

ORIGINAL ARTICLE

Sleep fragmentation promotes NADPH oxidase 2-mediated adipose tissue inflammation leading to insulin resistance in mice

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BACKGROUND: Short sleep has been implicated in higher risk of obesity in humans, and is associated with insulin resistance. However, the effects of fragmented sleep (SF) rather than curtailed sleep on glucose homeostasis are unknown.

METHODS: Wild-type and NADPH oxidase 2 (Nox2) null male mice were subjected to SF or sleep control conditions for 3 days to 3 weeks. Systemic and visceral adipose tissue (VAT) insulin sensitivity tests, glucose tolerance test, fluorescence-activated cell sorting and immunohistochemistry for macrophages and its sub-types (M1 and M2), and Nox expression and activity were examined.

RESULTS: Here we show that SF in the absence of sleep curtailment induces time-dependent insulin resistance, *in vivo* and also *in vitro* in VAT. Oxidative stress pathways were upregulated by SF in VAT, and were accompanied by M1 macrophage polarization. SF-induced oxidative stress, inflammation and insulin resistance in VAT were completely abrogated in genetically altered mice lacking Nox2 activity.

CONCLUSIONS: These studies imply that SF, a frequent occurrence in many disorders and more specifically in sleep apnea, is a potent inducer of insulin resistance via activation of oxidative stress and inflammatory pathways, thereby opening the way for therapeutic strategies.

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INTRODUCTION

Chronic sleep restriction and sleepiness are highly prevalent conditions in industrialized societies, and have been associated with an increased risk for development of metabolic dysfunction.^{1–6} However, another alteration of sleep, namely sleep fragmentation (SF), is also very frequently encountered in the context of multiple disorders, such as sleep apnea, depression, chronic obstructive pulmonary disease and asthma. Furthermore, excessive daytime sleepiness is a frequent occurrence in the context of SF, even though SF does not mandatorily lead to reduced sleep duration.

Experimentally imposed sleep restriction conditions have now repeatedly demonstrated the emergence of insulin resistance even in young healthy individuals.^{1–4,6} Initial preliminary studies in which SF was induced in the sleep laboratory in healthy human subjects showed the emergence of adverse metabolic consequences such as increased orexigenic behaviors and insulin resistance.^{7,8} However, the mechanisms underlying SF-associated metabolic dysfunction are unknown, and have yet to be investigated in animal models.

NADPH oxidase is a multi-subunit enzyme complex that localizes to both the plasma membrane and membranes of subcellular organelles, and catalyzes electron transfer from NADPH to molecular oxygen, producing superoxide.^{9,10} De-regulated activation of NADPH oxidase leads to ROS production in large quantities that are likely involved in oxidative cellular damage. In a previous study, we showed that SF elicited increased sleepiness in the absence of reductions in total sleep duration, and manifested

cognitive and behavioral alterations.^{11,12} In this context, Nox2-deficient mice were protected from SF-induced cognitive dysfunction, suggesting a role for NADPH oxidase in end-organ morbidities associated with disrupted sleep architecture. We therefore hypothesized that the previously demonstrated increases in NADPH oxidase (Nox) induced by chronic SF exposures would not be restricted to the central nervous system, and would potentially be activated in other metabolically active tissues such as visceral adipose tissue (VAT) in mice.¹³ We further posited that such increases in Nox2 activity would underlie potential alterations in insulin sensitivity associated with SF, and would further reflect changes in VAT macrophage populations and polarity within the VAT.^{13–15} We therefore took advantage of an SF-murine model, in which SF is applied during the light phase of the circadian cycle for extended periods of time and induces substantial increases in sleepiness without sleep restriction,^{11,12} and assessed changes in systemic and VAT-insulin sensitivity.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice and hemizygous *gp91^{phox-/-}* mice of similar age and weighing 22–25 g were purchased from Jackson Laboratories (Bar Harbor, ME, USA), were housed in a 12-h light/dark cycle (light on 0700–1900 hours) at a constant temperature (24 ± 1 °C) and were allowed access to food and water *ad libitum*. The experimental protocols were approved by the Institutional Animal Use and Care Committee and are in close agreement with the National Institutes of Health *Guide in the Care and Use*

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of Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. However, because the systemic tests below can be stressful, no animal underwent more than one experimental procedure.

