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RESEARCH PAPER

Diet-induced obesity increases NF-κB signaling in reporter mice

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Abstract The nuclear factor (NF)-κB is a primary regulator of inflammatory responses and may be linked to pathology associated with obesity. We investigated the progression of NF-κB activity during a 12-week feeding period on a high-fat diet (HFD) or a low-fat diet (LFD) using NF-κB luciferase reporter mice. In vivo imaging of luciferase activity showed that NF-κB activity was higher in the HFD mice compared with LFD-fed mice. Thorax region of HFD females displayed fourfold higher activity compared with LFD females, while no such increase was evident in males. In male HFD mice, abdominal NF-κB activity was increased twofold compared with the LFD males, while females had unchanged NF-κB activity in the abdomen by HFD. HFD males, but not females, exhibited evident glucose intolerance during the study. In conclusion, HFD increased NF-κB activity in both female and male mice. However, HFD differentially increased activity in males and females. The moderate increase in abdomen of male mice may be linked to glucose intolerance.

Keywords High-fat diet · Luciferase · Inflammation · Glucose intolerance · Molecular imaging

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Introduction

Obesity is a disease affecting increasing numbers of global populations, in some regions more than 30% of the adult population [1]. Increased adipose tissue mass, especially in the abdominal region, is associated with diseases such as type-2 diabetes and atherosclerosis [2]. It has been demonstrated that increased fat mass is associated with increased macrophage infiltration, increased release of cytokines, adipokines and free-fatty acids from adipocytes and/or activated macrophages, and local insulin resistance [3–6]. Adipose tissues also serve endocrine functions whereby adipokines and free-fatty acids are released into the circulation [7]. This allows transport to the liver and skeletal muscle, often promoting reduced insulin sensitivity in these organs [8]. Obesity and specifically the enlargement of the abdominal adipose depots are thus considered the major risk factors for the development of insulin resistance, a characteristic feature of type-2 diabetes and the metabolic syndrome [9].

A central mediator of inflammatory and stress responses is the NF-κB family of transcription factors. As a response to foreign pathogens and general stressful insults, NF-κB is activated in most cell types. In addition, NF-κB activity is linked to cancer development through its regulation of apoptosis, cell proliferation, angiogenesis, metastasis and cell survival [10]. Recent evidence also suggests that NF-κB activation is crucial for development of insulin resistance. For example, Arkan et al. [11] disabled the inflammatory pathway within macrophages by creating myeloid-specific IκB kinase β (IKKβ) knockout mice. These mice were more insulin sensitive and partially protected from high-fat diet (HFD)-induced glucose intolerance and hyperinsulinemia. Moreover, Cai et al. [12] reported that the activation of NF-κB in transgenic mice

expressing constitutively active IKKβ in hepatocytes (LIKK mice) lead to hyperglycemia and insulin resistance. Conversely, hepatocyte-specific deletion of NEMO (IKK-γ), which completely blocks NF-κB activation, protected against insulin resistance in mice-fed HFD [13]. Furthermore, pharmacological manipulations of the NF-κB system by administering salicylates revealed that these transcription factors are central to the obesity-induced proinflammatory state leading to metabolic syndrome, insulin resistance and type-2 diabetes [14, 15].

Although a number of studies have explored the effect of obesity on inflammatory mediators, surprisingly few studies have directly compared activation of NF-κB itself in obese individuals compared with lean controls. The dynamic regulation of NF-κB activity during weight gain is thus unknown, and it is not known whether increased NF-κB signaling is presented before, simultaneously, or after metabolic parameters are affected. More specifically, the time course of inflammation induced in parallel with obesity on an HFD has not been elucidated, including the organs involved, nor the dynamics of inflammation development.

