Detrimental metabolic effects of combining long term cigarette smoke exposure and high-fat diet in mice

Hui Chen¹, Michelle J Hansen²,³, Jessica E Jones²,³, Ross Vlahos²,³, Gary P Anderson²,³,⁴, Margaret J Morris¹

1. Department of Pharmacology, School of Medical Sciences, University of New South Wales, NSW 2052, Australia.
2. Department of Pharmacology, The University of Melbourne, Victoria 3010, Australia.
3. CRC for Chronic Inflammatory Diseases, The University of Melbourne, Victoria 3010, Australia.
4. Department of Medicine, The University of Melbourne, Royal Melbourne Hospital, Victoria 3050, Australia.

Running head: Combined effects of smoke exposure and high-fat diet

Corresponding author:

Professor Margaret J Morris
Department of Pharmacology,
University of New South Wales,
NSW 2052, Australia.
Telephone: 61 2 9385 1560
Fax: 61 2 9385 1059
Email: m.morris@unsw.edu.au
Abstract

Obesity and cigarette smoking are both important risk factors for insulin resistance, cardiovascular disease, and cancer. Smoking reduces appetite, which makes many people reluctant to quit. Few studies have documented the metabolic impact of combined smoke exposure (SE) and high-fat-diet (HFD). Neuropeptide Y (NPY) is a powerful hypothalamic feeding stimulator that promotes obesity. We investigated how chronic SE affects caloric intake, adiposity, plasma hormones, inflammatory mediators and hypothalamic NPY peptide in animals fed a palatable HFD.

Methods: Balb/c mice (5 weeks, male) were exposed to smoke (2 cigarettes, twice/day, 6 days/week, for 7 weeks) with or without HFD. Sham-exposed mice were handled similarly without SE. Plasma leptin, hypothalamic NPY, and adipose triglyceride lipase (ATGL) mRNA were measured.

Results: HFD induced a 2.3 fold increase in caloric intake, increased adiposity, and glucose in both sham and SE cohorts. SE decreased caloric intake by 23%, with reduced body weight in both dietary groups. Fat mass and glucose were only reduced by SE in the chow fed animals. ATGL mRNA was reduced by HFD in SE animals. Total hypothalamic NPY was reduced by HFD, but only in sham-exposed animals; SE increased arcuate NPY.

Conclusion: Although SE ameliorated hyperphagia and reversed the weight gain associated with HFD, it failed to reverse fat accumulation and hyperglycemia. The reduced ATGL mRNA expression induced by combined HFD and SE may contribute to fat retention. Our data support a powerful health message that smoking in the presence of an unhealthy western diet increases metabolic disorders and fat accumulation.

Key words: ATGL, appetite, leptin, NPY, TNFα
Introduction

Worldwide, 1.1 billion adults and 10% of children are now classified as overweight or obese (28). Excess bodyweight is the sixth most important risk factor contributing to the overall burden of disease. Overweight and obesity are predisposing factors for many metabolic diseases, including type 2 diabetes, gallbladder disease, non-alcoholic fatty liver, steatohepatitis, hypertension, cardiovascular disease and some forms of cancer associated with overweight (8). It has been suggested that poor diet and physical inactivity could soon replace smoking as the leading cause of avoidable death (36). However, human studies have shown that smoking itself is linked to the development of abdominal or central obesity (10).

Globally, about one third of male adults and one in five teenagers smoke, and smoking related diseases threaten the lives of one in ten adults; this is predicted to increase to one in six by 2030 if the current trend continues (55). Reduced appetite and body weight is one of the major motives for smoking cigarettes, especially among young women (2, 9, 23). Weight gain upon smoking cessation is a factor preventing people from giving up smoking, as over 75% of former smokers gain weight after quitting. Weight gain following cessation is primarily due to an increase in caloric intake, especially consumption of snack foods high in fat and sugar, a reduction in energy expenditure, and increased lipid accumulation (20, 25).

The health risks associated with diabetes and cardiovascular disease can be increased by smoking, as smokers display reduced high density lipoprotein cholesterol, higher triglyceride concentrations, and increased proinflammatory (e.g. TNFα, IL-6) and procoagulant markers (4, 21, 22, 31). However, in current heavy smokers, a lower mortality rate has been reported in
overweight, but not obese, subjects relative to those who never smoked (43). Furthermore, obesity has recently been recognized as a risk factor for inflammatory respiratory diseases (15, 35). TNFα causes insulin resistance in obese subjects by interfering with insulin receptor signaling, which may be partially due to the inhibition of adiponectin secretion and decreased glucose transporter, GLUT-4 (47, 50). A large proportion of circulating IL-6 is derived from adipose tissue, and levels correlate with fasting plasma glucose level and insulin resistance (5). Mobilization of fatty acids from triglyceride stores in adipose tissue requires lipolytic enzymes. Adipose triglyceride lipase (ATGL) is the only enzyme known to hydrolyze triglycerides expressed in both white and brown adipose tissue (58).

