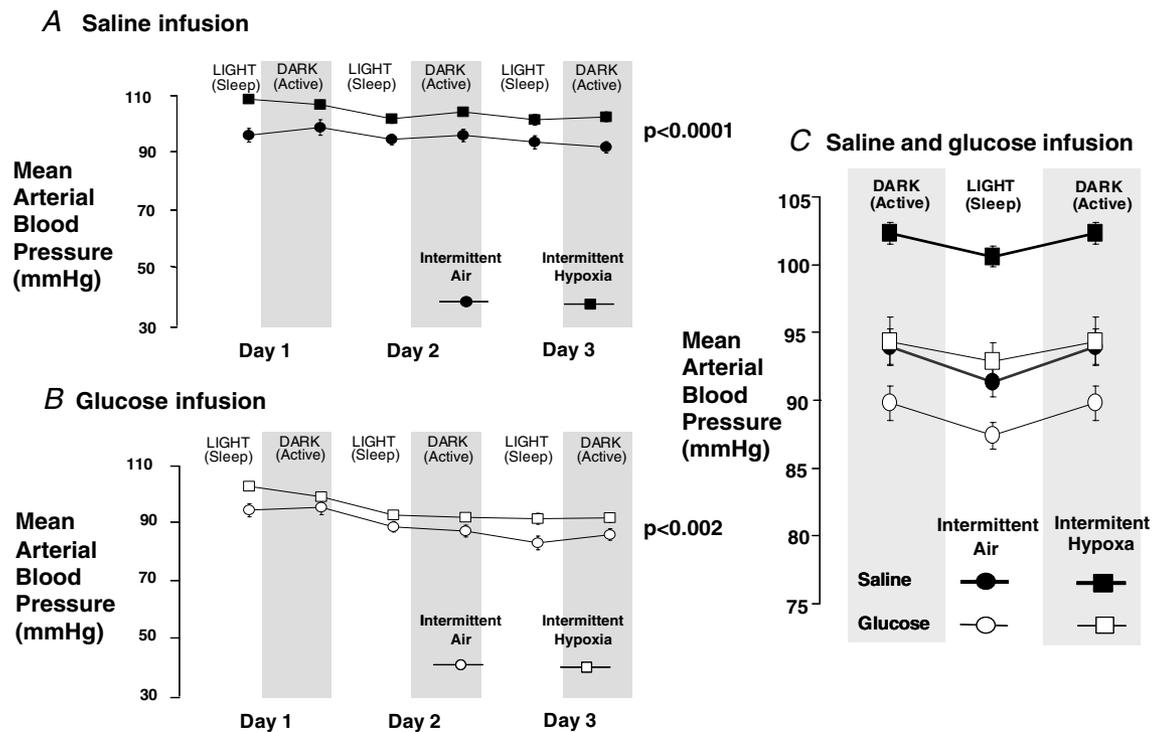


Figure 2. Intermittent hypoxia caused a sustained hypertension that is attenuated in the presence of hyperglycaemia

A, changes in mean arterial blood pressure over time during saline infusion (intermittent hypoxia, $n = 17$; intermittent air $n = 13$; statistical difference determined by one-way ANOVA). B, changes in mean arterial blood pressure over time during glucose infusion (intermittent hypoxia, $n = 14$; intermittent air $n = 14$; statistical difference determined by one-way ANOVA). C, averaged changes in mean arterial blood pressure during the light and dark periods for all four experimental groups (note: data for dark period is plotted twice to highlight the diurnal rhythm). Analysis by two-way ANOVA revealed a significant effect of exposure (intermittent hypoxia > intermittent air; $P < 0.0001$) and infusion (glucose < saline; $P < 0.0001$), and a significant interaction (glucose infusion reduced the hypertensive effects of intermittent hypoxia exposure; $P < 0.05$).

(109 ± 2 mg dl⁻¹; Fig. 3A), but no statistically detectable diurnal rhythm (Fig. 3B and C). However, in the IH-exposed group receiving glucose the reversed diurnal glucose rhythm seen under saline conditions was markedly accentuated with blood glucose levels averaging 135 ± 3 mg dl⁻¹ in the light period and falling to 109 ± 3 mg dl⁻¹ in the dark period (Fig. 3B and C; $P < 0.0001$). Again, this pattern of higher blood glucose levels during the light period with IH exposure was unrelated to either food intake (Table 1) or the plasma insulin levels that were doubled in both the IH and IA glucose-infused animals (Fig. 4B and C). The effective euglycaemia seen during the dark period, between exposure periods of IH, in the glucose-infused animals was a surprising finding and is consistent with a large rebound increase in insulin sensitivity after withdrawal of the hypoxic stress.

