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Regulation of S100B in white adipose tissue by obesity in mice

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S100B is a calcium binding protein found in adipose tissue; however, relatively little is known about the physiologic regulation or distribution of the protein within this organ. We examined plasma S100B concentration and white adipose tissue (WAT) *s100b* mRNA levels in lean and diet-induced obese (DIO) mice. Plasma S100B levels were increased by obesity. In WAT, *s100b* gene expression was also significantly increased by obesity and this increase was reversed following weight-loss. *s100b* gene expression was detected in both the adipocyte-enriched and stromal-vascular fractions of WAT; however, the increase in *s100b* gene expression in obese animals was only detected in the adipocyte-enriched fraction. Our results support published in vitro data indicating that WAT S100B may contribute to obesity-associated inflammation.

Introduction

S100B is a calcium binding protein found at high levels in the brain[1] and adipose tissue.[2-4] In the brain, S100B is localized largely within glial cells[1] and can act as either a neurotropic factor[5] or an inflammatory cytokine[6] via its activity at the receptor for advanced glycation end products (RAGE).[6] Brain S100B levels are increased during neurologic disease and trauma in humans and in animal models.[7] In contrast, much less is known about the distribution and physiologic regulation of S100B in adipose tissue.

Obesity is characterized by adipose tissue dysfunction. Chronic low-grade inflammation of white adipose tissue (WAT) is a key pathologic event that has been implicated in mediating insulin resistance and vascular changes associated with obesity.[8] Adipose tissue inflammation during obesity is mediated in part by peripheral immune cells, including pro-inflammatory "M1" polarized macrophages,[9] that are recruited to the tissue during disease progression. Recent in vitro data suggests that adipocyte-derived S100B can act as an inflammatory cytokine via RAGE, stimulating M1 polarization of macrophages in an in vitro co-culture system.[10] Further evidence for a role of S100B as an adipose-derived cytokine, or adipokine, comes from data demonstrating that circulating S100B levels are positively correlated with body mass index in human subjects.[11-14] Combined, these data suggest that the S100B-RAGE axis may be an important regulator of chronic low-grade adipose tissue inflammation during obesity; however, direct evidence for in vivo regulation of adipose tissue S100B during obesity is lacking. The objective of this study was to determine the distribution of S100B within adipose tissue and to examine its regulation in this organ by obesity.

Results

Plasma and white adipose tissue S100B levels were increased by diet-induced obesity in mice

We examined the regulation of plasma S100B protein levels and adipose tissue *s100b* gene expression in a diet-induced obese (DIO) mouse model. Fifteen weeks of high-fat feeding resulted in a statistically significant increase in body weight (lean: 30.9 ± 0.5 g; DIO: 44.7 ± 0.9 g; $P < 0.001$) and adiposity (lean: 2.2 ± 0.3 g; DIO: 14.3 ± 0.7 g; $P < 0.001$) compared with lean standard chow-fed control animals. DIO animals had a statistically significant increase in plasma S100B protein levels as measured by ELISA (**Fig. 1A**; lean: 7.2 ± 0.8 pg/mL; DIO: 19.5 ± 2.5 pg/mL; $P < 0.001$), supporting what has previously been shown in human subjects.[14] To determine if WAT S100B expression was also regulated by obesity we measured *S100b* gene expression in whole WAT by qPCR. There was a statistically significant increase in *S100b* gene expression in WAT of DIO animals compared with lean controls (**Fig. 1B**; $P < 0.05$). In addition, there was a statistically significant increase in WAT gene expression of *Tnf* (**Fig. 1C**; $P < 0.001$), *Ccl2* (**Fig. 1D**; $P < 0.001$) and *Emr1* (the gene for the macrophage marker F4/80; **Fig. 1D**; $P < 0.001$), indicating the presence of WAT inflammation in the DIO animals. WAT *Rage* gene expression was not significantly different between the lean and DIO groups (**Fig. 1E**). Together, these data indicate that,