In our previous studies, mice exposed to either short or long term cigarette smoke showed markedly decreased food intake and weight gain accompanied by lower white fat mass (11). This was likely due to a cigarette smoke exposure induced reduction in hypothalamic neuropeptide Y (NPY), a robust central appetite stimulator. Changes in the expression of genes relevant to energy expenditure (uncoupling proteins (UCPs)), insulin sensitivity (TNFα, IL-6) and ATGL in fat pads may participate in this process (11). When facing a palatable high fat diet (HFD), mice spontaneously display hyperphagia, resulting in marked fat accumulation, and reduced hypothalamic NPY (39). In this study, we investigated whether cigarette smoke exposure would alter the weight gain and metabolic risk induced by a HFD. We hypothesized that in mice fed a HFD, cigarette smoke exposure would reduce caloric intake and body weight, accompanied by reduced fat accumulation, thereby reducing metabolic risk. As NPY is regulated by both cigarette smoke exposure and HFD, in this study we examined how brain NPY was reprogrammed when animals were exposed to both cigarette smoke and HFD. Here we
demonstrate that while smoke exposure caused weight loss, it did not reduce the metabolic changes induced by HFD, and preserved fat mass.

Methods

Animals

Because of the strain dependence of the development of respiratory disease following cigarette smoke exposure (53), male Balb/C mice were chosen for this experiment. Mice (aged 5 weeks) were obtained from the Animal Resource Centre Pty. Ltd. (Perth, Australia), housed at 20 ± 2°C in sterile micro-isolator cages, and maintained on a 12:12 h light/dark cycle (lights on at 06:00 h). They were allowed a week to adapt to their new environment, with *ad libitum* access to standard laboratory chow and water. Animals were monitored daily. The current study was approved by the Animal Experimentation Ethics Committee of the University of Melbourne.

Treatment

After acclimatization, mice were randomly divided into 4 groups with similar average body weight: sham exposed fed chow (sham+chow), sham exposed fed HFD (sham+HFD), smoke exposed (SE) fed chow (SE+chow), and SE fed HFD (SE+HFD). For smoke exposure, animals were placed inside a perspex chamber (18 litres) and exposed to the smoke produced by 2 cigarettes (Winfield Red, 16 mg or less of tar, 1.2 mg or less of nicotine and 15 mg or less of CO, Philip Morris, Melbourne, Australia), twice a day (10:30 h and 16:30 h), 6 days a week for 7 weeks (53). The sham exposed animals were handled identically, but were not exposed to cigarette smoke. Mice consumed either laboratory chow (3.54 kcal/g, fat 12%, protein 22%, carbohydrate 66%, chow fed cohort), or a HFD (4.32 kcal/g, fat 32% (saturated fat 17%), protein
18%, carbohydrate 50%, HFD fed cohort) consisting of modified laboratory chow containing sweetened condensed milk and lard, which was supplemented with highly palatable cafeteria style food such as meat pies, cakes, and biscuits (26, 27, 39). Fresh food was provided at 17:00 h daily. Body weight and 24-hour caloric intake were measured twice a week.

**Sample collection**

At the conclusion of the experiment, tissue was harvested from 9:00 h to 11:00 h. Animals received their last exposure to chamber or cigarette smoke at 16:30 h the day before dissection. Mice were given an anaesthetic overdose (ketamine/xylazine 180/32 mg/kg, intraperitoneal), blood was collected from the abdominal vena cava, and then mice were decapitated for brain and tissue collection. Prior to dissection body weight, naso-anal (N-A) length and tibia length were recorded. Lee index was calculated as body weight (g^{0.33}) / N-A length (mm). About 10 µl blood was used to measure blood glucose (Accu-Chek® Advantage glucose meter; Roche Diagnostics, Castle Hill, NSW) and carboxyhemoglobin (CoHb) (54). Separated plasma was stored at -80°C for subsequent determination of plasma leptin, insulin, corticosterone, and NPY.

The brain was rapidly micro-dissected on ice into 6 regions containing the paraventricular nucleus (PVN), arcuate nucleus (Arc), anterior and posterior hypothalamus (AH and PH), medulla, and amygdala. Briefly, in the section (approximately Bregma +0.5 mm to -1.3 mm), containing the PVN, AH and preoptic areas, the hypothalamic area was dissected by cutting laterally at the hypothalamic sulcus and dorsally at the top of the third ventricle, and hemisected, with the dorsal region containing the PVN and the ventral region containing the AH. The next section of the hypothalamic area (approximately Bregma -1.3 mm to -2.7 mm) was removed and
the Arc was then taken as a triangle from the ventral surface (2 mm base and 1 mm sides). The remaining hypothalamic area was termed the PH. The area postrema was identified and two cuts were made, 1 mm caudal and 1 mm rostral to the area postrema, representing the medulla. In the section (approximately Bregma +1.5 mm to +0.5 mm), the area containing the nucleus accumbens was removed and kept. These were then weighed, frozen on dry ice and stored at -80°C for later determination of peptide content. Body fat (interscapular brown adipose tissue (BAT), left retroperitoneal (Rp) white adipose tissue (WAT), and testicular WAT), liver, spleen, heart, and kidney were dissected and weighed.