Analysis of all four experimental groups by two-way ANOVA showed an effect of glucose infusion to raise blood glucose ($P < 0.0001$) and IH to lower blood glucose

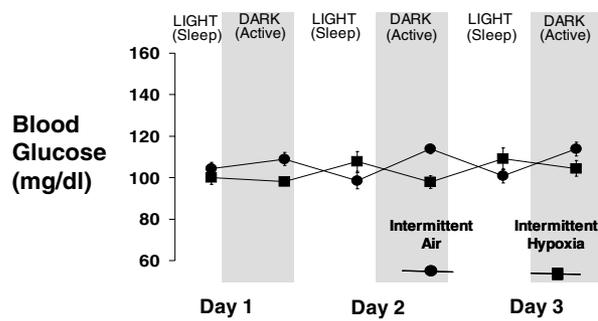
($P < 0.0001$), and also a significant interaction indicating that the hyperglycaemic effects of glucose infusion are reduced in the presence of IH ($P < 0.025$).

Intermittent hypoxia exacerbates peak corticosterone in light period

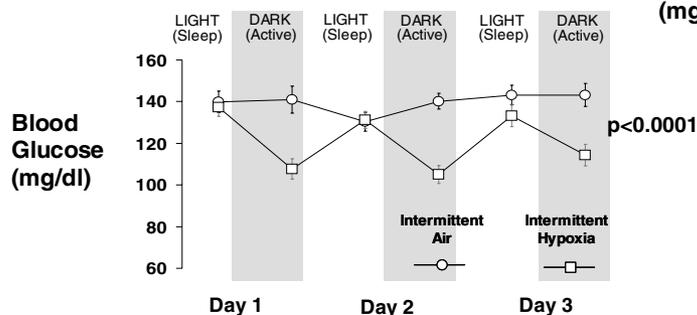
Under IA conditions, animals infused with saline demonstrated a significant diurnal corticosterone rhythm with higher levels during the light period (88 ± 12 ng ml⁻¹) compared to the dark period (36 ± 3 ng ml⁻¹; $P < 0.003$; Fig. 5A and C). During exposure to IH, the diurnal rhythm of corticosterone was markedly accentuated rising from 52 ± 4 ng ml⁻¹ in the dark period to 165 ± 13 ng ml⁻¹ in the light period. A quantitatively similar pattern of diurnal corticosterone changes occurred during glucose infusion compared to saline infusion for both IA and IH exposures.

Analysis of all four experimental groups by two-way ANOVA showed only a significant effect of IH ($P < 0.001$)

A Saline infusion



B Glucose infusion



C Saline and glucose infusion

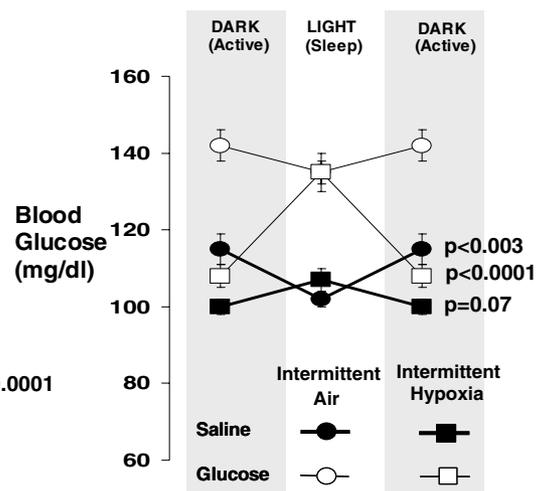


Figure 3. Intermittent hypoxia reversed the normal diurnal glucose rhythm and caused a relative hypoglycaemia during the dark period

A, changes in blood glucose over time during saline infusion (intermittent hypoxia, $n = 17$; intermittent air $n = 13$). B, changes in blood glucose over time during glucose infusion (intermittent hypoxia, $n = 14$; intermittent air $n = 14$; statistical difference determined by one-way ANOVA). C, averaged changes in blood glucose during the light and dark periods for all four experimental groups (note: data for dark period is plotted twice to highlight the diurnal rhythm). Significant differences between the light and dark periods within an experimental group are marked to the right of the figure. Analysis by two-way ANOVA revealed a significant effect of exposure (intermittent hypoxia < intermittent air; $P < 0.0001$) and infusion (glucose > saline; $P < 0.0001$), and a significant interaction (glucose infusion accentuated hypoglycaemia during intermittent hypoxia; $P < 0.025$).

to raise plasma corticosterone with no effect of glucose infusion and no interaction between IH exposure and glucose infusion.