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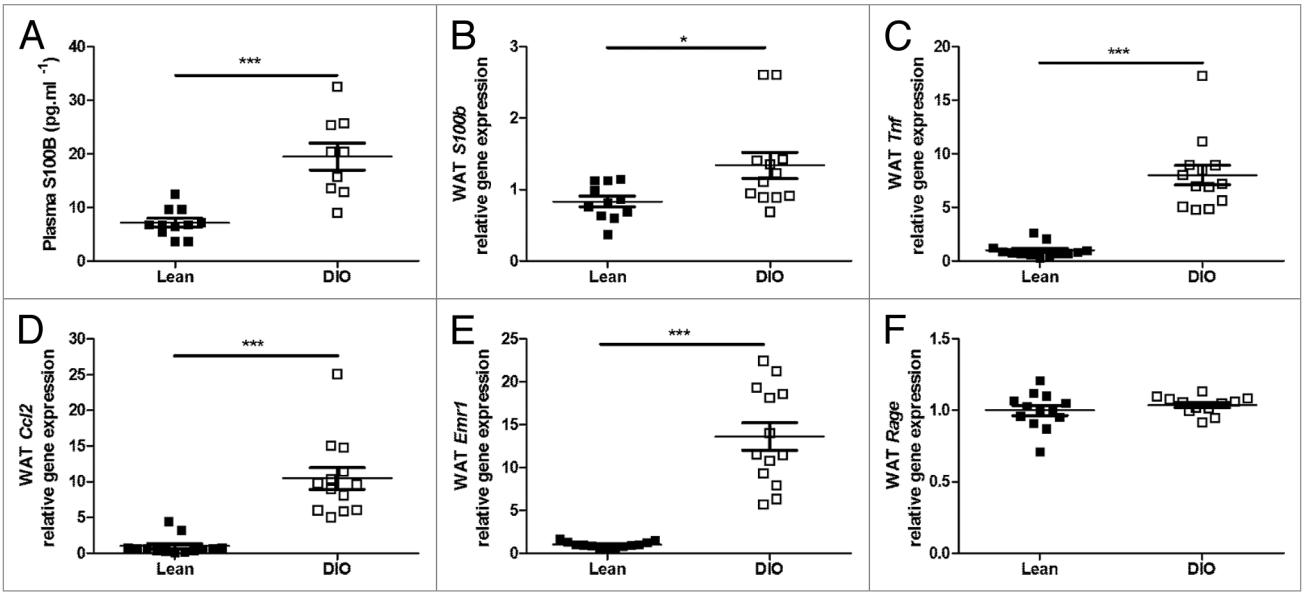

Figure 1. Plasma and white adipose tissue (WAT) S100B levels in diet-induced obese (DIO) mice. Plasma S100B protein (**A**) and WAT *s100b* gene expression (**B**) are increased in DIO mice compared with lean standard chow fed controls. DIO mice showed WAT inflammation as indicated by increased gene expression for *Tnf* (**C**), *Ccl2* (**D**), and *Emr1* (**E**). WAT gene expression for the S100B receptor, RAGE was not altered by diet-induced obesity (**F**). *$P < 0.05$; ***$P < 0.001$. $n = 12–13$/group.

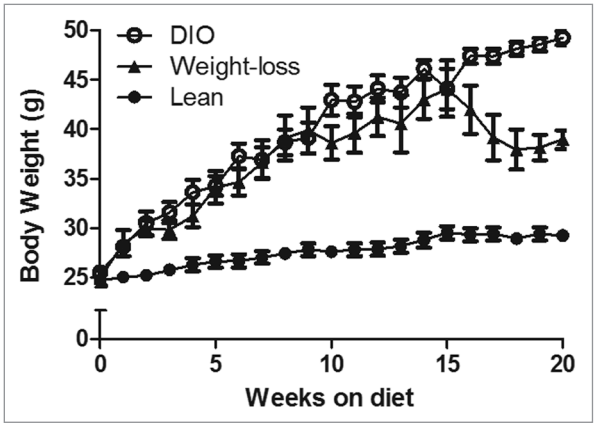

Figure 2. Body-weight curves of mice used for weight-loss study. Changing mice from high-fat diet back to standard chow for 5 wk resulted in weight loss (weight-loss group) compared with mice maintained on high-fat diet (DIO). Both groups remained heavier than the lean group that was maintained on standard chow throughout the study. $n = 7–9$/group

in common with markers of adipose tissue inflammation, WAT *s100b* gene expression was increased by diet-induced obesity.