BAT and RpWAT were snap frozen in liquid nitrogen and then stored at -80°C for later measurement of mRNA of UCP1, UCP3, TNFα, IL-6, and ATGL. A section of liver was fixed in 10% formalin for hematoxylin and eosin (HE) staining.

**HE staining of liver sections**

Sections of both left and middle liver lobes from each mouse were placed in formalin. Specimens were processed on an overnight cycle through graded ethanols and embedded in paraffin wax. Mounted tissues were then deparaffinised and rehydrated. Nuclei were stained with Harris haematoxylin, and cell cytoplasm was stained with eosin. Fatty change in the liver was graded by an observer blinded to the treatment groups, by assessing the amount and size of white vacuoles present throughout the stained sections (0 = normal; 1 = lipid vacuoles present within hepatocytes; 2 = increased vacuoles present within hepatocytes). The average of the grades within each treatment group was then calculated.
**Real-time PCR**

Total RNA was isolated from WAT and BAT using an RNeasy kit (Qiagen, Valencia USA) according to the manufacturer’s instructions. The purified total RNA was used as a template to generate first-strand cDNA synthesis using SuperScript III (Invitrogen, Carlsbad USA). Applied Biosystem probe/primers that were pre-optimised and validated were used for Quantitative real-time PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, Foster City USA) (7). Thus gene expression was quantified in a single multiplexing reaction, where our gene of interest (UCP1, UCP3, TNFα, IL-6, and ATGL) was standardized to control (18s rRNA). An individual BAT sample from the control sham group was then arbitrarily assigned as a calibrator against which all other samples are expressed as fold difference (7).

**Assays**

Endogenous NPY from the various brain regions was extracted by acetic acid. NPY-like immunoreactivity (LI) in brain and plasma was measured by radioimmunoassay using synthetic NPY as standard (10-1280 pg/tube, Auspep, Victoria, Australia) as described before (41). The detection limit for the radioimmunoassay was routinely 2 pg NPY per tube and the intra- and inter-assay coefficients of variation were 6% and 13%, respectively. NPY was expressed as ng NPY per mg tissue in each brain region and ng NPY in hypothalamus. Plasma leptin, insulin, and corticosterone concentrations were measured by using commercially available radioimmunoassay kits (leptin and insulin: Linco, Missouri, USA; corticosterone: MP Biomedicalals, Irvine, USA). Plasma triglyceride was measured using glycerol standard (equivalent to 0-8.46 mM triglyceride, Sigma, St. Louis, USA) and triglyceride reagent (Roche,
Nutley, USA). Briefly, samples and standards were incubated with triglyceride reagent at 37°C for 20 min, and read on a microplate reader (Bio-Rad 680XR, CA, USA) at 492 nm.

**Statistical analyses**

Results are expressed as mean ± S.E.M. Body weight and caloric intake over time were analyzed using one way analysis of variance (ANOVA) with repeated measures, followed by post hoc Fisher’s Least Significance Difference (LSD) tests. Differences in fat and organ weights, blood and plasma chemical concentrations, brain NPY concentration and content, and adipose mRNA expression were analyzed using two way ANOVA followed by post hoc Bonferroni tests. Difference in blood CoHb was analyzed using Student’s unpaired t-test. Difference in liver HE staining score was analyzed using Wilcoxon Signed Rank test.

**Results**

**Food intake and body weight trajectory**

During the run in period, there was no difference in caloric intake between experimental groups. Shortly after the dietary intervention started, the animals on a HFD (sham+HFD and SE+HFD groups) had a greater caloric intake than the chow fed animals (sham+chow and SE+chow). Smoke exposure reduced the caloric intake in both chow and HFD fed cohorts (Fig 1). A decrease in caloric intake occurred in the mice on chow diet within the first week of smoke exposure, and the intake of SE+chow appeared lower than the sham+chow group over the experimental period (Fig 1). However, this failed to reach statistical significance using ANOVA with repeated measures. In the animals consuming a HFD, cigarette smoke exposure led to a significant reduction in caloric intake from the fourth week, and a 36% reduction was observed.
at 7 weeks (SE+HFD group, Fig 1). Over the 7 week experimental period, HFD increased the average caloric intake by 2.29 fold in both the sham and SE cohorts (p< 0.05, compared with appropriate chow control groups, Table 1), while smoke exposure reduced the caloric intake by 23% in both SE+chow and SE+HFD animals (p< 0.05, compared with appropriate sham exposed control groups, Table 1). Caloric intake per gram of body weight was also significantly lower in smoke exposed animals, and higher in animals consuming HFD (p< 0.05, Table 1) over the 7 weeks. However, smoke exposed animals on HFD consumed 16% more fat than sham exposed animals on the same diet as a percentage of their total caloric intake (p< 0.05). Consumption of sweet foods (cake, biscuits) accounted for 26% (± 2.8%) of total energy intake of SE+HFD mice, while it was only 16% (± 2.4%) in sham+HFD animals (p< 0.05).