To address these questions, we have utilized transgenic mice harboring a luciferase gene specifically controlled by 3 NF-κB DNA-binding sites [16–18]. Thus, the luciferase activity directly reflects NF-κB transactivation due to the activation of the NF-κB signaling pathways. One group of NF-κB luciferase mice was switched to HFD, whereas another control group was maintained on LFD. NF-κB activity was monitored as luciferase activity by non-invasive in vivo molecular imaging. In this paper, we analyzed for the first time in a non-invasive manner the in vivo NF-κB response to HFD over time.

Materials and methods

NF-κB luciferase reporter mice

Transgenic mice carrying a transgene with three binding sites for NF-κB (5′GGGACTTTCC′3) coupled to the luciferase gene were used in this study. The transgene is flanked by insulator sequences from the chicken [β]-globin gene [19] to reduce interference from the genome. The resulting transgenic founder, which was the basis of these experiments, was the result of screening of several founders. The original genetic background was a mix of C57BL/6J and CBA. Subsequently, the mice were backcrossed four times with C57BL/6J prior to this experiment. The mice were housed in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

Animal experiments

All animal experiments were performed according to the national guidelines for animal welfare. Mice were matched according to age (average age 17 weeks), and dedicated to two dietary groups fed either HFD or LFD. Both groups were initially maintained on the LFD, a regular chow diet from SDS diets (RM3-SDS Diets, Scanbur, Nittedal, Norway). Eight mice (4 females, 4 males) were designated to the HFD group and nine mice (5 females, 4 males) to the LFD group. HFD contained 23% fat (45% of energy intake) and LFD contained ~3% fat (Table 1). We performed glucose tolerance tests after 6 and 11 weeks, by injecting D-glucose (2 g/kg body weight), i.p., D-glucose was dissolved in phosphate buffered saline (30% solution) and injected once at $t = 0$. Blood glucose concentrations were measured with a blood glucose meter (Accu-Chek®, Aviva, Roche Diagnostics, Mannheim, Germany) in blood obtained from the tail vein of anesthetized mice every 30 min up to 2 h.

In vivo imaging of NF-κB activity

In vivo imaging was performed in an IVIS 100 System (Xenogen. Alameda, CA). Mice were anesthetized using 2.5% isoflurane, and ventral fur was removed by shaving. D-luciferin (Biosynth, Staad, Switzerland) (160 mg/kg) in PBS was injected, i.p., and mice were placed in a light-proof chamber under a light-sensitive camera. After 8–10 min of luciferin injection, the luminescence emitted ventrally from the mice was monitored for typically 2 min. The luminescence (photons/s/cm^2/steradian) was quantified

Table 1 Composition of diets (% of food weight; w/w)

	HFD	LFD
Carbohydrates	35.4	39.7
Starch	15	33.9
Sugar	20.4[a]	5.8
Fat	23.0	4.2
C12:0	0.072	0.05
C14:0	0.81	0.2
C16:0	6.2	0.36
C18:0	5.1	0.009
C16:1	0.77	0.013
C18:1	8.88	1.03
C18:2 ($n-6$)	0.77	1.15
C18:3 ($n-3$)	0.19	0.17
C20:4 ($n-6$)	0.26	0.22
Protein	21.4	22.4

[a] Cerelose® (dextrose), *HFD* high-fat diet, *LFD* low-fat diet

using the Living Image Software (Xenogen). Females and males in the LFD and HFD groups were imaged separately and each time in the same order, once every 7 days for 12 weeks. Thus, each capture contained the in vivo image of four to five mice. Luminescence emitted from the whole body of the mice, as well as the thoracic and abdominal body regions, was quantified by defining three different regions of interest (ROIs) (Fig. 1).

