Sympathetic but not sensory denervation stimulates white adipocyte proliferation

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Foster, Michelle T., and Timothy J. Bartness. Sympathetic but not sensory denervation stimulates white adipocyte proliferation. Am J Physiol Regul Integr Comp Physiol 291: R1630–R1637, 2006. First published August 3, 2006; doi:10.1152/ajpregu.00197.2006.—White adipocyte proliferation is a hallmark of obesity, but it largely remains a mechanistic mystery. We and others previously demonstrated that surgical denervation of white adipose tissue (WAT) triggers increases in fat cell number, but it is unknown whether this was due to preadipocyte proliferation or maturation of existing preadipocytes that allowed them to be counted. In addition, surgical denervation severs not only sympathetic but also sensory innervation of WAT. Therefore, we tested whether sympathetic WAT denervation triggers adipocyte proliferation using 5-bromo-2′-deoxyuridine (BrdU) as a marker of proliferation and quantified BrdU-immunoreactive (ir) cells that were colabeled with AD-3-ir, an adipocyte-specific membrane protein marker. The unilateral denervation model was used for all experiments where Siberian hamster inguinal WAT (IWAT) was unilaterally denervated, the contralateral pad was sham denervated serving as a within-animal control, and then BrdU was injected systemically for 6 days. When IWAT was surgically denervated, severing both sympathetic and sensory nerves, tyrosine hydroxylase (TH)-ir, a sympathetic nerve marker, and calcitonin gene-related peptide (CGRP)-ir, a sensory nerve marker, were significantly decreased, and BrdU was increased by 300%. When IWAT was selectively senorally denervated via local microinjections of capsaicin, a sensory nerve-specific toxin, CGRP-ir, but not TH-ir, was decreased, and BrdU+AD-3-ir adipocytes were unchanged. When IWAT was selectively sympathetically denervated via local microinjections of 6-hydroxy-dopamine, a catecholaminergic-specific toxin, TH-ir, but not CGRP-ir, was significantly decreased, and BrdU+AD-3-ir adipocytes were decreased by 400%. Collectively, these data provide the first direct evidence that sympathetic nerves inhibit white adipocyte proliferation in vivo.

Adipose; obesity; capsaicin; 6-hydroxy-dopamine; 3-bromo-2′-deoxyuridine; innervation; Siberian hamster

Obesity literally is an expanding disease in the United States, affecting over 60% of the population (27). Obesity is associated with comorbidities such as type 2 diabetes, high blood pressure, heart disease, some cancers, and compromised immune responses (6, 23, 44, 51–53). A hallmark of obesity is an increase in fat cell number (FCN) (17), yet adipocyte hypercellularity is poorly understood mechanistically. The severity of the increase in adiposity might be limited and pose much less of a clinical problem if the degree of obesity were constrained by the filling of only the existing white adipocytes (fat cell hypertrophy); however, the ability of new adipocytes to form and then fill, apparently endlessly, demonstrates the need to understand the factors that promote fat cell prolifera-
WAT (IWAT) was either surgically denervated, to destroy both sympathetic and sensory innervation, chemically sensory denervated using local microinjections of capsaicin, a toxin for small unmyelinated sensory nerves (32, 33), or chemically sympathetically denervated using local microinjections of 6-hydroxy-dopamine (6-OHDA) (45), a neurotoxin that destroys catecholaminergic nerves. The contralateral IWAT pad received either sham surgery or vehicle injections and thus served as a within-animal control. The efficacy and selectivity of the surgical, sensory, and sympathetic denervation was demonstrated immunohistochemically by quantifying the number of immunoreactive staining sites for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis and a marker of sympathetic nerves (11), and CGRP, the sensory nerve-associated neuropeptide (50).