Sleep fragmentation

The SF device that is used to induce SF in rodents has been previously described^{11,12,16} and employs intermittent tactile stimulation of freely behaving mice in a standard laboratory mouse cage using a near-silent motorized mechanical sweeper. This method prevents the need for human contact and intervention, introduction of foreign objects or touching of the animals during sleep, and is therefore superior to other existing methods of sleep disruption. To induce moderate to severe SF, we chose a 2-min interval between each sweep, implemented during the light period (0700–1900 hours). Of note, four mice were housed in each sleep fragmenter cage to prevent isolation stress, and had *ad libitum* access to food and water. We should also point out that imposition of SF for extended periods of time is not associated with any measurable changes in sleep duration, such that sleep disruption is imposed in the absence of sleep curtailment or deprivation.^{11,12}

Surgical procedure and implantation of telemetric transmitter and electrodes for sleep recordings. All surgical procedures were performed under sterile conditions and isoflurane general anesthesia, as previously described.^{11,12,16} Briefly, a telemetric transmitter weighing 3.5 g, F20-EET (DSI, New Brighton, MN, USA), which allows simultaneous monitoring of two biopotential channels, temperature and locomotor activity, was inserted, biopotential leads were exteriorized and the abdominal wall was closed. The two pairs of biopotential leads were then advanced to the dorsal neck incision: the first pair of biopotential leads was fixed to the skull above the frontal area (1 mm anterior to bregma and 2 mm lateral to mid-sagittal suture for one of the leads, and 1 mm anterior to lambda and 2.5 mm lateral to mid-sagittal suture for the other lead). The other pair of biopotential leads was placed within the same bundle of dorsal neck muscles for the recording of nuchal electromyogram.

Acclimatization, sleep recording and SF. After complete recovery from surgery (7–10 days), mice were transferred to the sleep fragmenter device for 1-week acclimatization. The recording cages were mounted on a DSI telemetry receiver (RPC-1), which was in turn connected to an acquisition computer through a data exchange matrix. Physiological data were continuously acquired for 24 h using Dataquest ART acquisition software (DSI; version 3.1) at a sampling rate of 500 Hz. Data were first scored automatically using Sleepsign software (Kissei Comtec, Nagano, Japan), and records were visually confirmed or corrected as needed.

Behavior was classified into three different states: wake, slow-wave sleep (SWS) and rapid eye movement sleep.^{11,12,16} To determine the number of wake events during SF, the mean number of wake episodes of a 2-h bin was calculated. Furthermore, to determine the time elapsed following a wake episode to initiation of SWS, the latency in seconds

was calculated for each arousal throughout the 24-h period. The time was measured from the beginning of each wake episode to the beginning of the next SWS episode and the mean calculated.¹⁷

GTT and ITT

Both tests were performed following 2 weeks of either sleep control (SC) or SF conditions, using the protocols described previously.¹⁸ Briefly, the animals were fasted for 3 h with water available *ad libitum*. Sterile glucose (2 mg g⁻¹ of body weight for glucose tolerance test (GTT)) or sterile humulin (0.25 units kg⁻¹ of body weight for insulin tolerance test (ITT)) was injected intraperitoneally. Blood was sampled from a nick at the tip of the tail at various time points after injection and blood glucose was assessed using an OneTouch Ultra2 glucometer (Life Scan, Inc., Milpitas, CA, USA). Slopes A and B were calculated for GTT using the glucose levels between 0 and 15 min after glucose injection and those between the peak (30 min) and 120 min after glucose injection, respectively. On the other hand, only Slope A was calculated for ITT using glucose levels between 0 min and the nadir (60 min) after insulin injection. In addition, area under the curve (AUC) was calculated for GTT.

Insulin sensitivity assay

Adipocyte insulin sensitivity was assessed as described previously.¹⁹ Briefly, primary VAT adipocytes were isolated from epididymal fat pads by collagenase digestion and flotation centrifugation. They were then incubated with insulin at various concentrations at 37 °C for 10 min with gentle vortexing every 2 min. After two washes with cold Krebs–Ringer buffer (KRB), cells were lysed in Laemmli buffer and assessed using western blotting analysis for phosphorylated and total Akt (antiphospho-Akt (Ser473) and anti-Akt; Cell Signaling Technology, Danvers, MA, USA).