Plasma analyses

Blood samples were taken at $t = 0$, 5 and 9 weeks after the start of the experiment. The saphena vein was first exposed by shaving the skin and then punctured allowing the blood to be collected from the surface of the skin into EDTA-coated capillaries. Plasma was isolated after centrifugation for 10 min at 6,000*g* and stored at −70°C. Concentrations of MCP-1, IL-6, TNF-α insulin, leptin, PAI-1 and resistin were determined in the isolated plasma with a multiplexed immunoassay (Mouse Adipokine LINCOplex kit; Millipore, Billerica, MA) according to the manufacturer's instructions on a Luminex instrument (BioRad, Hercules, CA). Plasma TNF-α levels were below the detection limit of the assay.

Statistical analysis

Data were analyzed using the SPSS software package (SPSS 15 for Windows, Chicago, IL). Comparisons of repeated measurements between groups were conducted with mixed model analysis using the Toeplitz covariance structure. Student's *t* test was used to compare groups at individual time points. The direction and strength of linear relationships between variables were evaluated using Pearson correlation coefficient and two-tailed test of significance.

Results

Elevated whole body NF-κB activity in mice-fed HFD

Male and female NF-κB reporter mice were separated in two groups, and fed either HFD (4 females and 4 males) or LFD (5 females and 4 males) for 12 weeks. The LFD was a regular chow control diet, and both groups were maintained on this diet prior to the onset of the experiment. To test whether HFD might increase NF-κB activity in vivo**,** we assessed NF-κB reporter gene activity longitudinally by non-invasive imaging. Ventral assessment of photon flux from the whole body showed that NF-κB activity in mice-fed HFD as compared to LFD was significantly higher ($P = 0.001$) (Fig. 2), although both HFD and LFD displayed a persistent increase in NF-κB-mediated luminescence (Fig 2). After 12 weeks, NF-κB activity in mice on HFD increased 3.5-fold compared with baseline ($t = 0$) values, whereas LFD increased 2.3-fold. Thus, NF-κB had increased more in the HFD group as compared to the LFD group at 12 weeks ($P = 0.012$). These results suggest that NF-κB activity increases in a time-dependent manner, and we cannot exclude that this increase represents an aging effect (cf. LFD group), and that HFD feeding enhances whole body NF-κB activity.

To discriminate various body regions of the mice, we assessed the photon flux from the abdominal and the thoracic regions. Furthermore, we sub-grouped the data and analyzed female and male mice separately. The largest

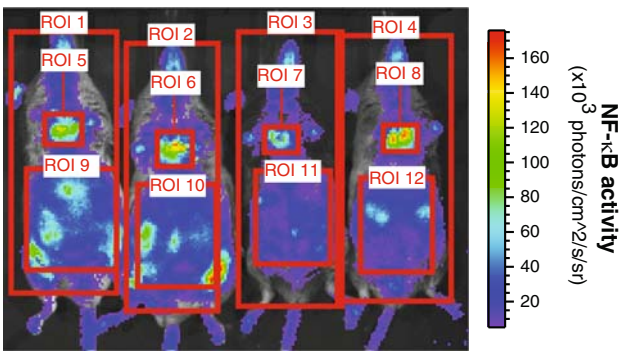

Fig. 1 In vivo imaging analysis of anesthetized mice harboring a luciferase transgene controlled by NF-κB DNA-binding sites. The figure shows a representative capture of four reporter mice. The heat map is a two-dimensional representation of light emitted ventrally from the mice after luciferin injection. The *red squares* are examples of regions of interest (ROIs) set during the image analysis to quantify NF-κB activity in different body regions: whole body (ROI 1–4); thorax (ROI 5–8), and abdomen (ROI 9–12)