Pancreatic β -cell replication is increased by intermittent hypoxia

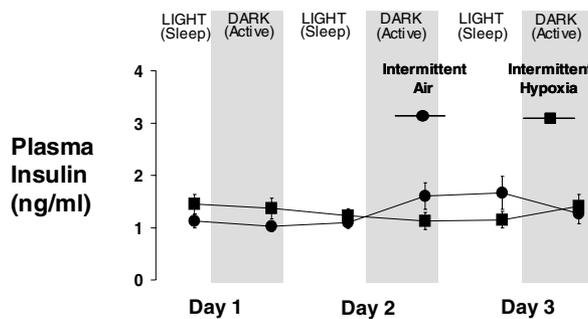
As expected, 4 days of IA exposure and moderate hyperglycaemia ($139 \pm 3 \text{ mg dl}^{-1}$) caused an increase in pancreatic β -cell replication to $4.4 \pm 0.8\%$ BrdU-positive β -cells, a rate of replication similar to that which we published recently (Alonso *et al.* 2007) using the same glucose infusion protocol as in the current study (Fig. 6A and B). However, we did not anticipate that during saline infusion, IH would induce a comparable rate of β -cell replication of $4.0 \pm 0.8\%$ BrdU-positive β -cells to that observed with glucose infusion (Fig. 6A and B), despite the overall blood glucose level being in the euglycaemic range ($109 \pm 2 \text{ mg dl}^{-1}$; Fig. 3A and C). The combined stimulus of IH and glucose infusion resulted in a replication rate of $3.6 \pm 0.4\%$ BrdU-positive β -cells, suggesting a lack of an additive or synergistic effect of glucose and IH.

β -Cell size was unaffected by glucose infusion (Fig. 6C), a finding consistent with our recently published study (Alonso *et al.* 2007). We now extend these findings to show that during IH exposure with either saline or glucose infusion, there is also no change in β -cell size.

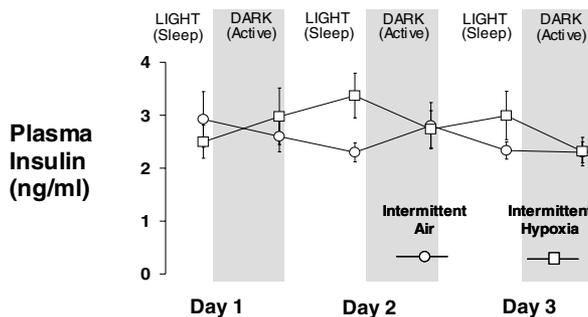
There was no statistical difference in apoptosis as assessed by TUNEL staining across all four experimental groups by one-way ANOVA (Fig. 6D; note, the one-way ANOVA did exhibit a strong statistical trend ($P = 0.085$), and application of Dunnett's *post hoc* assessment showed an increase in apoptosis in the glucose + IH group relative to the saline + IA control group ($P = 0.032$)). By two-way ANOVA there was a significant effect of IH exposure to increase apoptosis ($P < 0.05$), but there was no significant effect of glucose infusion or an interaction between the two factors. Regardless, the rates of apoptosis in all four groups were relatively low (0.05–0.15% TUNEL-positive β -cells). The per cent of cells with pyknotic nuclei was also low (Fig. 6E) and did not statistically differ between groups by either one-way or two-way ANOVA.

In animals exposed to 4 weeks of IH there were statistically significant increases in β -cell area per pancreas

A Saline infusion



B Glucose infusion



C Saline and glucose infusion

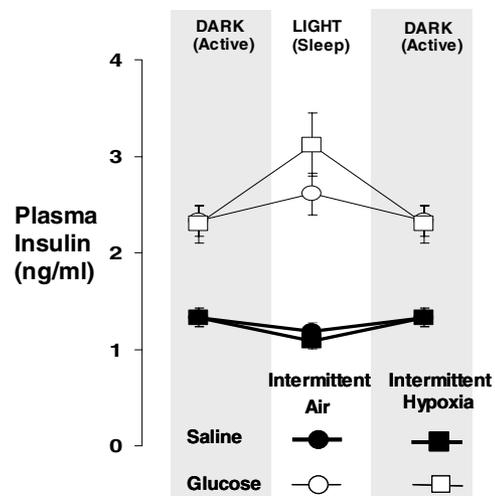


Figure 4. Glucose infusion, but not intermittent hypoxia, increased plasma insulin levels

A, changes in plasma insulin over time during saline infusion (intermittent hypoxia, $n = 17$; intermittent air $n = 13$). B, changes in plasma insulin over time during glucose infusion (intermittent hypoxia, $n = 14$; intermittent air $n = 14$). C, averaged changes in plasma insulin during the light and dark periods for all four experimental groups (note: data for dark period is plotted twice to highlight the diurnal rhythm). Analysis by two-way ANOVA revealed a significant effect of infusion (glucose > saline; $P < 0.0001$), but no effect of intermittent hypoxia exposure and no interaction.

area ($P < 0.025$) and β -cell mass per body weight ($P < 0.025$), suggesting that during longer-term exposure to IH there is no evidence that β -cell death by apoptosis dominates over β -cell replication (Table 3).