Body weight of all groups was well matched at the beginning of the experiment (Fig 2). Sham chow animals gained 30% body weight over the experiment. Body weight was significantly increased by HFD in both sham+HFD and SE+HFD groups. The net weight gain effect of HFD was greater in animals exposed to cigarette smoke (Fig 2). Body weight gain was decreased within the first week of cigarette smoke exposure in both SE+chow and SE+HFD groups, and this remained significant until the end of the experiment (p< 0.05, compared with appropriate sham exposed group, Fig 2). The body weight trajectory of the SE+HFD group was maintained below that of the sham+chow group (Fig 2).

**Body weight and organ weight at death**

At 7 weeks, body weight of the sham+HFD group was 8% greater than the sham+chow animals (p< 0.05, Table 1), and SE+HFD group was 13% greater in the SE+chow animals (p< 0.05,
Table 1). Smoke exposure caused a 15% reduction in body weight in the chow fed animals (p< 0.05, sham+chow vs. SE+chow groups), and an 11% reduction in the HFD fed animals (p< 0.05, sham+HFD vs. SE+HFD groups, Table 1).

N-A length was significantly increased by HFD in both sham exposed and SE animals (p< 0.05, Table1), while it was significantly decreased by smoke exposure in both dietary groups (p< 0.05, Table 1). Tibia length was significantly increased by HFD in sham exposed animals (p< 0.05, sham+chow vs. sham+HFD group, Table 1), while smoke exposure only reduced tibia length in the HFD fed animals (p< 0.05 sham+HFD vs. SE+HFD groups, Table 1). The Lee index was only reduced in SE + chow animals (p< 0.05, Table 1), suggesting growth is differentially affected by smoke exposure in low and high fat fed animals. HFD alone had no significant effect on the Lee index in this strain.

Liver mass was significantly reduced by smoke exposure in both chow and HFD fed cohorts (p< 0.05, Table 1). It was not obviously altered by HFD. HFD exposure increased kidney, heart, and spleen weights in both sham exposed and SE cohorts, while smoke exposure decreased the weights of these organs in both dietary cohorts (p< 0.05, Table 1). When organ weights were standardized by body weight, there were no differences between the experimental groups, except for a significantly reduced liver weight in the SE+HFD group compared with the SE+chow group (4.22 ± 0.06 vs. 4.52 ± 0.08% respectively, p< 0.05).

Consumption of HFD increased WAT mass in both sham exposed and SE cohorts, while smoke exposure only significantly reduced fat mass in the chow fed animals (p< 0.05, Table 1). The fat
mass determined using this method has been previously shown to be correlated with DXA fat mass and carcass lipid (46). When results were standardized for body weight, both RpWAT and testicular WAT masses were lower in the SE+chow animals compared to both sham+chow and SE+HFD groups (p< 0.05) (RpWAT: sham+chow 0.77 ± 0.05%, sham+HFD 0.90 ± 0.08%, SE+chow 0.48 ± 0.04%, SE+HFD 0.87 ± 0.05%; testicular WAT: sham+chow 3.04 ± 0.16%, sham+HFD 3.61 ± 0.32%, SE+chow 1.87 ± 0.17%, SE+HFD 3.52 ± 0.20%). BAT mass was increased by HFD and reduced by smoke exposure (p < 0.05, Table 1). The percentage of BAT was significantly increased by HFD, and was not altered by smoke exposure (sham+chow 0.34 ± 0.01%, sham+HFD 0.44 ± 0.02 %, SE+chow 0.36 ± 0.01%, SE+HFD 0.43 ± 0.02%).

**Blood CoHb and glucose and plasma triglyceride, leptin, insulin, corticosterone, and NPY concentrations**

Blood CoHb levels were increased by 9.6 fold in the SE animals (10.93 ± 0.01 vs. 1.03 ± 0.01% in the SE and sham exposed animals respectively, p< 0.05). Increased blood CoHb content in the SE mice suggested an appropriate level of smoke exposure (6).

The blood glucose levels were increased by HFD in animals that were either sham or smoke exposed (p< 0.05, Table 2); smoke exposure significantly reduced the blood glucose level only in the chow fed animals (p< 0.05, Table 2).

Plasma triglyceride levels were not significantly altered by either HFD or smoke exposure (Table 2). The HFD increased plasma leptin concentration by 46% in the sham+HFD animals (p< 0.05, sham+chow vs. sham+HFD groups), and almost doubled it in the SE animals (p< 0.05, SE+chow
vs. SE+HFD groups). Smoke exposure markedly reduced plasma leptin levels across both diet groups (p< 0.05, Table 2). Plasma insulin was not affected by HFD at 7 weeks, while it was significantly reduced by cigarette smoke exposure in the chow fed animals (p< 0.05, sham+chow vs. SE+chow groups, Table 2). Plasma corticosterone levels were not significantly altered by either HFD or smoke exposure (Table 2), suggesting the mice had no extra stress from exposure to cigarette smoke or cafeteria HFD. NPY is released from the sympathetic nerves and the adrenal medulla, and plasma levels change in response to various stress conditions (38, 40, 41, 59). The similar plasma NPY levels observed suggest 7 weeks of cigarette smoke and HFD exposure did not significantly affect the degree of sympathetic activation.