Obesity-associated increases in plasma and white adipose tissue S100B levels were reversed by weight loss in mice

In obese animals, adipose tissue dysfunction and WAT inflammation decrease following weight loss.[15] To further examine the potential contribution of S100B to adipose tissue dysfunction during obesity we determined whether the increased plasma and WAT S100B levels in DIO mice could be reversed by weight loss. Switching DIO mice to standard chow for 5 wk after 15 wk of HFD (weight loss group) resulted in weight loss compared with the animals that remained on HFD (DIO; **Fig. 2**); however, at euthanasia the animals in the weight-loss group were still significantly heavier than the control lean animals that were fed standard chow throughout the study (lean: 29.9 ± 0.6 g; weight-loss: 36.3 ± 1.5 g; $P < 0.001$). The difference in terminal body weight between groups was due to differences in adiposity, with all three groups having statistically significant differences in body fat content (lean: 2.5 ± 0.4 g; weight-loss: 7.6 ± 1.2 g; DIO: 16.8 ± 0.5 g; $P < 0.001$).

In agreement with our first study (**Fig. 1**), we found that in DIO animals there was a significant increase in plasma S100B (**Fig. 3A**; lean: 15.7 ± 2.4 pg/mL; DIO: 35.9 ± 5.0 pg/mL; $P < 0.01$), WAT gene expression of *s100b* (**Fig. 3B**; $P < 0.01$) and markers of inflammation including *Tnf* (**Fig. 3C**; $P < 0.001$), *Ccl2* (**Fig. 3D**; $P < 0.001$) and *Emr1* (**Fig. 3E**; $P < 0.001$) compared with lean control mice. Weight loss reversed the obesity-associated increases in plasma S100B, WAT *s100b* gene expression and WAT inflammation (**Fig. 3A–E**; $P > 0.05$). In contrast, there were no significant differences in WAT gene expression of the S100B receptor *Rage* between groups (**Fig. 3F**; $P > 0.05$). These data demonstrate that, in common with markers of adipose tissue inflammation, *s100b* gene expression is reversed following weight loss in mice.

S100B immunoreactivity was detected in both adipocytes and adipose tissue macrophages

Immunohistochemistry was performed on a whole mount WAT preparation to determine the localization of S100B within adipose tissue from lean and DIO animals. Adipocytes in DIO animals were hypertrophied (**Fig. 4A**) compared with lean controls (**Fig. 4F**). In agreement with our qPCR data,