Discussion

OSA is an established risk factor for the development of cardiovascular disease (Nieto *et al.* 2000; Peppard *et al.* 2000; Moore *et al.* 2001; Gami *et al.* 2005; Marin *et al.* 2005; Yaggi *et al.* 2005), and numerous animal models have shown a causal pathway from airway obstruction, through IH stress, to sustained systemic hypertension (Fletcher *et al.* 1992; Brooks *et al.* 1997; Campen *et al.* 2005). More recently studies suggest that OSA and hypoxic stress may also independently contribute to insulin resistance, hyperglycaemia and type 2 diabetes (Ip *et al.* 2002; Punjabi *et al.* 2002, 2004; Harsch *et al.* 2004). In the present study we examined the relationship between IH and experimentally induced hyperglycaemia

on arterial blood pressure, blood glucose homeostasis, and replication and apoptosis of pancreatic β -cells. Utilizing a chronically double-catheterized, unhandled, murine model we determined that IH produced a sustained hypertensive response, as has been previously described in rats (Tahawi *et al.* 2001; Lai *et al.* 2006). In contrast, IH reversed the diurnal pattern of glucose regulation, which now peaked during the light period of IH exposure and reached a nadir during subsequent room air exposure during the dark period, despite unchanging levels of plasma insulin. Surprisingly, this apparent 'rebound increase in insulin sensitivity' in the dark period after IH exposure was of sufficient magnitude that blood glucose reached euglycaemic levels even during infusion of 50% dextrose at $100 \mu\text{l h}^{-1}$. Therefore, blood glucose levels fluctuated diurnally based on the presence *versus* the absence of IH stress, whereas elevations in arterial blood pressure were continuous and sustained throughout the 4 day protocol. The presence of hyperglycaemia is a strong physiological stimulus for pancreatic β -cells to

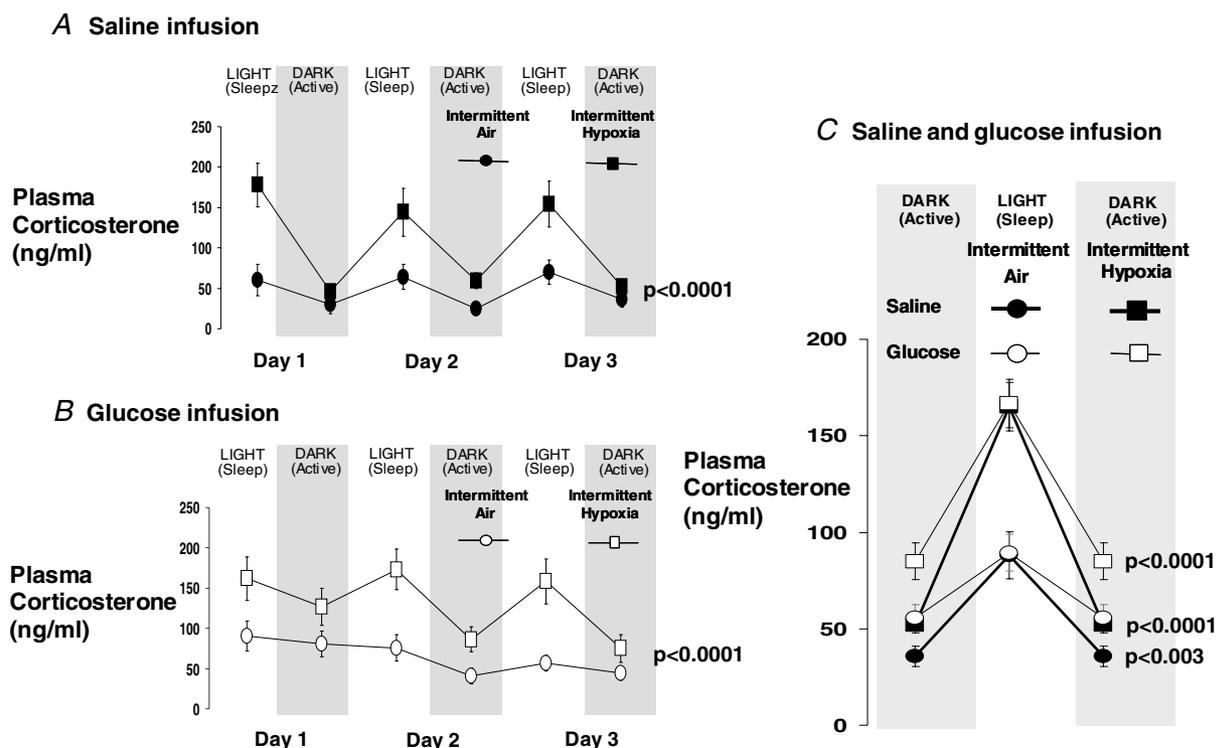


Figure 5. Intermittent hypoxia, but not glucose infusion, accentuated the light period spike in plasma corticosterone