Liver HE staining

Some fat infiltration was observed in the liver from all four experimental groups, regardless of diet type and level of smoke exposure. However, livers from SE animals had higher scores than the appropriate sham exposed control animals (p< 0.05; SE+chow 1.17 ± 0.58 vs. sham+chow 0.70 ± 0.26, SE+HFD 0.92 ± 0.19 vs. sham+HFD 0.60 ± 0.27 arbitrary unit; n=16 per group). HFD did not increase lipid infiltration in either the sham or SE animals.

Gene expression

The mRNA expression of UCP1, UCP3 in BAT, and IL-6 in WAT were not significantly affected by either smoke exposure or HFD (Table 3). WAT TNFα mRNA expression was moderately increased in the SE+HFD animals (Table 3). BAT and WAT ATGL mRNA expression was significantly reduced by HFD in SE animals (p< 0.05, Fig 3). This trend was also observed in the sham exposed cohort.
**Brain NPY concentration and content**

Within the sham exposed cohort, NPY concentrations in the PVN and AH were significantly reduced in animals consuming the HFD, by 14% and 16% respectively \((p< 0.05, \text{Fig } 4)\), with an increase in the medulla \((p< 0.05, \text{Fig } 4)\). On the other hand, cigarette smoke exposure caused a 25% increase in Arc NPY concentration in the chow fed animals, and a 20% increase in the HFD fed animals \((p< 0.05, \text{Fig } 4)\). However, in the SE mice, consumption of a HFD did not result in any obvious change in NPY levels in the hypothalamus, medulla or amygdala \((\text{Fig } 4)\).

Thus smoke exposure appeared to partially reverse the reduction in hypothalamic NPY peptide in response to HFD \((\text{Fig } 5)\) as reflected in total hypothalamic NPY content, which was significantly decreased in the sham+HFD animals compared to the sham+chow group \((p< 0.05, \text{Fig } 5)\). Smoke exposure increased it by 13% within the HFD cohort \((p< 0.05, \text{Fig } 5)\).

**Discussion**

In the developed world, tobacco smoking and obesity are generally recognized as the most important modifiable factors responsible for excess mortality at the population level. This occurs across the lifespan, and a higher incidence of cardiovascular risk linked to insulin resistance was observed in young male smokers compared to non-smokers \((49)\). Both obesity and cigarette smoking are also important risk factors in many age related diseases, accelerating the ageing process via increasing oxidative stress and inflammation \((51)\). It has been hypothesized that weight loss associated with chronic obstructive pulmonary disease commonly observed in long term smokers is actually due to lean body mass loss \((32, 52)\). This study, for the first time,
examined the combined effects of these two detrimental behaviors, cigarette smoking and consumption of an energy dense, high fat diet.

Smoke exposure reduced linear growth regardless of diet type, whereas HFD feeding increased body length in both sham and SE animals. Cigarette smoke exposure also reduced the adiposity in chow fed animals as evaluated by the Lee Index, and this was reversed by HFD feeding. Rather than reducing the detrimental impact of HFD induced obesity, smoke exposure reduced weight gain without significantly reducing fat mass in the regions assessed. Nor was blood glucose reduced by smoking in the HFD fed animals. The lower body weight of SE mice on chow was accompanied by significantly smaller fat and organ masses, and lower plasma leptin and insulin concentrations, whereas hyperphagia, adipose accumulation, hyperleptinemia, and hyperglycemia were present in the SE mice eating the HFD, relative to control mice.

However, weight change in the smoke exposed animals does not seem to be merely secondary to a reduction in food intake, as our previous long term pair-feeding experiment in chow-fed animals showed that smoke exposure caused a greater reduction in weight gain compared with equivalent food restriction (12). The impact of smoke exposure on feeding behavior appeared independent of diet type, since the same proportional reduction in caloric intake was observed in both chow and HFD fed animals over 7 weeks. Furthermore, SE+chow animals consumed fewer calories both as net and proportional to their body weight, suggesting a state of negative energy balance. Interestingly, animals consuming the HFD exposed to smoke consumed 2.5 times the energy of the sham+chow animals, yet weighed a similar amount, probably reflecting fat accretion (Table 1). In other words, smoke exposure reduced WAT masses by 47% in chow fed
animals, but only 15% in HFD fed animals. Nicotine infusion stimulates lipolysis to increase fasting triglyceride levels in both humans and animals (3, 17, 48). However, in the current study, triglyceride level was not significantly affected by smoke exposure. Another explanation may be that the liver acts to buffer circulating lipid level through uptake (24), thus a difference in triglyceride level across the groups may not be observed. For the first time, enhanced liver lipid accumulation in response to smoke exposure was observed, regardless of diet type. The degree of fat loss induced by smoke exposure was discrepant between chow and HFD fed animals. Only the combination of smoke exposure and HFD significantly reduced ATGL gene expression in both WAT and BAT. In the long run, this might reduce the capacity for lipolysis, which may result in excessive fat accumulation. The lack of impact of HFD on triglyceride levels may be related to the strain of the mouse used in this study, as observed in a previous study (42).