Isolation of stromal-vascular fraction and flow cytometry analysis

Epididymal fat pads representing VAT were minced in KRB supplemented with 1% bovine serum albumin and incubated with collagenase (1 mg ml⁻¹; Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37 °C for 45 min with shaking. Cell suspensions were filtered through a 100-µm mesh and centrifuged at 500 g for 5 min to separate floating adipocytes from the stromal-vascular fraction (SVF) pellet. SVF pellets were then resuspended in fluorescence-activated cell sorting buffer (PBS plus 2% FBS) and 10⁶ cells were used for staining with fluorescence-conjugate-primary antibodies or control IgGs at 4 °C for 30 min. Cells were then washed twice and analyzed with a flow cytometer (Canto II; BD Biosciences, San Jose, CA, USA). Data analysis was performed using the FlowJo software (Tree Star, Ashland, OR, USA). Adipose tissue macrophages (ATMs) were defined as F4/80+ and CD11b+ cells, from which M1 and M2 macrophages were identified as CD11c+ or CD206+ cells, respectively. All antibodies were from Biolegend (San Diego, CA, USA).

Table 1. Sleep state distribution, mean sleep latency, awakenings and body weight in WT and Nox2 null mice subjected to SF for 15 days and controls

State	Time of day	WT SC	WT SF	WT SC vs SF	Nox2-SC	Nox2-SF	Nox2 SC vs SF
		Percentage time spent	Percentage time spent	Significance	Percentage time spent	Percentage time spent	Significance
Wake	0700–1900 hours	31.78 ± 4.18	27.56 ± 3.78	P > 0.05	34.29 ± 4.87	31.17 ± 4.70	P > 0.05
	1900–0700 hours	67.9 ± 5.67	70.29 ± 6.83	P > 0.05	66.5 ± 6.16	69.85 ± 6.74	P > 0.05
SWS	0700–1900 hours	51.25 ± 5.42	54.18 ± 6.79	P > 0.05	55.08 ± 7.7	54.4 ± 7.4	P > 0.05
	1900–0700 hours	21.48 ± 4.46	24.46 ± 4.80	P > 0.05	20.56 ± 4.52	22.11 ± 4.16	P > 0.05
REMS awakenings (#/2 h)	0700–1900 hours	14.96 ± 2.39	15.27 ± 3.17	P > 0.05	13.73 ± 2.83	14.9 ± 2.92	P > 0.05
	1900–0700 hours	6.5 ± 1.57	7.24 ± 2.13	P > 0.05	7.5 ± 2.05	8.1 ± 2.92	P > 0.05
MSL (min)	0700–1900 hours	12.3 ± 3.57	42.3 ± 4.63	P < 0.0001	11.7 ± 2.93	41.9 ± 3.92	P < 0.0001
	1900–0700 hours	7.4 ± 1.2	2.1 ± 0.9	P < 0.0001	7.4 ± 1.2	2.1 ± 0.9	P < 0.0001
Body weight (g) ^a		26.8 ± 1.7	27.2 ± 1.8	P > 0.05	25.8 ± 1.6	26.0 ± 1.8	P > 0.05

Abbreviations: MSL, mean sleep latency; REMS, rapid eye movement sleep; SWS, slow-wave sleep; WT, wild type. WT vs Nox2, no significant differences for all measures. ^aRefers to n = 24–36/group.

Immunohistochemistry

Sections of formalin-fixed paraffin-embedded adipose tissue were deparaffinized and rehydrated prior to antigen unmasking by boiling in 10 mM sodium citrate, pH 6.0, with 0.05% Tween-20 for 30 min. Sections were blocked in 5% Donkey/Goat serum in PBS with 0.5% Triton X-100 for 1 h followed by incubation with F4/80

and CD11C antibodies at 4°C overnight. Secondary antibody staining was performed using donkey anti-rat daylight 488 and goat anti-hamster daylight 649 (Jackson ImmunoResearch, West Grove, PA, USA) and cell nuclei were labeled with the Hoechst dye. Images were captured with a Nikon Eclipse Ti microscope (Nikon Instruments, Melville, NY, USA).