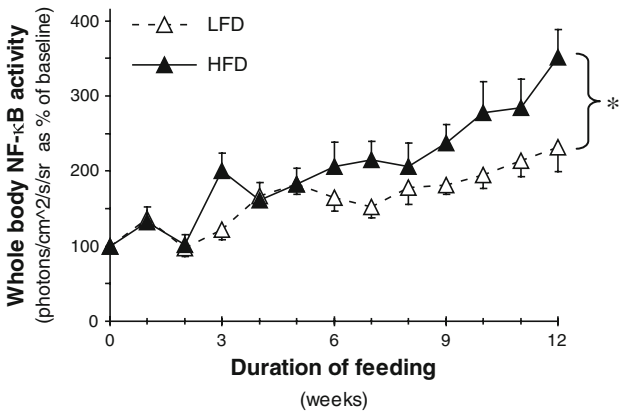

Fig. 2 Whole body in vivo NF-κB activity as measured from the entire ventral side every week in both female and male reporter mice fed either high-fat diet (HFD; *closed triangles*; $n = 8$; mean + SEM) or a low-fat diet (LFD; *open triangles*; $n = 9$; mean–SEM) for 12 weeks. *Significantly different by mixed model analysis taking the whole feeding period into account

effect of high-fat feeding was observed in the thoracic region of HFD female mice, which displayed a significant ($P = 0.012$) increase in NF-κB activity as compared to the LFD females (Fig. 3a). The difference in mean NF-κB activity increased gradually during the feeding period and from week 5 until week 12 there was a fourfold difference between the HFD and LFD female mice. Interestingly, in male mice, the thoracic NF-κB activity of HFD mice was slightly lower than in the LFD group.

In the abdominal region, HFD male mice displayed a twofold increase in NF-κB activity compared with LFD male mice ($P = 0.002$). In female mice, however, no such difference was found in the abdominal region between the two feeding groups (Fig. 3b). Thus, these results indicate that the high-fat feeding increases NF-κB activity differentially in the abdominal and thoracic region in a sex-specific manner.

To assess whether the observed local inflammatory NF-κB response translates into a systemic inflammatory response, we also measured the concentrations of plasma markers of inflammation after 5 and 9 weeks of diet feeding. After 5 weeks, mean plasma IL-6 concentration in HFD mice tended to be higher (fourfold; $P = 0.172$) than in mice-fed LFD (Fig. 4), an effect that was sex-independent and transient because IL-6 levels were similar for LFD and HFD after 9 weeks (Fig. 4). Furthermore, MCP-1 plasma concentrations were unaltered after 5 and 9 weeks of high-fat feeding (Fig. 4) suggesting that the enhanced tissue-associated NF-κB activity is only partially reflected in systemic increases in plasma cytokine levels.

Thoracic NF-κB activity associated with relative body weight gain in HFD mice

Female as well as male mice in both feeding groups gained body weight during the experimental period (Fig. 5a). After 12 weeks, male and female mice in the HFD group had increased their body weight compared with the baseline by 16 and 30%, respectively (Fig. 5a). Because HFD females increased their body weight relatively more than HFD males; and at the same time showed an increased NF-κB activity in the thoracic region, we tested if there was a relationship between relative body weight gain and thoracic NF-κB activity at the end of the experimental period. A positive correlation ($R = 0.886$, $P = 0.003$) was observed between relative increase in body weight from baseline and thoracic NF-κB activity in HFD (Fig. 5b).

The enhanced body weight was also manifested in two- to threefold ($P < 0.01$) increased plasma leptin levels in HFD compared with LFD after 5 and 9 weeks of feeding in both sexes (Fig. 5c).