METHODS

Animals and housing conditions. Fifteen 3-mo-old adult male Siberian hamsters were obtained from our breeding colony. Our laboratory’s hamster colony was described recently (5). Hamsters were weaned at 21 days of age, housed with same-sex siblings in groups of 8–12 in polyvinyl cages (48 × 27 × 15 cm) with corn cob bedding and nestlets, and kept in a long “summer-like day” (16:8-h light-dark cycle with lights on at 0300) until they were used in the present experiment. Temperature was kept constant at 20°C, relative humidity was maintained at 50 ± 5% throughout the experiment, and all animals had ad libitum access to a standard laboratory chow (PMI no. 5001, Purina, St. Louis, MO) and tap water. Two weeks before the start of the experiments, animals were housed individually in polypropylene cages (27.8 × 17.5 × 13.0 cm), and body mass was monitored daily for the duration of the experiment. All experimental procedures were approved by Georgia State University Institutional Animal Care and Use Committee in accordance with Public Health Service and United States Department of Agriculture guidelines.

Denervation experimental design. We used the unilateral denervation model (for review, see Ref. 4) made possible because most WAT groups of 8–12 in polyvinyl cages (48 × 27 × 15 cm), and body mass was monitored daily for the duration of the experiment. All experimental procedures were approved by Georgia State University Institutional Animal Care and Use Committee in accordance with Public Health Service and United States Department of Agriculture guidelines. Animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The hair was removed from the incision area, and then the area was wiped with 95% ethanol-soaked gauze. An incision was made at the dorsal hindlimb of the animal and lateral to the spinal column that continued rostrally and then ventrally to the ventral hindlimb. Care was taken with the depth of the incision to avoid the underlying blood vessels and musculature. Using a dissecting microscope, the IWAT pad was separated from the skin and abdominal wall, while care was taken not to damage the blood vessels leading into or through the pads. A drop of 1% toluidine blue was applied to the fat pad to facilitate visualization of the nerves. The nerves were then freed from the surrounding tissue and vasculature. Small nerves were cut in two or more locations, and, for major nerves, ~3-mm segments were removed to prevent possible, although unlikely, reconnection. For sham surgeries, fat pads were gently lifted or pushed with tissue forceps to visualize the nerves without damaging either the nerves or the blood vessels. Fat pads were kept moist with 0.15 M NaCl-soaked gauze throughout denervation and sham surgeries. The incision was closed using wound clips, and nitrofurazone powder was applied to minimize infection.

Capsaicin sensory denervation. Capsaicin (Sigma St. Louis, MO) stock solution was made at a concentration of 200 μg/μl in 100% ethanol and was diluted to a final concentration of 20 μg/μl using 10% of stock solution, 80% of 0.15 M NaCl, and 10% of Tween 80, before injection. Capsaicin microinjections were done according to our laboratory’s previous protocol (48, 49). In brief, the left IWAT pads received 20 microinjections of capsaicin (250 nl per injection), whereas the right pads were injected with equivolumic injections of the vehicle and served as a within-animal control (n = 5). Reflux of the solutions was reduced by holding the needle at each injection site for 60 s before removal. Effort was made to inject capsaicin and vehicle across the full extent of the pads.

6-OHDA sympathetic denervation. Based on pilot experiments, and our laboratory’s work in WAT of mice (45), the parameters of the 6-OHDA injections used here yield maximal NE depletion. Specifically, animals (n = 5) received 40 unilateral microinjections (250 nl per injection) of 6-OHDA (Sigma Chemical; 4 mg/ml in 0.15 M NaCl that contained 1% ascorbic acid, the latter gassed in a light-tight container with N2 for 10 min before 6-OHDA was added) in the left IWAT pads. The right pads were given equivolumic vehicle injections and served as within-animal controls. Reflux of the solutions was reduced by holding the needle at each injection site for 60 s before removing the microsyringe. The 6-OHDA and vehicle solutions were kept on ice between animals. Effort was made to inject the 6-OHDA and vehicle across the full extent of the pads.