In chow fed animals, smoke exposure was associated with lower circulating glucose and insulin levels, which may be linked to the observed weight loss. However, smoke exposure had minimal impact on the changes in plasma glucose induced by HFD. Exposure to environmental tobacco smoke has been shown to be associated with low insulin sensitivity, which was improved by smoking cessation (18, 29). Nicotine, carbon monoxide, or other agents in tobacco smoke have been speculated to directly contribute to impaired insulin sensitivity among smokers (19). Reduced blood flow due to prolonged smoking induced vascular changes, as well as muscle wasting, might decrease insulin-mediated glucose uptake in skeletal muscles (19). TNFα is a well known inflammatory factor that is linked to the onset of insulin resistance (30, 37). A modest elevation in WAT TNFα expression was observed, which may act to amplify this effect. The mRNA expression of IL-6, which is also involved in insulin resistance (45), showed a
similar trend as TNFα in SE+HFD animals. Whether these changes affect insulin sensitivity and glucose uptake needs further investigation. No changes in UCP1 and UCP3 mRNA expression in BAT were observed in this study, although significant changes in BAT mass were apparent. However, in our previous studies, BAT UCP1 was significantly reduced in pair fed mice with equivalent food restriction (11, 12), but this was partially restored by smoke exposure. This may suggest energy expenditure was enhanced in smoke exposed animals, which may also contribute to reduced weight gain.

The regulation of energy homeostasis involves several brain regions, including the hypothalamus, cortex, brain stem, and amygdala; however it is the hypothalamus that integrates many of the humoral and neural signals involved in appetite regulation (56). The Arc is one of the “circumventricular” organs in which the blood-brain barrier is specially modified and able to sense circulating peptides and proteins, including insulin and leptin. NPY-expressing neurons in the rodent hypothalamus are concentrated in the Arc, which send dense projections to other hypothalamic areas, such as the PVN, lateral hypothalamus, and dorsomedial hypothalamic nucleus. The PVN is a major integration site for inputs related to food intake and energy homeostasis (56). In the current study, reduced PVN and AH NPY concentration in the animals on a HFD alone was consistent with previous studies in mice and rats (26, 27, 39). Compared with our previous 4 week study (11), the SE+chow animals had lower caloric intake (23% reduction at 7 weeks vs 19% reduction at 4 weeks), greater weight loss (15% at 7 weeks vs 9% at 4 weeks), and larger plasma leptin reduction (38% at 7 weeks vs 18% at 4 week). This may explain why an increase in Arc NPY production was observed in the current study. Even in animals fed HFD, those exposed to smoke were hypophagic compared with their sham exposed
counterparts. In the state of long term negative energy balance observed in the current study, Arc NPY responses were activated in smoke exposed animals, which may also be a response to low plasma leptin concentrations. However, chronic smoke exposure reduced food intake despite increased Arc NPY. In a previous long term (12 weeks) pair feeding study, the increase in hypothalamic NPY in animals on equivalent food restriction was higher than that in the smoke exposed animals (12), which suggests smoke exposure blunted the normal alteration of NPY in response to caloric restriction. It has also been shown by others that the orexigenic effect of NPY can be blunted by nicotine injection (33). Downregulation of NPY Y1 receptor expression in both anterior and posterior hypothalamus was also observed upon nicotine injection with reduced binding sensitivity in the anterior hypothalamus (34), which may help to explain why increased Arc NPY in our study failed to reverse hypophagia in the smoke exposed animals, NPY in the amygdala is linked to anxiolytic-like effects (44), thus the unchanged NPY levels here might indicate no change in stress or anxiety responses across groups. The NTS in the medulla receives afferent inputs from the vagus nerve arising from the gastrointestinal tract, transferring peripheral satiety signals (1, 16, 57). The increased medullary NPY concentration induced by either HFD feeding or cigarette smoke exposure alone might reflect altered peripheral input in response to altered feeding states, or direct effects of nicotine or some other component of cigarette smoke.

Conclusion

The hyperphagia and weight gain induced by long term cafeteria HFD in mice were reduced by concomitant cigarette smoke exposure. In humans, the weight loss associated with smoking has been linked to reduced mortality, but only in overweight subjects (BMI < 30) (43). However,
results from the current study suggest that in obese mice, smoke exposure does not affect the other changes that would potentially lead to disorders of glucose and lipid metabolism. The weight loss induced by smoke exposure in animals consuming a HFD was not accompanied by marked fat loss when compared with chow fed animals; lean body mass, including skeletal muscle loss, may be the real contributor to their weight reduction. Muscle wasting is a critical issue in patients with long term respiratory disease, which exacerbates the disease and indicates a negative prognosis. Here smoking led to increased fat accumulation in the liver, suggesting a link with nonalcoholic fatty liver disease. This helps to explain the findings in human studies, where fasting glucose and lipid levels were significantly higher in smokers than in nonsmokers with similar BMI, and the duration of smoking and the number of cigarettes were positively correlated with the incidence of cardiovascular disease in smokers (17). Smoking and HFD are both risk factors for insulin resistance and type 2 diabetes, and putting these two behaviours together can increase the risk instead of ameliorating it. Smoking cessation is not only beneficial for lung function (13), but also reduces the risk of subsequent mortality by 36% among people with coronary disease (14). Our data suggest preferential retention of fat when smoking is combined with HFD, and this may provide a powerful health message.