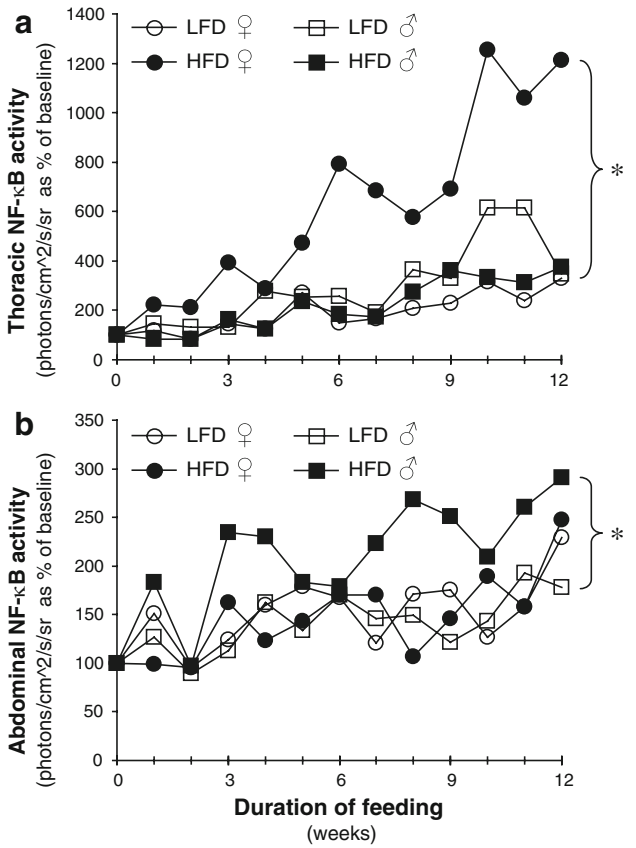

Fig. 3 Analysis of sex-specific NF-κB activity in different anatomical regions: **a** thorax and **b** abdomen. Each data point represents means at a given time for either the female (*circles*) or male (*squares*) reporter mice fed with high-fat diet (HFD; *closed symbols*) or low-fat diet (LFD; *open symbols*). *Significantly different by mixed model analysis taking the whole feeding period into account

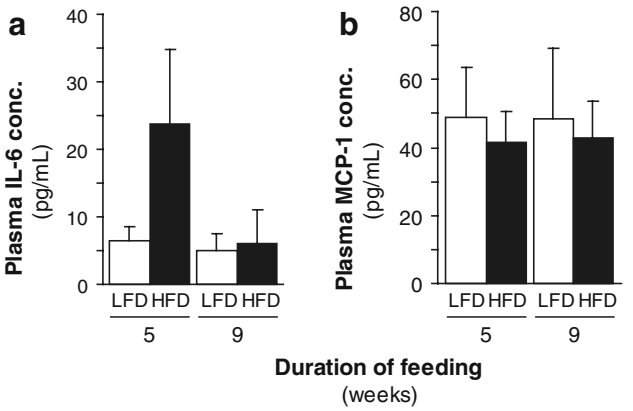

Fig. 4 Effect of high-fat diet (HFD; *closed bars*) versus low-fat diet (LFD; *open bars*) on plasma. **a** IL-6 and **b** MCP-1 concentrations (mean ± SEM) in the NF-κB reporter mice after 5 and 9 weeks of feeding