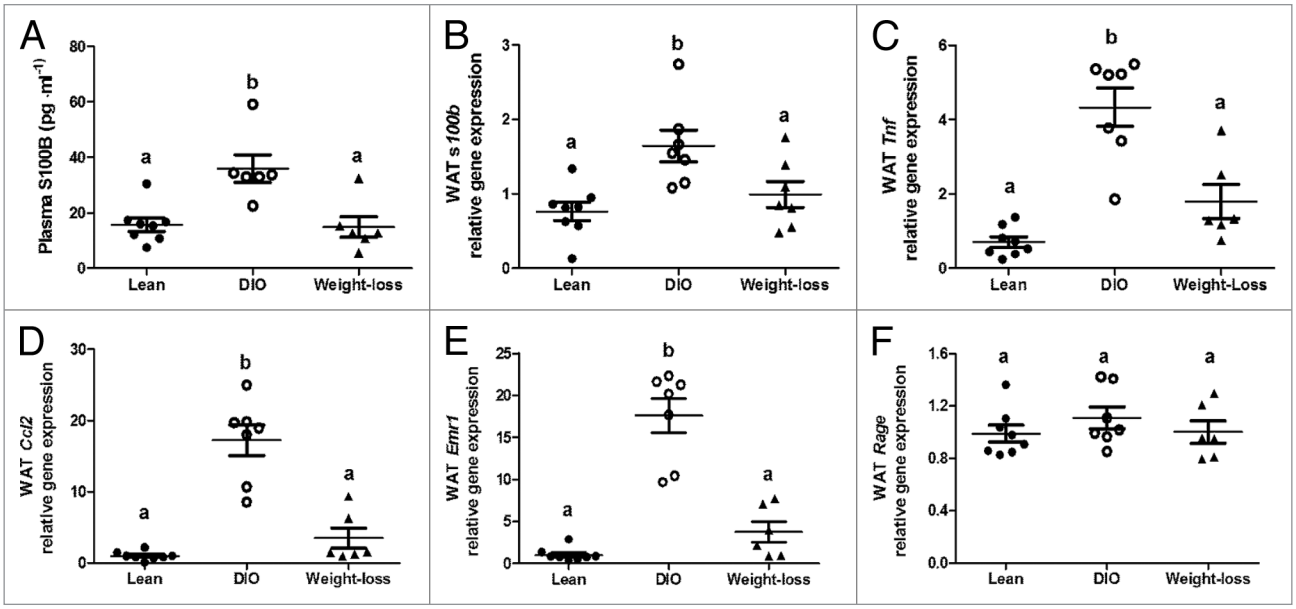

Figure 3. Plasma and white adipose tissue (WAT) S100B levels after weight loss in mice. Weight loss reversed the diet-induced obesity-associated increase in plasma S100B protein (**A**), WAT *s100b* gene expression (**B**), and markers of WAT inflammation (*Tnf* [**C**], *Ccl2* [**D**], and *Emr1* [**E**]). Gene expression for the S100B receptor RAGE was not significantly different between the groups (**F**). Data sets with different letters are significantly different from each other. *n* = 7–9/group. DIO, diet-induced obese.

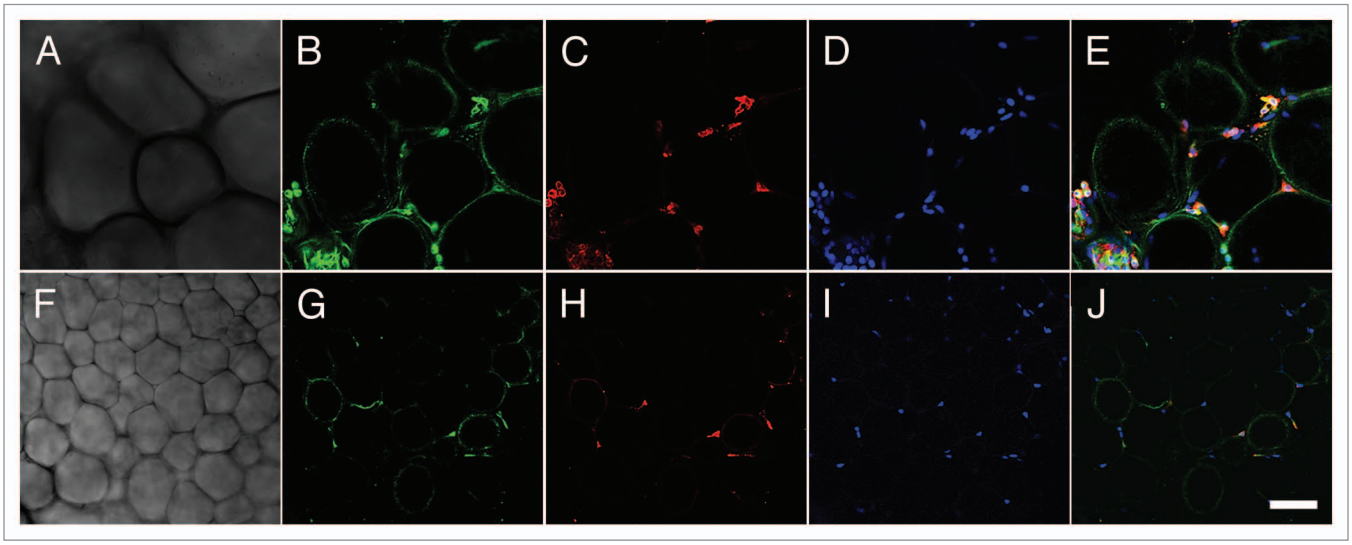

Figure 4. Immunohistochemistry for S100B in adipose tissue from lean and diet-induced obese mice. Differential interference contrast (DIC) microscopy revealed that diet-induced obesity was associated with adipocyte hypertrophy (**A**) compared with lean controls (**F**). Immunoreactivity for S100B (green) was seen in both adipocytes and non-adipocytes in both diet-induced obese (DIO) (**B**) and lean control mice (**G**) with levels increased in the DIO animals. Increased F4/80 immunoreactivity (red) was seen in DIO (**C**) compared with lean control mice (**H**). Co-localization of S100B and F4/80 immunoreactivity (yellow/white) revealed expression of S100B in both adipocytes and F4/80-positive macrophages in both DIO (**E**) and lean (**J**) mice. DAPI (blue) was used to indicate cell nuclei. Upper panels (**A–E**) are representative images from a DIO mouse. Lower panels (**F–J**) are representative images from a lean control. Scale bar = 50 μm.

immunohistochemistry reveled increased immunoreactivity for S100B (**Fig. 4B**) and F4/80 (**Fig. 4C**) in the DIO animals compared with lean controls (**Fig. 4G and H**). Co-localization of S100B immunoreactivity with F4/80 immunoreactivity revealed expression of S100B in adipose tissue macrophages in lean (**Fig. 4J**) and DIO animals (**Fig. 4E**).