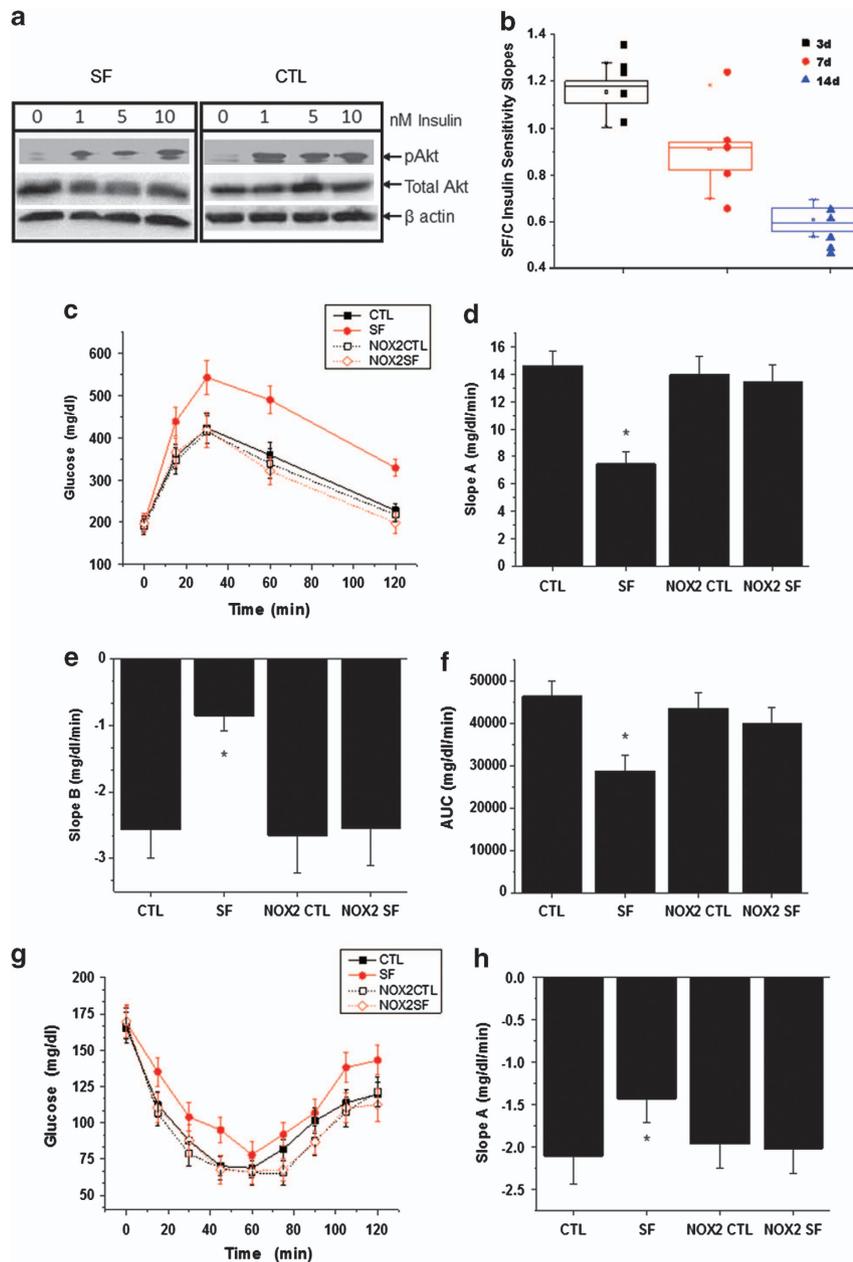


Figure 1. SF-induced insulin resistance in WT, but not in Nox2-deficient mice. (a) Adipocyte insulin sensitivity in WT mice assessed by insulin-induced Akt phosphorylation in adipocytes isolated from epididymal fat pads. Shown are representative western blots. (b) Boxplots of SF-induced temporal alterations in *in vitro* adipocyte insulin sensitivity in WT mice, calculated as the SF vs control ratio of insulin dose-response slopes. $N = 5$ for each group. Slopes of mice exposed to SF for 3 days were significantly increased ($P < 0.01$) compared with timed controls. After 7 days of SF no significant changes emerged ($P > 0.1$), while after 14 days a dramatic decrease ($P < 0.00003$) in insulin sensitivity became apparent. (c) SF-induced glucose intolerance as shown by GTT. Data are mean \pm s.e.; $n = 6$ for each experimental group. (d) SF-induced glucose intolerance in WT but not Nox2-deficient mice, as shown by a steeper slope A calculated using the glucose levels between 0 and 15 min after glucose injection. Data are mean \pm s.e.; $n = 6$ for each group; $*P < 0.05$ vs all other three groups. (e) SF-induced reduced glucose clearance in WT but not Nox2-deficient mice, as shown by a flatter slope B calculated using glucose levels between the peak (30 min) and 120 min after glucose injection. Data are mean \pm s.e.; $n = 6$ for each group; $*P < 0.05$ vs all other three groups. (f) SF-induced glucose intolerance in WT but not Nox2-deficient mice, as shown by a greater AUC after glucose injection. Data are mean \pm s.e.; $n = 6$ for each group; $*P < 0.05$ vs all other three groups. (g) SF-induced insulin insensitivity as shown by ITT. Data are mean \pm s.e.; $n = 6$ for each group. (h) SF-induced insulin insensitivity in WT but not Nox2-deficient mice, as shown by a flatter slope A calculated using the glucose levels between 0 min and the nadir (60 min) after insulin injection. Data are mean \pm s.e.; $n = 6$ for each group; $*P < 0.05$ vs all other three groups.

Inflammatory genes and NADPH oxidase expression