A, changes in plasma corticosterone over time during saline infusion (intermittent hypoxia, $n = 17$; intermittent air $n = 13$; statistical difference determined by one-way ANOVA). *B*, changes in plasma corticosterone over time during glucose infusion (intermittent hypoxia, $n = 14$; intermittent air $n = 14$; statistical difference determined by one-way ANOVA). *C*, averaged changes in plasma corticosterone during the light and dark periods for all four experimental groups (note: data for dark period is plotted twice to highlight the diurnal rhythm). Significant differences between the light and dark periods within an experimental group are marked to the right of the figure. Analysis by two-way ANOVA revealed a significant effect of exposure (intermittent hypoxia > intermittent air; $P < 0.0001$), but no effect of glucose infusion and no interaction.

Table 3. The effect of four weeks exposure to intermittent hypoxia (IH) on pancreatic β -cell mass

	IA + saline	IH + saline	P value
Sample size (<i>n</i>)	4	4	—
Body weight at end of IH exposure (g)	24.7 ± 0.43	21.3 ± 0.68*	<i>P</i> = 0.005
Pancreas weight at end of IH exposure (g)	309.0 ± 32.6	276.6 ± 27.0	<i>P</i> = 0.475
β -Cell area per pancreas area (%)	0.2526 ± 0.028	0.375 ± 0.030*	<i>P</i> = 0.023
β -Cell mass (mg)	0.78 ± 0.12	1.02 ± 0.05	<i>P</i> = 0.106
β -Cell mass per body weight (mg kg ⁻¹)	31.4 ± 4.4	47.9 ± 3.3*	<i>P</i> = 0.013

IH, intermittent hypoxia; IA, intermittent air; statistical significance determined by unpaired, two-tailed, *t* test; **P* < 0.05.