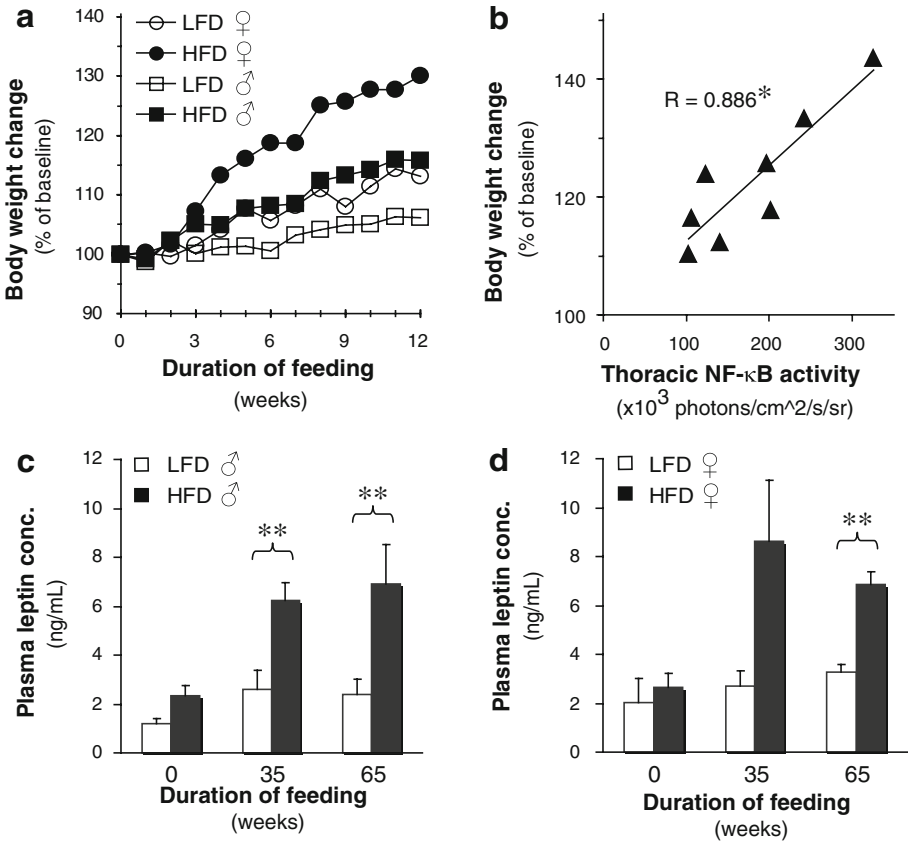

Fig. 5 **a** Body weight change in the NF-κB reporter mice from weeks 1–12, relative to body weight at baseline (week 0; set to 100%) Female (*circles*) and male (*squares*) mice were fed either the high-fat diet (HFD; *closed symbols*) or the low-fat diet (LFD; *open symbols*). **b** There was a significant positive correlation between body weight change and thoracic NF-κB activity in both female and male mice in the HFD group. Effect of the high-fat diet (HFD; *closed bars*) versus the low-fat diet (LFD; *open bars*) on plasma leptin concentrations (mean ± SEM) in **c** male and **d** female NF-κB reporter mice after 0, 5 and 9 weeks of feeding. $*P < 0.01$

Abdominal NF-κB activity in male HFD mice associated with glucose intolerance

Numerous studies have suggested a link between increased NF-κB activity and the development of insulin resistance. By week 6 of feeding, HFD male mice were glucose intolerant ($P = 0.001$), as inferred from intra-peritoneal glucose tolerance testing (IP-GTT) (Fig. 6a). A similarly pronounced glucose intolerance in the HFD males was also observed after 11 weeks of feeding ($P = 0.005$; data not shown). In contrast, HFD female mice did not develop glucose intolerance after 6 weeks ($P = 0.981$) or 11 weeks ($P = 0.293$; data not shown) of feeding (Fig. 6b).

We next investigated whether the elevated NF-κB activity in male mice given HFD correlated with development of insulin resistance, but no correlation was found between abdominal NF-κB activity and glucose intolerance in HFD after 6 ($R = -0.222$; $P = 0.598$) or 11 ($R = 0.111$; $P = 0.793$) weeks, as inferred from the area under the curve of the IP-GTT. The difference in metabolic status between males and females was further supported by data showing that HFD male mice had twofold higher resistin levels as compared to LFD males ($P = 0.005$), an effect not paralleled in female mice (Fig. 6c). Taken together, these data indicate that only males fed with HFD developed glucose intolerance (Fig. 6a), and that the individual NF-κB activity does not correlate with the individual degree of glucose intolerance achieved by HFD feeding.

Discussion

In our present study, we have for the first time followed the in vivo NF-κB activity non-invasively over time in experimental animals fed with HFD and LFD. We observed that mice-fed LFD increased their whole body NF-κB activity 2.3-fold above the baseline values after 12 weeks. At the same time point, mice-fed HFD displayed about 3.5-fold increased whole body NF-κB activity. This shows that basal NF-κB activity increases in a time-dependent manner in mice ingesting a regular laboratory diet containing low amounts of fat, whereas a diet high in fat content adds to this effect.