BrdU injections. After 2 days of recovery from surgical or chemical denervations, animals were injected subcutaneously between the scapulae with BrdU (50 mg/kg; Sigma Chemical) once per day for 6 days. Animals were killed 2 days after the last injection, as described directly below. This schedule of BrdU injections was chosen based on pilot experiments showing that, during this time frame, surgically induced adipocyte proliferation was at a high level.

Tissue harvesting. Animals were deeply anesthetized with pentobarbital sodium (70 mg/kg), and IWAT pads were dissected, weighed, and stored in 4% paraformaldehyde until processing for immunohistochemical identification of TH, CGRP, BrdU, and AD-3. To confirm the ability of the antibodies to detect the antigens in contemporarily harvested tissues, we removed positive control tissues [brain for TH (47); small intestine for BrdU (28); skeletal muscle for CGRP (49)] at this time.

General immunohistochemical tissue processing. Immunohistochemical tissue processing. Immunohistochemical processing was performed on WAT according to the method of Giordano et al. (22), as our laboratory has done previously (21, 48, 49). Briefly, the WAT pads were washed with 0.015 M PBS (pH 7.4) and then dehydrated with a series of increasing concentrations of ethanol. Specifically, tissues were incubated into 75% ethanol (60 min), 95% ethanol (2 × 75 min), and 100% ethanol (3 × 60 min). After dehydration, fat pads were incubated in xylene (2 × 60 min). Tissues were then infiltrated with paraffin, a paraplast embedding media (Sigma Chemical), at 60°C overnight and then embedded with fresh paraffin. Each pad was sliced across its extent at 4 μm using a rotary microtome (American Optical Instrument, Buffalo, NY). Sections were placed in a tissue-floating water bath (37°C) immediately after being sliced and were mounted on glass slides. Slides were left after being sliced and were mounted on glass slides. Slides were left.
with 1:10 normal serum in 0.15 M PBS for 20 min. Sections were then incubated with the primary polyclonal antibodies, mouse anti-TH (1:300; Chemicon) or rabbit anti-CGRP (1:500; Chemicon) in a humid chamber for 48 h at 4°C. Antibody specificity was demonstrated by incubating sections without the primary antibody and by preadsorption of the primary antibody with its antigen; each procedure resulted in no immunoreactivity. After incubation with the primary antibody, the slides were rinsed with 0.015 M PBS (×3) and then incubated with 1:200 anti-mouse biotinylated IgG (for TH and CGRP, Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.015 M PBS for 30 min at room temperature. The slides were washed with 0.015 M PBS (×3) and then incubated in a 1:100 solution of the avidin-biotin horseradish peroxidase (HRP) complex (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The HRP was made visible with 0.075% diaminobenzidine (DAB) and 0.02% H2O2 in 0.05 M Tris buffer (pH = 7.6) for 3 min. The slides were washed with tap water and counterstained with hematoxylin (Vector Laboratories). The slides were covered with Histomount (National Diagnostics, Atlanta, GA) after dehydration with a series of increasing concentrations of ethanol and xylene. The DAB chromagen product was visualized using light microscopy. Antibody specificity was demonstrated by incubating sections without the primary antibody and by preadsorption of the primary antibody with its antigen; each procedure resulted in no immunoreactivity.