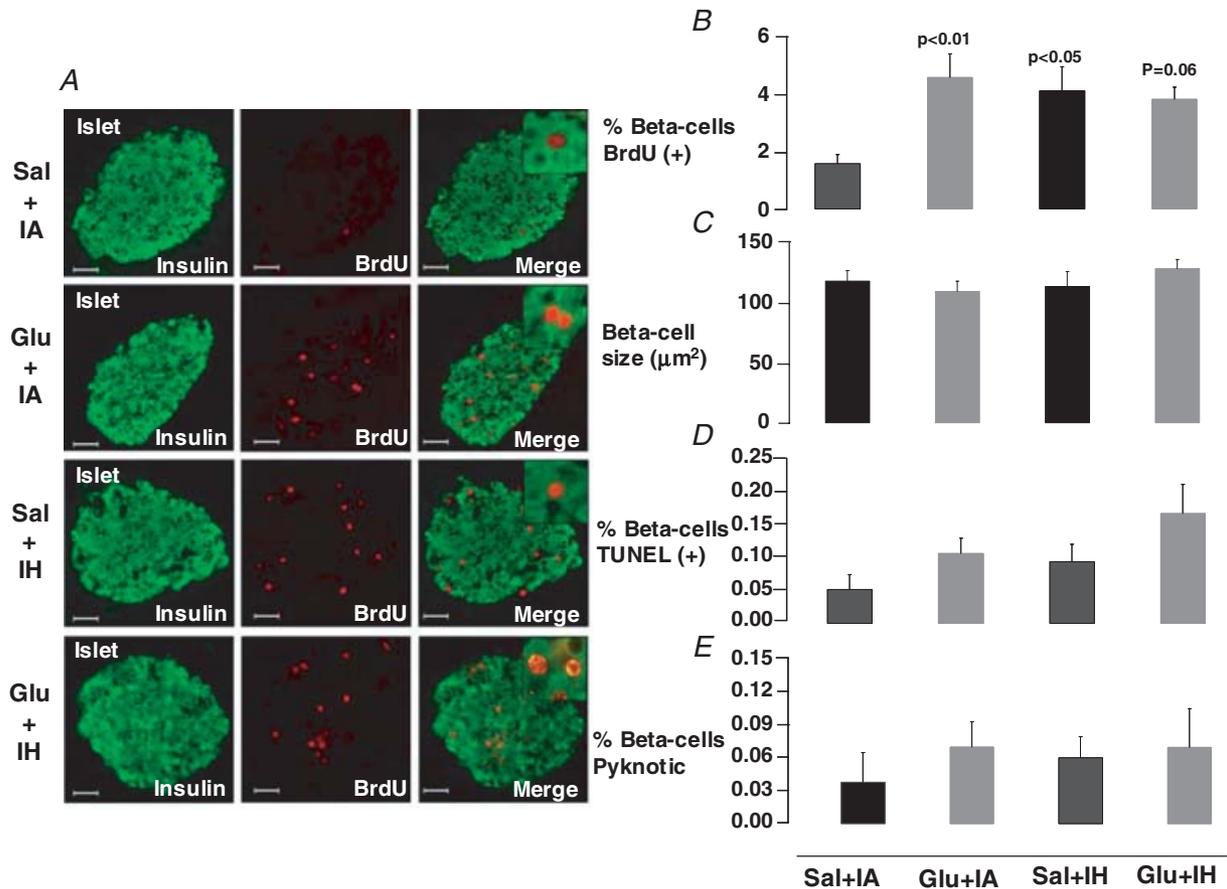


Figure 6. Glucose infusion and intermittent hypoxia increased pancreatic β -cell replication

A, representative islets stained for BrdU and insulin show glucose-induced and hypoxia-induced replication. Insets: high magnification confirms BrdU-positive nuclei belong to insulin-positive cells. Scale bars, 20 μ m. B, mean percentage β -cell replication during intermittent air exposure (saline, *n* = 9; glucose *n* = 10) and intermittent hypoxia exposure (saline, *n* = 9; glucose *n* = 9). Statistical difference determined by one-way ANOVA and Dunnett's *post hoc* assessment relative to the saline + intermittent air control group are shown above bars. Analysis by two-way ANOVA revealed a significant effect of infusion (glucose > saline; *P* < 0.05), no effect of intermittent hypoxia, but a significant interaction (glucose infusion reduced the rate of replication due to intermittent hypoxia exposure; *P* < 0.025). C, mean β -cell size during intermittent air exposure and intermittent hypoxia exposure (sample size same as B). D, mean changes in β -cells positive for TUNEL during intermittent air exposure and intermittent hypoxia exposure (sample size same as B). One-way ANOVA showed a trend for statistical significance (*P* = 0.085) and Dunnett's *post hoc* assessment showed an increase in apoptosis in the glucose + intermittent hypoxia group relative to the saline + intermittent air control group (*P* = 0.032). Analysis by two-way ANOVA revealed a significant effect of exposure (intermittent hypoxia > intermittent air; *P* < 0.05), but no effect of glucose infusion and no interaction. E, mean percentage β -cells that were pyknotic during intermittent air exposure and intermittent hypoxia exposure (sample size same as B). Sal + IA, saline infusion and intermittent air exposure; Glu + IA, glucose infusion and intermittent air exposure; Sal + IH, saline infusion and intermittent hypoxia exposure; Glu + IH, glucose infusion and intermittent hypoxia exposure.

replicate, a compensatory response that may provide more insulin to counteract hyperglycaemia. Here we show that 4 days exposure to IH can cause a comparable degree of β -cell replication as hyperglycaemia, potentially acting as a compensatory response to increase insulin supply to counteract the presence of insulin resistance (Iiyori *et al.* 2007). Although this increase in replication is tempered by the finding that IH may independently increase the rate of β -cell apoptosis, after 4 weeks of IH exposure there was no evidence of an overall reduction in β -cell mass.

Chronically catheterized model

The model system utilized in our 7 day study was designed to account for the average lifespan of the very small femoral artery catheter used for repeated blood sampling and continuous monitoring of arterial blood pressure. As in our previous studies (Alonso *et al.* 2007; Iiyori *et al.* 2007), we preferred the more technically difficult femoral arterial catheterization over the commonly used carotid arterial catheterization, to maintain an intact circulation to both carotid bodies. Animals in all groups had re-established normal food intake, mean arterial blood pressure and blood glucose levels by the third post-operative day, leaving approximately 4 days of reliable catheter patency to conduct the IH exposure and glucose infusion studies. The restoration of appetite control mechanisms was evident during protocols with 50% dextrose infusion in which the animals appropriately reduced their food intake of dry chow to maintain a normal daily calorie intake. The light–dark cycle was strictly controlled by maintaining the customized housing chambers delivering the IH or IA stimulus inside a larger sound-restricting cubicle with its own internal low wattage light source. Within this environment the animals established a highly predictable and consistent daily rhythm of blood glucose and plasma corticosterone levels (Laposky *et al.* 2007). Finally, the ability to sample arterial blood without handling the animal resulted in an unstressed animal as determined by the very low plasma corticosterone and unfasted blood glucose levels in the IA exposed, saline-infused control group.