S100b gene expression was increased in the adipocyte-enriched fraction of adipose tissue by obesity

WAT is a heterogeneous organ that can be separated into an adipocyte-enriched fraction and a stromal–vascular fraction (SVF), containing pre-adipocytes, endothelial, and immune cells. Using a subset of the animals from the weight-loss study we

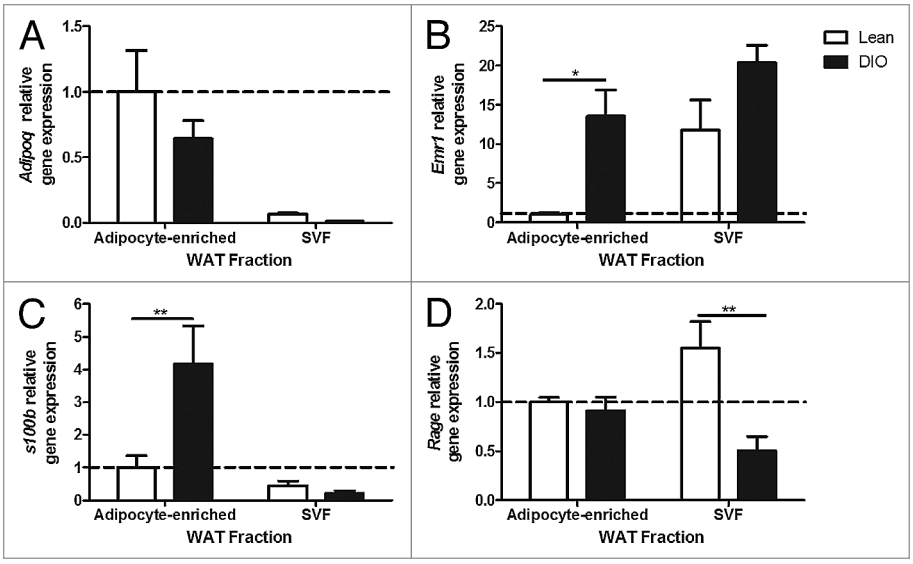

Figure 5. Regulation of S100B gene expression in different white adipose tissue (WAT) compartments. Fractionation of WAT into adipocyte-enriched and stromal–vascular fractions (SVF) was verified by examining gene expression for the adipocyte marker adiponectin (**A**) and the macrophage marker *emr1* (**B**). In diet-induced obese (DIO) mice, *s100b* gene expression was selectively increased in the adipocyte-enriched fraction (**C**) while *Rage* gene expression was selectively decreased in the SVF fraction. $n = 3–5$/group. *$P < 0.05$; **$P < 0.01$.

quantified the relative gene expression of *s100b* and *Rage* in the adipocyte-enriched and SVFs of lean and DIO mice by qPCR. First we determined the efficiency of our cellular fractionation by examining the relative gene expression of the adipocyte marker, adiponectin (*Adipoq*), and the macrophage marker, *Emr1,* in the different fractions. *Adipoq* gene expression was high in the adipocyte-enriched fractions but barely detectable in the SVFs, independent of diet (**Fig. 5A**; $P_{(fraction)} < 0.01$, $P_{(diet)} > 0.05$, $P_{(interaction)} > 0.05$), indicating efficient separation of the adipocytes into the adipocyte-enriched fraction. As expected, the SVF contained higher levels of the macrophage marker *Emr1* compared with the adipocyte-enriched fractions across all groups (**Fig. 5B**; $P_{(fraction)} < 0.05$, $P_{(diet)} < 0.01$, $P_{(interaction)} > 0.05$); however, likely due to the presence of lipid-laden macrophages in the DIO animals,[16] the efficiency of the separation was reduced and statistically significant levels of the macrophage marker *Emr1* were still detected in the adipocyte-enriched fractions from DIO animals (**Fig. 5B**; $P < 0.05$). In agreement with our immunhistochemical study, using qPCR we detected *s100b* gene expression in both the adipocyte-enriched fractions and SVFs in lean and DIO mice (**Fig. 5C**). In response to high fat feeding there was a statistically significant increase in *s100b* gene expression in the adipocyte-enriched fraction but not the SVF fraction (**Fig. 5C**; $P_{(fraction)} < 0.001$, $P_{(diet)} < 0.05$, $P_{(interaction)} < 0.01$). Gene expression of *Rage* was also detected in both adipocyte-enriched and SVF (**Fig. 5D**; $P_{(fraction)} < 0.01$, $P_{(diet)} > 0.05$, $P_{(interaction)} < 0.05$). Bonferroni post-hoc analysis revealed a statistically significant decrease in *Rage* gene expression in the SVF fraction from DIO mice (**Fig. 5D**; $P < 0.01$); however, there were no obesity-associated changes in *Rage* gene expression in the adipocyte-enriched fraction. These data suggest that obesity-associated changes in WAT *s100b* gene expression are due to increased expression in adipocytes and/or the lipid-laden macrophages that fractionate with the adipocytes in DIO animals.