The HFD-evoked increase in NF-κB activity was not uniform, but was sex-dependently localized to different body regions. Females, but not males, displayed increased NF-κB activity in the thoracic region. Conversely, the abdominal area of males, but not females, showed enhanced NF-κB activity in response to HFD. Plasma levels of inflammatory cytokines were not enhanced by HFD suggesting that these inflammatory processes are

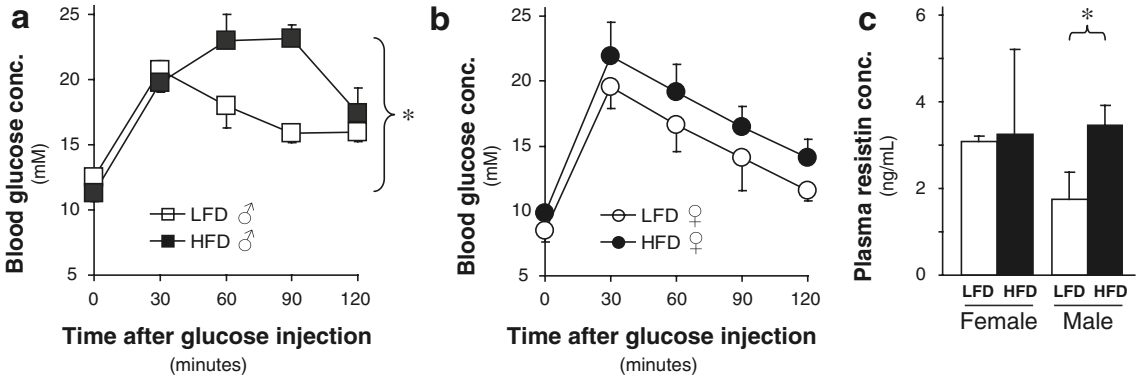

Fig. 6 Glucose tolerance test performed in **a** male and **b** female NF-κB mice after 6 weeks on the high-fat diet (HFD; *closed symbols*) or the low-fat diet (LFD; *open symbols*). Blood glucose concentrations were measured at different time points after a single bolus of glucose, injected, i.p., immediately after the baseline measurement (0 min). **c** Effect of the high-fat diet (HFD; *closed bars*) versus the low-fat diet (LFD; *open bars*) on plasma resistin concentrations (mean ± SEM) in the NF-κB reporter mice after 9 weeks of feeding. *Significantly different by mixed model analysis taking all time points into account. $^{**}P < 0.01$ using *t* test

localized to individual tissues rather than at the systemic level.

We observed increased NF-κB activity in the thoracic region of HFD-fed female mice, indicative of increased activity in the thymus and/or thoracic lymph nodes (i.e. axillary, brachial lymph nodes). To what extent HFD may influence and even increase NF-κB activity in these lymphoid tissues is not clear from our studies. Previous experiments indicate that mice-fed HFD have larger thymuses than mice fed with a control diet [20]. Moreover, leptin-deficient ob/ob mice have highly involuted thymuses, and leptin can attenuate LPS-induced and starvation-induced thymic involution [21–23]. Thus, one possibility is that the enhanced thoracic NF-κB activity in the female HFD groups may be explained by elevated leptin levels. In the current study, females gained more weight relative to baseline values than males and it was evident that NF-κB activity showed a stronger association with body weight gain, rather than a high-fat intake per se.

It has been suggested that the ingestion of HFD may induce low-grade inflammation in adipose tissue [24]. Especially, the adipose tissue located in the abdominal region has been linked to the development of diseases such as type-2 diabetes and atherosclerosis [2]. We hypothesized, therefore, that the abdominal region would be the major site of HFD-induced NF-κB activity associated with glucose intolerance. We did observe an increase in abdominal NF-κB activity in response to HFD, but the effect was restricted to male mice and was rather modest compared with the thoracic activity in females. We also found that only males developed glucose intolerance and displayed elevated levels of plasma resistin, indicative of the development of diabetes type 2. Altogether, these data suggest a link between increased NF-κB activity and glucose intolerance. However, we could not find any intra-individual correlation between abdominal NF-κB and parameters of glucose intolerance, possibly due to the limited number of animals.