Acknowledgment

This work was funded by the National Health and Medical Research Council of Australia
References:


35. **Mannino DM, Mott J, Ferdinands JM, Camargo CA, Friedman M, Greves HM, and Redd SC.** Boys with high body masses have an increased risk of developing asthma: findings


**Figure and table legend**

Fig 1: Caloric intake (kcal/mouse/24h) of the sham+chow (▲ n = 16), sham+HFD (■ n = 16), SE+chow (△ n = 16), and SE+HFD (□ n = 16) groups during the experimental period. Results are expressed as mean ± S.E.M. Data were analysed by one-way ANOVA with repeated measures followed by post hoc LSD tests.

* P< 0.05, diet effect, relative to chow fed animals.
‡ P< 0.05, SE effect, relative to sham exposed animals on the same diet.

Fig 2: Body weight of the sham+chow (▲ n = 16), sham+HFD (■ n = 16), SE+chow (△ n = 16), and SE+HFD (□ n = 16) groups during the experimental period. Results are expressed as mean ± S.E.M. Data were analysed by one-way ANOVA with repeated measures followed by post hoc LSD tests.

* P< 0.05, diet effect, relative to chow fed animals.
‡ P< 0.05, SE effect, relative to sham exposed animals on the same diet.

Fig 3: mRNA expression of ATGL (adipose triglyceride lipase) in WAT and BAT in sham+chow (open bar), sham+HFD (grey bar), SE+chow (stippled bar), and SE+HFD (black solid bar) groups at 7 weeks. Results are expressed as fold difference relative to a control BAT sample. Results are expressed as mean ± S.E.M. Data were analysed by two way ANOVA followed by a post hoc Bonferroni test.

* P< 0.05, diet effect, relative to chow fed animals.
Fig 4: Brain NPY-LI (expressed as ng/mg tissue) in the sham+chow (open bars n = 16), sham+HFD (grey filled bars n = 16), SE+chow (stippled bars n = 16), and SE+HFD (black filled bars n = 16) groups at 7 weeks. Areas shown are paraventricular nucleus (PVN), anterior hypothalamus (AH), arcuate nucleus (Arc), posterior hypothalamus (PH), medulla (Med), and amygdala (AMYG). Results are expressed as mean ± S.E.M. Data were analysed by two way ANOVA followed by post hoc Bonferroni tests.

* P< 0.05, diet effect, relative to chow fed animals.
‡ P< 0.05, SE effect, relative to sham exposed animals on the same diet.

Fig 5: Total hypothalamic NPY-LI in the sham+chow (open bars n = 16), sham+HFD (grey filled bars n = 16), SE+chow (stippled bars n = 16), and SE+HFD (black filled bars n = 16) groups at 7 weeks. Results are expressed as mean ± S.E.M. Data were analysed by two way ANOVA followed by a post hoc Bonferroni test.

* P< 0.05, diet effect, relative to chow fed animals.
‡ P< 0.05, SE effect, relative to sham exposed animals on the same diet.

Table 1: Results are expressed as mean ± S.E.M. Data were analysed by two way ANOVA with post hoc Bonferroni tests.

* p< 0.05, diet effect, relative to chow fed animals. ‡‡ p< 0.05, SE effect, relative to sham exposed animals on the same diet.

Lee index: body weight (g^{0.33})/N-A length (mm)

Table 2: Results are expressed as mean ± S.E.M. Data were analysed by two way ANOVA with post hoc Bonferroni tests.
* p< 0.05, diet effect, relative to chow fed animals.†
‡ p< 0.05, SE effect, relative to sham exposed animals on the same diet.

Table 3: Results are expressed as fold difference relative to a control sample, mean ± S.E.M.
Data were analysed by two way ANOVA.
BAT: brown adipose tissue; UCP: uncoupling protein; WAT: white adipose tissue.
Total hypothalamic NPY content (ng)

- sham+chow
- sham+HFD
- SE+chow
- SE+HFD

* denotes statistical significance.
Table 1 Body weight, food intake, organ and fat mass, and length in mice