Discussion

WAT dysfunction during obesity contributes to the development of insulin resistance and cardiovascular disease.[8] An improved understanding of factors that mediate WAT dysfunction will enable the development of novel therapeutics for these detrimental comorbidities. In this paper we demonstrate that plasma S100B concentration and whole WAT *s100b* gene expression are increased by obesity in mice and that these increases can be reversed by weight-loss. Further we provide evidence that obesity-associated increases in WAT S100B expression are likely specific to adipocytes and/or the lipid-laden macrophages that fractionate with the adipocytes from obese animals.

One limitation of this work stems from the WAT fraction study. Due to the presence of lipid-laden macrophages that fractionate with adipocytes in DIO animals[16] we were unable to generate an adipocyte-enriched fraction that did not contain a statistically significant increase in macrophages compared with the lean group. This means that we cannot unequivocally say that the DIO-associated increase in *s100b* gene expression in the adipocyte-enriched fraction is due to changes in adipocytes rather than the lipid-laden macrophages that fractionate with the adipocytes in the obese animals.

While the presence of S100B was first reported in adipose tissue in 1983[4] its physiologic function in this organ is not well characterized. In the CNS, S100B is documented to act as an inflammatory cytokine[6] via its interaction with RAGE. Due to the known proinflammatory role of S100B in the CNS it has been proposed that adipose-derived S100B may play a role in the recruitment and subsequent activation of innate immune cells in adipose tissue.[17] This hypothesis is supported by our findings which provide evidence of an increase in adipose *S100b* gene expression in WAT during obesity, which, in common with markers of adipose tissue inflammation, can be reversed following weight loss. Our findings provide an in vivo complement to a recent a study by Fujiya and colleagues[10] who demonstrated in vitro that S100B released from adipocytes acts as a proinflammatory cytokine promoting "M1" polarization of macrophages via the RAGE receptor. Interestingly, we also noted a reduction in *Rage* gene expression in the SVFs from obese animals, which may represent a compensatory mechanism to reduce inflammation in the face of increased S100B and other RAGE ligands[18] in adipose tissue during obesity. Together our data and published studies provide evidence suggesting that the activation of the

S100B–RAGE axis may contribute to the chronic low-grade adipose tissue inflammation during obesity.

In addition to increased adipose tissue *s100b* gene expression we also demonstrated that obesity is associated with elevated plasma S100B in mice. This is in agreement with published data reporting a positive correlation between plasma S100B levels and BMI in humans.[11-14] However, this finding has been disputed by others.[19] The source of the elevated plasma S100B during obesity remains to be determined. In addition to WAT, another potential source is the brain, as increased gene expression for *s100b*[20]and other markers of inflammation[21,22] and glial cell activation[20,22] have been reported in response to chronic diet-induced obesity in rodents. The elevated plasma S100B levels seen in obesity suggest the potential for endocrine activation of the S100B-RAGE axis. Inhibition of RAGE following administration of soluble RAGE or neutralizing antibodies has been shown to be protective in mouse models of atherosclerosis[23,24] and diabetes-associated kidney disease[25] suggesting that elevated plasma S100B during obesity, whatever its source, may contribute to the well-characterized vascular inflammation and compromise associated with obesity.