Development of sustained hypertension

In the saline-infused animals, IH caused a sustained 10 mmHg increase in mean arterial blood pressure. A comparable hypertensive response to IH has been previously reported by us in C57BL/6J mice exposed to 4 weeks of IH (Campen *et al.* 2005), as well as in many other rodent studies of IH (Kanagy *et al.* 2001; Tahawi *et al.* 2001; Thongboonkerd *et al.* 2002; Lai *et al.* 2006), and also in response to 4 weeks of experimentally induced airway obstruction in a canine model of OSA (Brooks *et al.*

1997). Multiple studies in normal subjects, OSA patients and animal models suggest that the hypoxic stress of OSA is dependent on increased sympathetic nerve activity (SNA) (Leuenberger *et al.* 1995; Somers *et al.* 1995; O'Donnell *et al.* 1996; Xie *et al.* 2001), that is maintained through the ensuing daytime, or non-hypoxic, period (Somers *et al.* 1995; Xie *et al.* 2001). Although we do not know whether increased sympathetic nerve activity contributed to hypertension in the present study, the rapid onset of hypertension we observed would be consistent with a neurally mediated mechanism.

IH reverses diurnal glucose rhythm

In the unfasted mouse, blood glucose levels are higher during the dark, or active, period when the majority of the daily calorie intake is consumed. A major finding of the current study was that daily 12 h exposures to IH in the light, or sleeping, period reversed the normal diurnal rhythm of blood glucose. The reversed diurnal rhythm was not the result of any alteration in daily food consumption patterns, but may be related in part to fluctuating periods of decreased and increased insulin sensitivity. The higher blood glucose levels attained during the IH exposure occurred in the face of unchanged insulin levels, consistent with our recent report (Iiyori *et al.* 2007) of reduced insulin sensitivity during periods of IH stress. It is also possible that hepatic glucose output may have contributed to elevations in blood glucose during the hypoxic exposure period, as our previous study showed a non-significant trend for IH to increase basal hepatic glucose output (Iiyori *et al.* 2007). Moreover, the magnitude of the diurnal variation in blood glucose during IH exposure was accentuated by the presence of simultaneous glucose infusion. This pattern of fluctuating blood glucose levels was clearly different from the sustained increases seen in arterial blood pressure in response to IH, and suggests the absence of a common mechanism linking cardiovascular and metabolic sequelae in IH or OSA. Indeed, we previously reported (Iiyori *et al.* 2007) that the increase in SNA that is integral in the development of IH-induced hypertension, is not required for IH to reduce insulin sensitivity. Thus, the pathways that elevate blood glucose levels during IH, including a potential role for fragmented sleep, remain to be determined.

One possible mechanism for IH to reduce insulin sensitivity and increase blood glucose levels is an elevation in plasma corticosterone, the predominant glucocorticoid in mice. In our recent study (Iiyori *et al.* 2007) we showed that IH elevated corticosterone levels, but this was based on a single measurement under anaesthesia at time of kill. The present study extends this finding by first establishing a distinct corticosterone rhythm in IA-exposed, saline-infused, control animals with higher corticosterone levels during the light period than the dark

period. During IH exposure in the light period there was an approximately threefold increase in plasma corticosterone for both saline-infused and glucose-infused animals that was temporally associated with the peak in blood glucose. Future experiments that either abolish corticosterone release during IH, or mimic the diurnal pattern of corticosterone in the absence of IH, will be necessary to determine the specific impact of glucocorticoids on the glucose rhythm. However, it is possible that other counter-regulatory hormones such as glucagon, adrenaline or growth hormone may also play a role (note: due to limitations in the volume of blood that could be withdrawn twice daily we only had sufficient plasma to assay for glucose, insulin and corticosterone at each time point). Similarly, pathways of lipotoxicity including hyperlipidaemia, ectopic fat deposition, inflammatory cytokines and generation of reactive oxygen species are all potentially activated in IH or OSA and may negatively impact on insulin sensitivity (Newman *et al.* 2001; Yokoe *et al.* 2003; Li *et al.* 2005; Minoguchi *et al.* 2006; Li *et al.* 2007; Savransky *et al.* 2007). Although our data cannot discriminate between these potential mechanisms, the unexpected low levels of blood glucose during the dark period in the IH-exposed, glucose-infused, animals suggests that the pattern of blood glucose changes observed was not solely dependent on factors worsening insulin sensitivity.

In glucose-infused mice, blood glucose was markedly reduced to euglycaemic levels in the dark period following exposure to IH. Relative to the glucose-infused IA group, the blood glucose during the dark period was 33 mg dl^{-1} lower in the IH-exposed animals, yet insulin levels were identical. These data suggest that when assessing the impact of IH or OSA on insulin sensitivity, it is important to consider not only factors that worsen insulin sensitivity during the exposure period, but also factors that may increase insulin sensitivity when the hypoxic stress is removed. Potentially, such factors may include biochemical changes within the insulin-signalling pathway in peripheral organs, and would be an interesting focus of future studies. The ability of insulin sensitivity to oscillate above and below a baseline level in response to the absence *versus* presence of IH under experimental conditions suggests that clinical studies examining glucose regulation in OSA patients should consist of multiple time-points, including during sleep when the hypoxic stress is manifest.