mRNA levels of the p47^{phox} subunit in visceral fat were analyzed with quantitative RT-PCR in triplicates, using primers 5'-CAGCCAGCAC TATGTGTACA-3' and 5'-GAACTCGTAGATCTCGGTGAA-3' (Applied Biosystems, Foster City, CA, USA). Ribosomal 18S rRNA was used as an internal control. The mRNA expression in ATMs of tumor necrosis α (TNF- α), interferon γ (IFN), interleukin-1 (IL-1) and interleukin 6 (IL-6) was determined by quantitative RT-PCR using commercially available specific Taqman primers (Applied Biosystems).

-NADPH oxidase activity

1×10^6 SVF cells were incubated in RPMI-1640 medium containing 2% FBS, 20 mM HEPES and 5 μ M lucigenin. Superoxide-dependent reduction of lucigenin was initialized by the addition of phorbol 12-myristate 13-acetate (100 nM). Light emission was recorded using a VICTOR3V plate reader (Perkin Elmer, Waltham, MA, USA) at 30-s intervals for 30 min and specific NOX activities were determined by subtracting the background level of luminescence of paired samples containing 10 μ M diphenyliodonium.

Data analysis

All data are reported as mean \pm s.e. Comparison of glucose and p47^{phox} mRNA levels among SF and SC conditions was performed using one-way ANOVA followed by unpaired Student's *T*-test with Bonferroni correction. Comparison of all other quantitative data between SF and SC conditions was performed using unpaired Student's *T*-tests. For all comparisons, a *P*-value < 0.05 was considered as statistically significant.

RESULTS

Long-term SF increases sleep propensity without changes in sleep duration

Sleep recordings following 15 days of SF exposures were conducted in a separate subset of 5–7 mice/group, and revealed an increased number of awakenings while the SF device was activated, as well as reduced mean sleep latencies among both wild-type (WT) mice and Nox2 null mice, when compared with corresponding controls. However, no significant changes in total sleep duration of sleep state distribution were apparent (Table 1).

SF promotes systemic and visceral fat insulin resistance

The temporal trajectory of changes in insulin sensitivity in visceral fat, as assessed by Akt phosphorylation in response to insulin, indicated an initial increase in insulin sensitivity after 3 days of SF, followed by subsequent reductions in insulin responsiveness, ultimately manifesting as insulin resistance (Figures 1a and b), in the absence of any significant changes in body weight (Table 1).