*Brdu immunohistochemistry.* For Brdu immunohistochemistry, sections were deparaffinized with xylene and hydrated with a series of decreasing concentrations of ethanol and distilled water immediately before beginning the assay. Sections were first incubated in methanol with 0.3% H2O2 for 10 min at room temperature to block endogenous peroxidase activities. Sections were washed (2 × 10 min) in Dulbecco’s PBS (139 mM NaCl, 2.7 mM KCl, 0.75 mM CaCl2, 0.48 mM MgCl2·H2O, 8.8 mM Na2HPO4·H2O, 1.48 mM KH2PO4), pH 7.6. To recover immunoreactivity of BrdU, heat-induced epitope retrieval was used (for a review, see Ref. 42). To label the BrdU incorporated into the cell DNA, the tissue was microwaved to open the cell, denature the DNA, and thereby permit the BrdU monoclonal antibody to bind to the incorporated BrdU in the DNA of the proliferating cells. In brief, after rinsing, sections were placed into a microwave container (Microwave Tender Cooker, Nordic Ware, Minneapolis, MN) and submerged in 0.01 M citrate buffer, pH 6.0, before the top was placed on the container. The container was placed in a 650-W microwave for 23 min on high. Full pressure was reached in ~13 min and was allowed to continue for an additional 10 min at full pressure. Upon removal from the microwave, the container was allowed to cool for 10 min before the lid was removed. The contents were allowed to cool at room temperature until the liquid reached 60°C. Sections were rinsed in Dulbecco’s PBS (2 × 10 min) and then incubated with the mouse monoclonal anti-BrdU (1:100; Sigma) in Dulbecco’s PBS with 1% goat serum in a humid chamber for 24 h at 23°C. After incubation with the primary antibody, the slides were rinsed with Dulbecco’s PBS (2 × 10 min) and then incubated with 1:200 anti-mouse biotinylated IgG (Jackson ImmunoResearch Laboratories) in Dulbecco’s PBS 30 min at room temperature. The slides were then incubated in Dulbecco’s PBS (2 × 10 min) and then incubated in a 1:100 solution of the avidin-biotin HRP complex (Vectastain ABC Elite Kit; Vector Laboratories) for 1 h at room temperature. The HRP was made visible with 0.01% DAB and 0.02% H2O2 in 0.05 M Tris buffer (pH = 7.6) for 3 min. The slides were then washed with tap water and placed into dH2O until processing for AD-3 immunohistochemistry.

*AD-3 immunohistochemistry.* Given that the AD-3 monoclonal antibody has not been used extensively (7, 26, 36, 39, 54, 56), it seems necessary to provide information as to its specificity as an adipocyte/preadipocyte marker. As noted above, AD-3 is an adipocyte membrane protein-specific marker present at all stages of adipocyte development, including the preadipocyte stage (54, 56). Moreover, the antigen material for making the AD-3 antibody is derived from a crude membrane preparation of isolated adipocytes (not preadipo-
ring in isolated adipocytes in vitro (54) or in tissue culture (7, 26, 36, 39, 56), not all double-labeled cells exhibit this outer ring in vivo at this early stage of lipid filling, making it difficult to capture this histological signature; however, minimally bringing the cells in and out of focus made the AD-3-ir readily visible. Frequent areas excluded during counting were the outer edges of the tissue that sometimes was damaged due to handling and folding of the tissue. The total BrdU \(\times AD-3\)-ir count for each of the 10 representative fat pad slides was added and represents adipocyte proliferation number; a multiplicative function was not applied to estimate cell counts for the entire pad.

Statistical analysis. Fat pad mass, BrdU \(\times AD3\)-, TH-, and CGRP-immunoreactivity for experiments 1 and 2 were analyzed using one-way between-subjects ANOVA (SPSS for Windows, release 11.5.0; SPSS. Chicago, IL) followed by Fisher’s least significant difference post hoc tests. For both experiments, differences among groups were considered statistically different if \(P < 0.05\). Exact probabilities and test values were omitted for simplicity and clarity of presentation of results.

RESULTS

Control immunohistochemistry. Positive controls for each antibody were verified as our laboratory [TH-ir in brain (47); CGRP-ir in skeletal muscle (49)] or others [BrdU-ir in small intestine (28)] as we have shown previously (data not shown).