Short-term IH exposure causes pancreatic β -cell replication

The current study is a first attempt to examine the impact of IH on the ability of pancreatic β -cells to replicate and potentially slow the progression of hyperglycaemia.

Under basal conditions, adult pancreatic β -cells are very slow to divide (Teta *et al.* 2005). However, in rodent models, constantly infusing glucose to produce a hyperglycaemic milieu can increase the replication of β -cells up to approximately 4–5% over a 4 day period (Bonner-Weir *et al.* 1989; Steil *et al.* 2001; Alonso *et al.* 2007). A surprising finding of the current study was that in saline-infused animals, 4 days of exposure to IH also resulted in a β -cell replication rate of approximately 4%; a rate of replication 2- to 3-times higher than the IA-exposed, saline-infused, control group. The increased replication rate in response to IH was not related to blood glucose levels that overall remained in the euglycaemic range ($109 \pm 2 \text{ mg dl}^{-1}$). In the short term, these data suggest that exposure to IH causes a compensatory response in β -cell replication. Interestingly, in the field of pancreatic islet transplantation, sustained hypoxia is considered to have deleterious effects on β -cell viability (Wang *et al.* 2006). This is in part due to the known effects of hypoxia to cause apoptosis in β -cells (Moritz *et al.* 2002). Consequently, the replication benefits of IH must be balanced against its potential apoptotic effects. IH has been shown to produce apoptosis in specific areas of the brain, leading to learning and memory deficits (Row *et al.* 2003). However, the rates of apoptosis that we observed in pancreatic β -cells were in general low, although there was an overall statistically significant effect of IH by two-way ANOVA to increase the rate of apoptosis. The absolute rates of replication and apoptosis in pancreatic β -cells cannot be directly compared, since replication rate is determined over the entire 4 day period, whereas the rate of apoptosis is relevant to only the time of kill. Our 28 day measurements of β -cell mass suggest that during IH exposure the rate of apoptosis does not exceed the rate of replication of β -cells, and, if anything, β -cell mass may be increased. Thus, the potential compensatory increase in β -cell replication during IH exposure may be sustained for at least 4 weeks, although longer periods of exposure may increase apoptosis and ultimately lead to β -cell failure.

The addition of glucose infusion to IH exposure did not cause any further increase in β -cell replication to that seen with IH (+ saline infusion) or glucose infusion (+ IA exposure) alone. In fact there was a negative interaction by two-way ANOVA, with the combined stimulus of IH exposure and glucose infusion resulting in a replication rate of only 3.6%. The lack of an additive effect between IH and glucose on pancreatic β -cell replication could potentially be due to: (1) a ceiling effect that limits *in vivo* pancreatic β -cell replication to a maximum of 4–5%; (2) IH and glucose sharing a common biological pathway for inducing replication that was maximally utilized; (3) overall lower blood glucose levels ($122 \pm 3 \text{ mg dl}^{-1}$) in the IH + glucose group, thereby reducing the replication signal due to hyperglycaemia. Whatever factors are limiting replication, the combination of IH exposure and glucose

infusion also produced a strong trend for increased apoptosis (note: it was not technically feasible to assess changes in β -cell mass over 28 days with a combined IH exposure and glucose infusion). Thus, the presence of hypoxic stress and hyperglycaemia, a combination often seen in obese OSA patients, potentially provides the least favourable environment for increasing pancreatic β -cell mass.

Summary

We have developed a chronically catheterized, unhandled murine model to characterize the diurnal rhythms of arterial blood pressure and glucose homeostasis, as well as to assess pancreatic β -cell replication, in response to IH and hyperglycaemia. Arterial blood pressure was elevated and sustained by IH exposure, whereas glucose regulation exhibited a diurnal rhythm that was reversed by IH exposure, with the highest blood glucose, and possibly lowest insulin sensitivity, occurring during the period of hypoxic stress. Simultaneous glucose infusion attenuated the hypertension, but exacerbated the reversed diurnal blood glucose fluctuations, in response to IH. Finally, IH acted to stimulate pancreatic β -cell replication, a compensatory response expected to combat insulin resistance and hyperglycaemia, but the combined stimulus of IH and glucose infusion produced the highest rate of apoptosis. Our demonstration of metabolic abnormalities in a murine model of glucose infusion and IH exposure will provide a basis for future studies that use transgenic manipulations to explore underlying mechanisms as well as to determine the interactive metabolic effects of common comorbidities in OSA, including obesity, insulin resistance and type 2 diabetes.

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