In WT mice, GTTs after 2 weeks of SF showed higher glycemic levels and greater AUC responses (Figures 1c–f), and ITTs showed clear evidence of reduced insulin sensitivity (Figures 1g and h),

despite no evidence of increased body weight gains at this time point of SF exposures. In contrast, Nox2-deficient mice exposed to SF for 2 weeks showed no changes in either GTT or ITT responses when compared with littermates exposed to SC conditions (Figures 1c–h).

SF is associated with increased macrophage number and polarization in visceral fat

SF for a period of 2–3 weeks induced increases in the global number of macrophages present in the SVF of visceral fat in WT mice, but not in Nox2-deficient mice (Figure 2). Such increases consisted primarily of macrophages exhibiting the pro-inflammatory M1 phenotype (Figure 3), which localized mainly in perivascular areas (Figure 4). Interestingly, SF seemed to cause a concurrent reduction in M2 macrophages in SVF in comparison with SCs, although the difference between the two groups did not reach significance at this early stage ($P = 0.078$; Figure 3b). SF-induced changes in both M1 and M2 macrophages were conspicuously absent in Nox2-deficient mice exposed to SF. In addition, when compared with control WT mice, macrophages harvested from visceral fat in mice exposed to SF exhibited increased mRNA expression of TNF- α (3.4-fold; $n = 6$; $P < 0.01$), IL-6 (4.2-fold; $n = 7$; $P < 0.01$) and interferon- γ (2.9-fold; $n = 6$; $P < 0.03$), while no significant changes in IL-1 emerged ($P > 0.05$; $n = 7$). Such changes were absent for all these cytokines in SF-exposed Nox2-deficient mice.

SF induces Nox2 expression and activity in visceral fat

WT mice exposed to SF showed time-dependent increases in mRNA expression levels of the p47^{phox} subunit of the NOX2 complex in VATs (Figure 5a). Furthermore, these mice manifested marked increases in NOX enzymatic activities in SVF cells, which were absent in Nox2-deficient mice (Figures 5b and c).

DISCUSSION

In the present study, we show that mice that are subjected to sleep disruption during the course of their natural sleep period develop reductions in both systemic and visceral adipocyte cell insulin sensitivity. Similarly, SF induces increased activity of Nox2 in VAT, along with polarization and infiltration of M1-type macrophages in visceral fat. Furthermore, both systemic and VAT SF-induced changes are either markedly attenuated or altogether absent in Nox2-deficient mice.

Systemic and adipose tissue insulin resistance will develop after sleep restriction or even after acute SF in humans.^{1,7,20,21} Such changes in insulin sensitivity have been attributed to altered autonomic nervous system balance, particularly to increases in

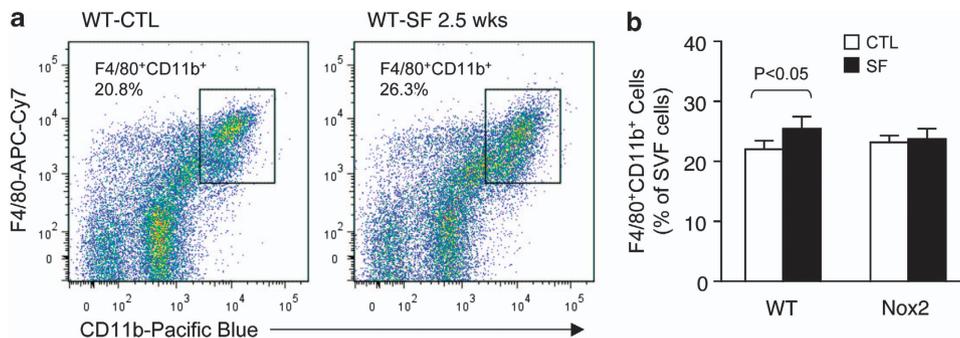


Figure 2. SF-induced increases in global macrophage numbers in the visceral fat of WT, but not Nox2-deficient mice. (a) A representative flow cytometry analysis showing a moderate increase in the number of F4/80⁺CD11b⁺ macrophages in the visceral fat of a WT mouse exposed to SF for 2.5 weeks. This phenomenon was absent in Nox2-deficient gp91^{phox} mice. (b) Summary of four independent flow cytometry experiments. Data are mean \pm s.e.; $n = 4$ for each group.