Owing to the scattering of light from the inmost organs, it is difficult to judge the exact origin of the luciferase signal. By visual inspection, it is most likely that the activity arises in visceral fat and/or mesenteric lymph nodes, and not from liver. Abdominal NF-κB activity may be due to immune cells invading the adipose tissue depots or an alteration of the microbial environment of the colon [25, 26]. Alternatively, enhanced NF-κB activity may be due to HFD-induced activation of T cells in the mesenteric lymph nodes surrounded by visceral fat. A recent work demonstrated that HFD induces atrophy of mesenteric lymph nodes, explained by inflammation-induced stimulation of T cells [27].

Previous studies utilizing electrophoresis mobility shift assays (i.e. NF-κB DNA binding assay) or phosphorylation assays (NF-κB activation) have suggested enhanced NF-κB activation in obese individuals and experimental animals fed with HFD. For example, NF-κB DNA binding is increased about twofold in the liver, hypothalamus and skin of rodents fed with HFD for 6 months compared with animals fed with a control diet [12, 28]. NF-κB was similarly elevated in the liver and skin in common genetic obesity models of genetic hyperphagia (*ob/ob* mice and *fa/fa* rats) [12, 29]. Furthermore, peripheral blood mononuclear cells from obese human subjects have been shown to express enhanced nuclear NF-κB DNA binding [30].

It is important to note that HFD and obesity typically induce activation of NF-κB about twofold, which is much lower that the 10–100-fold activation typical of acute inflammatory reactions. This is consistent with the view of obesity as a chronic low-grade inflammatory condition.

Increased NF-κB activation in aged animals has been observed previously. Helenius [31] detected increased NF-κB DNA binding in heart, liver, kidney and brain of older mice and rats, as compared to younger animals, whereas Spencer et al. found increased NF-κB activation in splenic macrophages and lymphocytes of aged mice [32].

Although these previously published studies have assessed NF-κB activation measured as NF-κB DNA binding or phosphorylation of NF-κB components, none of the previous studies have analyzed NF-κB transactivation. The use of reporter constructs, such as the NF-κB luciferase transgene, we have used in the present reporter mice enables direct analysis of NF-κB transactivation. Thus, the luciferase reporter measures the integrated effects of different protein modifications regulating the NF-κB signal transduction pathway leading to DNA binding and transcriptional regulation, as well as the effects of other genetic and epigenetic factors affecting NF-κB signaling.

Reporter mice are particularly useful for analyzing gene regulation over time in a physiological context as opposed to cell cultures. Our in vivo model is also ideally suited to take into account absorption efficiency, transport in blood or other extracellular fluids, and cellular uptake, metabolism and degradation. Furthermore, the present technology also provides the possibility for elucidating the full anatomical expression profile of the regulatory module of interest. The recent description and validation of reporter mice open new horizons for nutrition research and drug discovery because these novel animal models provide a global view of gene expression following acute, repeated or chronic dietary or pharmacological treatment.

In summary, we are the first to report a dynamic assessment of NF-κB activity as a function of high versus low-fat feeding. The results show that NF-κB activity is more elevated in mice-fed HFD. We find that weight gain in HFD mice may be a strong predictor of NF-κB activity in the thoracic region of female mice. Moreover, male mice displayed a modest, but significant increase in abdominal NF-κB activity possibly derived from abdominal adipose tissue depots.

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Conflict of interest statement H.C. and R.B. are shareholders in the company Cgene with the commercial rights to the NF-κB-luciferase reporter mice. The authors have full control of all primary data and agree to allow the journal to review the data if requested.

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