In summary, our data suggests that the activation of the S100B–RAGE axis may be a previously undescribed mechanism contributing to WAT dysfunction during obesity that warrants further study. As such, the S100B–RAGE axis may be a potential therapeutic target for obesity-associated comorbidities.

Materials and Methods

Animals

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Vanderbilt University. The animals used in these experiments were male C57BL/6J mice (000664; The Jackson Laboratory). Animals were housed at 21 ± 2 °C with ad libitum access to standard laboratory chow (13% kcal from fat; Picolab rodent diet 20, PMI Nutrition International) and water, unless stated otherwise. For the diet-induced obesity (DIO) study, mice were randomly divided into two groups (n = 12–13 mice/diet) at 8 wk of age and placed on a 60% high-fat diet (HFD; 60% kcal from fat; D12532, Research Diets) or maintained on standard laboratory chow (Chow) for 15 wk. For the weight-loss study, mice were randomly divided into three groups (n = 7–9) at 8 wk of age: (1) Lean: mice maintained on standard chow for 20 wk; (2) DIO: mice maintained on a HFD for 20 wk; or (3) Weight-loss group: mice fed HFD for 15 wk then switched back to standard chow for 5 wk resulting in weight loss. Mice were weighed weekly and their body composition assessed immediately prior to sacrifice using a Bruker Minispec Analyzer (Bruker Optics) in the Vanderbilt Mouse Metabolic Phenotyping Center. At the time of sacrifice blood was collected by cardiac puncture and then mice were perfused transcardially with 0.9% saline before epididymal WAT was collected and stored at –80 °C until use.

Laboratory assays

ELISA

S100B protein concentrations were measured using an enzyme-linked immunosorbent assay (ELISA; Millipore) in citrated plasma. Assays were performed in duplicate with a detection limit of 2.7 pg/mL and had intra- and inter-assay coefficient of variances (CV) lower than 5%.

Immunohistochemistry

Immunohistochemistry in whole adipose tissue from lean and DIO mice was performed as previously described[26] (n = 3 animals per group) with the following primary-secondary antibody combinations: 1:200 rabbit monoclonal anti-S100B (52642; Abcam) followed by 1:500 donkey anti-rabbit Alexa 488 (Life Technologies Corp.) and 1:250 rat monoclonal anti-F4/80 (ab109497; Abcam) followed by 1:500 donkey anti-rat Alexa 594 (Life Technologies Corp.). Imaging with laser confocal microscopy (Zeiss LSM 710, Carl Zeiss International) was performed in the Cell Imaging Shared Resource at Vanderbilt Medical Center. Images shown are representative of three independent fields examined from each animal.

Adipose tissue fractionation

Adipose tissue fractionation was performed on lean and DIO mice as previously described[27] and the final pellets resuspended in Trizol (Life Technologies Corp.) for RNA extraction.

Quantitative real-time RT-PCR (qPCR)

Total RNA was extracted from WAT or adipose tissue fractions using Trizol reagent (Life Technologies Corp.) and cDNA (cDNA) was synthesized using iScript cDNA synthesis kits (Bio-Rad Laboratories), according to manufacturer's instructions. Real-time RT-PCR reactions were performed using a CFX96 thermal cycler (Bio-Rad Laboratories) with FAM-conjugated primer/probe sets (TaqMan Gene Expression Assays; Life Technologies Corp., Carlsbad, NY) against the following targets: *s100b* (Mm00485897_m1); *tnf* (Mm00443258_m1), *Ccl2* (Mm00441242_m1); *emr1* (Mm00802529_m1) and normalized to *gapdh* (Mm99999915_g1). All samples were run in duplicate. After verifying equal amplification efficiency of the primer probe sets, data were analyzed using the $\Delta\Delta C_t$ method. Whole WAT data are presented as gene expression relative to the lean standard chow-fed controls. For the adipose tissue fractionation study data are presented as gene expression relative to the adipocyte-enriched fraction of lean standard chow fed controls.

Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M.). Unpaired t tests were used to assess differences between the lean and obese group (**Fig. 1**). A one-way ANOVA was used to assess differences between lean, obese and weight-loss groups (**Figs. 2 and 3**). Two-way ANOVA was used to assess differences in gene expression between adipose tissue fractions of lean and DIO mice (**Fig. 5**). All data analyses were performed using GraphPad Prism version 5.04 (GraphPad Software), and a significance level of 0.05 was used for statistical inference.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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