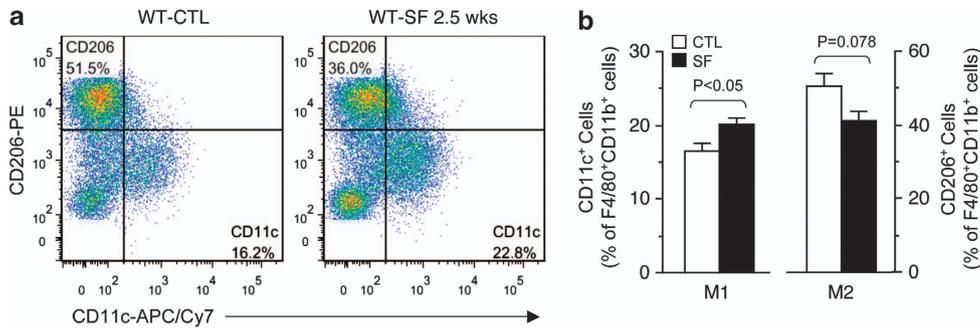


Figure 3. SF-induced selective increase in the number of pro-inflammatory M1 macrophages in the visceral fat of WT, but not Nox2-deficient mice. **(a)** A representative flow cytometry analysis showing an increase in the number of CD11c⁺ M1 macrophages in the visceral fat of a WT mouse exposed to SF for 2.5 weeks. Such SF-induced effect on the pro-inflammatory M1 subset was not observed in Nox2-deficient gp91^{phox-/-} mice. **(b)** Summary of five independent flow cytometry experiments. Note that SF-induced decrease in the M2 subset was not significant at this stage of chronic SF. Data are mean ± s.e.; n = 5 for each group.

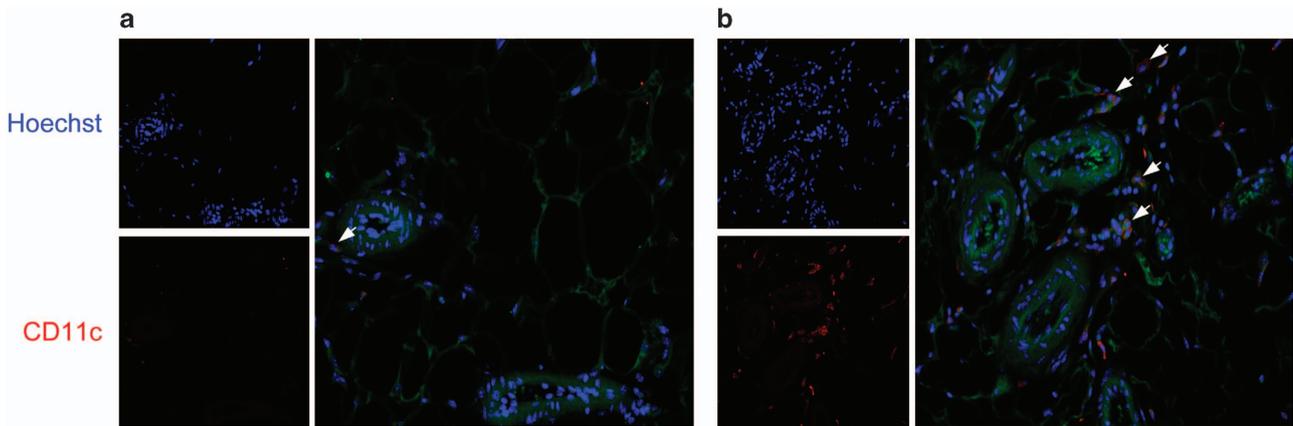


Figure 4. SF-induced pro-inflammatory M1 macrophage infiltration in visceral fat. **(a)** Visceral fat from a WT control mouse immunostained with the M1 marker CD11c. Nuclei were co-stained with the Hoechst dye. Sporadic CD11c⁺ M1 cells are shown in perivascular areas. **(b)** Visceral fat from a WT mouse exposed to SF for 3 weeks, showing clusters of CD11c⁺ M1 cells in perivascular areas.