<table>
<thead>
<tr>
<th></th>
<th>Sham+chow (n = 16)</th>
<th>Sham+HFD (n = 16)</th>
<th>SE+chow (n = 16)</th>
<th>SE+HFD (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>26.4 ± 0.3</td>
<td>28.3 ± 0.6 *</td>
<td>22.4 ± 0.3 ‡</td>
<td>25.2 ± 0.3 *‡</td>
</tr>
<tr>
<td>Average food intake</td>
<td>12.2 ± 0.4</td>
<td>39.9 ± 1.2 *</td>
<td>9.4 ± 0.4 ‡</td>
<td>30.7 ± 1.3 *‡</td>
</tr>
<tr>
<td>(kcal/mouse/24h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food intake/BW</td>
<td>0.47 ± 0.02</td>
<td>1.56 ± 0.06 *</td>
<td>0.41 ± 0.02 ‡</td>
<td>1.30 ± 0.08 *‡</td>
</tr>
<tr>
<td>(kcal/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (mg)</td>
<td>1179.6 ± 33.2</td>
<td>1234.6 ± 31.1</td>
<td>1012.8 ± 22.7 ‡</td>
<td>1062.8 ± 17.4 ‡</td>
</tr>
<tr>
<td>Kidney (mg)</td>
<td>177.4 ± 4.6</td>
<td>194.0 ± 4.2 *</td>
<td>152.6 ± 5.0 ‡</td>
<td>165.8 ± 2.8 *‡</td>
</tr>
<tr>
<td>Heart (mg)</td>
<td>108.9 ± 2.6</td>
<td>118.7 ± 1.9 *</td>
<td>94.5 ± 1.8 ‡</td>
<td>103.2 ± 1.7 *‡</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>85.4 ± 1.6</td>
<td>101.0 ± 2.0 *</td>
<td>72.6 ± 2.1 ‡</td>
<td>79.4 ± 2.0 *‡</td>
</tr>
<tr>
<td>BAT (mg)</td>
<td>90.3 ± 2.2</td>
<td>126.4 ± 6.3 *</td>
<td>80.5 ± 3.2 ‡</td>
<td>109.1 ± 4.4 *‡</td>
</tr>
<tr>
<td>RpWAT (mg)</td>
<td>203.8 ± 12.2</td>
<td>261.6 ± 27.4 *</td>
<td>108.2 ± 10.2 ‡</td>
<td>219.6 ± 14.8 *</td>
</tr>
<tr>
<td>Testicular WAT (mg)</td>
<td>801.1 ± 45.1</td>
<td>1047.5 ± 108.0 *</td>
<td>419.5 ± 39.0 ‡</td>
<td>893.0 ± 58.1 *</td>
</tr>
<tr>
<td>N-A length (cm)</td>
<td>9.46 ± 0.04</td>
<td>9.69 ± 0.05 *</td>
<td>9.16 ± 0.04 ‡</td>
<td>9.35 ± 0.04 *‡</td>
</tr>
<tr>
<td>Tibia (cm)</td>
<td>1.92 ± 0.02</td>
<td>2.00 ± 0.02 *</td>
<td>1.87 ± 0.02</td>
<td>1.88 ± 0.02 ‡</td>
</tr>
<tr>
<td>Lee index</td>
<td>3.14 ± 0.01</td>
<td>3.15 ± 0.02</td>
<td>3.07 ± 0.01 ‡</td>
<td>3.14 ± 0.01*</td>
</tr>
</tbody>
</table>
Table 2 Blood glucose, plasma leptin, insulin, corticosterone, and NPY concentrations in mice

<table>
<thead>
<tr>
<th></th>
<th>Sham+chow (n=16)</th>
<th>Sham+HFD (n=16)</th>
<th>SE+chow (n=16)</th>
<th>SE+HFD (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>9.29 ± 0.32</td>
<td>10.66 ± 0.37 *</td>
<td>7.88 ± 0.39 ‡</td>
<td>9.88 ± 0.35 *</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>0.77 ± 0.03</td>
<td>0.67 ± 0.04</td>
<td>0.77 ± 0.04</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td>Plasma leptin (ng/ml)</td>
<td>6.20 ± 0.58</td>
<td>9.04 ± 0.84 *</td>
<td>3.83 ± 0.39 ‡</td>
<td>7.40 ± 0.44 *‡</td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>0.55 ± 0.07</td>
<td>0.49 ± 0.03</td>
<td>0.41 ± 0.04 ‡</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>Plasma corticosterone (ng/ml)</td>
<td>221.2 ± 25.9</td>
<td>198.2 ± 26.9</td>
<td>281.8 ± 45.9</td>
<td>231.1 ± 34.5</td>
</tr>
<tr>
<td>Plasma NPY (ng/ml)</td>
<td>8.33 ± 1.12</td>
<td>9.78 ± 1.15</td>
<td>7.20 ± 0.47</td>
<td>8.15 ± 0.48</td>
</tr>
</tbody>
</table>
Table 3 mRNA expression of UCP1 and UCP3 in BAT, TNFα, and IL-6 in WAT at 7 weeks

<table>
<thead>
<tr>
<th></th>
<th>Sham+chow (n = 4)</th>
<th>Sham+HFD (n = 4)</th>
<th>SE+chow (n = 4)</th>
<th>SE+HFD (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAT UCP1</td>
<td>1.18 ± 0.15</td>
<td>1.25 ± 0.09</td>
<td>0.98 ± 0.09</td>
<td>1.20 ± 0.10</td>
</tr>
<tr>
<td>BAT UCP3</td>
<td>1.37 ± 0.25</td>
<td>1.37 ± 0.26</td>
<td>2.05 ± 0.44</td>
<td>1.63 ± 0.31</td>
</tr>
<tr>
<td>WAT TNFα</td>
<td>2.54 ± 0.56</td>
<td>1.86 ± 0.33</td>
<td>1.76 ± 0.32</td>
<td>4.19 ± 1.14</td>
</tr>
<tr>
<td>WAT IL-6</td>
<td>2.04 ± 0.65</td>
<td>2.23 ± 0.53</td>
<td>2.23 ± 0.47</td>
<td>3.26 ± 0.57</td>
</tr>
</tbody>
</table>