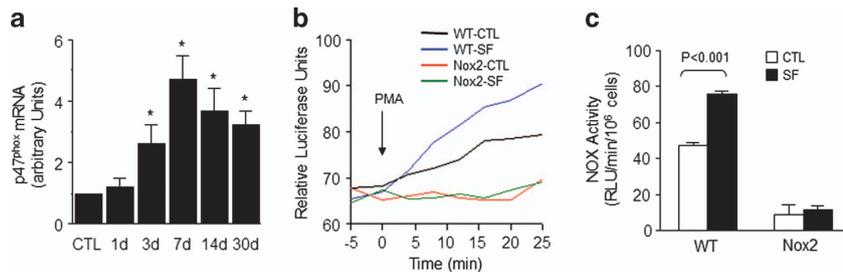


Figure 5. SF-induced increases in Nox subunit expression and enzymatic activity in VAT. **(a)** SF increased p47^{phox} mRNA expression in visceral fat from WT mice. Data are mean ± s.d.; n = 6 for each group. *P < 0.05 vs the control group. **(b)** SVF cells isolated from WT but not Nox2-deficient gp91^{phox-/-} mice exposed to SF for 3–4 weeks possessed elevated Nox enzymatic activity. Shown are representative kinetic tracings. **(c)** Summary of six enzymatic activity experiments. Data are mean ± s.e.; n = 6 for each group.

sympatho-excitatory balance, as well as ascribed to increased activity of the hypothalamic–pituitary–adrenal axis, both of which develop after acute sleep restriction,²² as exogenous administration of cortisol and norepinephrine will induce systemic or tissue insulin resistance.^{23,24} However, the evidence supporting the activation of the hypothalamic–pituitary–adrenal axis has been conflictive.^{25,26} Furthermore, we have previously shown that no increases in systemic cortisol or in norepinephrine (data not shown) are detectable in the context of chronic SF exposures,¹⁶ suggesting that the effects of chronic SF are most likely mediated by increased oxidative stress.²⁷

There is now compelling evidence that high fat diet leads to ATM infiltration in white adipose tissue in both humans and mice, and that the magnitude of such infiltration is tightly associated with the degree of adiposity as well as with insulin resistance.^{28–30} Concurrent with the increased presence of ATM in adipose tissues, there is also a shift in their phenotypes, such that increased populations of M1 ATMs are apparent, and most likely represent specific migration of inflammatory monocytes from the circulation.³⁰ Here we show that SF alone (that is, without sleep restriction and in the absence of high-fat diet) is sufficient to induce the polarization of ATM in vascular fraction, concurrent

with the development of insulin resistance, and that such events precede the onset of accelerated somatic weight accrual. Although our findings do not intend to challenge the potential contributions of sleep restriction to obesity and inflammation,^{31,32} they provide initial evidence that inflammatory and metabolic changes may develop during disrupted sleep conditions, even in the absence of sleep curtailment. The absence of such changes in Nox2-deficient mice further supports the concept that initial activation of oxidative stress pathways in the context of SF is a critical and necessary early event that is temporally followed by ATM polarization and onset of insulin resistance.³³

In summary, the present study provides compelling evidence in support of an intimate mechanistic link between sleep integrity, in addition to sleep duration, and metabolic regulatory pathways. The current findings further highlight the role of preserved and intact sleep architecture in the regulation of oxidative stress pathways, whose homeostatic preservation emerges as an essential component for maintaining physiological metabolic functions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

SXLZ and AK performed the experiments and analyzed the data. YW conducted the experiments, analyzed the data, drafted portions of the manuscript and served as blinded observer. AC and FH performed GTT and ITT assays. AC and CH performed sleep recordings. BAN and MJB assisted with technical aspects of adipose tissue insulin sensitivity assays. ZQ performed the experiments, particularly immunohistochemistry. DG provided the conceptual design of the project, analyzed the data, drafted the manuscript and is responsible for the financial support of the project and the manuscript content. All authors have reviewed and approved the final version of the manuscript. DG is supported by National Institutes of Health grants HL-65270, HL-086662 and HL-107160. Dr Gozal is the guarantor of this work, had full access to all the data and takes full responsibility for the integrity of data and the accuracy of data analysis.

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