IWAT surgical denervation increased BrdU \(\times AD-3\)-ir, but not fat pad mass and decreased TH- and CGRP-ir. Both innervated and denervated IWAT fat pads had positive BrdU \(\times AD-3\)-ir (Figs. 1–3), most prevalently in regions of the WAT containing existing small adipocytes, which often occurred as clusters in these so-called “proliferating zones” (9) (Fig. 1), with areas of relatively low proliferation coexisting within the same fat pad (Fig. 2). Unlike the stimulation of
WAT growth after more chronic surgical denervation (e.g., 12 wk) (5, 49, 55), surgically denervated IWAT mass was not significantly increased compared with their contralateral sham-denervated counterparts, likely because of the short postdenervation time period (10 days; Fig. 4A). Surgical denervation was apparent by the statistically significant decrease (70%) in immunoreactive sites for the markers of both sympathetic (TH) and sensory (CGRP) innervation compared with sham-denervated intact IWAT (P < 0.05; Fig. 4C).

IWAT capsaicin sensory denervation did not affect IWAT mass, BrdU+AD-3-ir, and TH-ir, but decreased CGRP-ir. Capsaicin- and vehicle-treated IWAT pad masses were not significantly different from each other (Fig. 5A). There was no effect of capsaicin injection on BrdU+AD-3-ir (Fig. 5B). Capsaicin treatment disrupted sensory, but not sympathetic,

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**Fig. 4. Surgical denervation.**

A: IWAT pad mass. B: BrdU-ir positive staining. C: tyrosine hydroxylase (TH)-ir and calcitonin gene-related peptide (CGRP)-ir positive staining. Values are means ± SE. *P < 0.05, surgical denervated vs. innervated (sham denervated) IWAT pad.

**Fig. 5. Capsaicin denervation.**

A: IWAT pad mass. B: BrdU-ir positive staining. There was no difference in BrdU-ir positive cells between capsaicin-denervated and -innervated IWAT pads. C: TH-ir and CGRP-ir positive staining. *P < 0.05 capsaicin injected (sensory denervated) vs. vehicle injected (innervated) IWAT pad. Values are means ± SE.
IWAT innervation, as shown by the significantly decreased (~70%) immunoreactivity for the sensory nerve marker CGRP (P < 0.05; Fig. 5C) compared with the vehicle-injected control pads, whereas immunoreactivity for the sympathetic marker TH was not affected (Fig. 5C) compared with its contralateral control mates.

**IWAT sympathetic denervation by 6OHDA increased BrdU+AD-3-ir and decreased TH-ir, but did not affect IWAT mass or CGRP-ir.** 6OHDA- and vehicle-treated IWAT pad masses were not significantly different from each other (Fig. 6A). As occurred with surgical denervation, IWAT 6OHDA treatment significantly increased BrdU+AD-3-ir cells (~400%) compared with the contralateral vehicle-injected pads (P < 0.05; Fig. 6B). The efficacy of the chemical sympathetic denervation was evident by the significant decrease (~70%) in TH-ir sites compared with the vehicle-injected control pads (P < 0.05; Fig. 6C) and was selective because CGRP-ir was not affected (Fig. 6C) compared with its contralateral control mates.

**DISCUSSION**

The results of the present experiments support the findings of previous in vitro (34) and in vivo studies, suggesting that the SNS inhibits FCN (5, 12, 49, 55). Moreover, the present study significantly extends the previous studies, showing for the first time that the surgical denervation-induced increase in FCN was due exclusively to severing of the sympathetic nerves, rather than the cosevering of sensory nerves, resulting in bona fide adipocyte proliferation, rather than simply lipid filling of existing adipocytes that allowed them to be counted by automated methods (i.e., Coulter counter). That is, surgical IWAT denervation destroyed both sympathetic and sensory innervation and triggered an ~300% increase in BrdU+AD-3-ir adipocytes compared with the within-animal sham-denervated control pads. This result was mimicked by local injection of the catecholaminergic neurotoxin 6OHDA that only destroyed sympathetic, but not sensory, innervation and that triggered an ~400% increase in BrdU+AD-3-ir adipocytes compared with the within-animal, vehicle-injected control pads. In contrast, selective local IWAT sensory denervation, accomplished by capsaicin injections, spared sympathetic innervation and did not affect the number of BrdU+AD-3-ir adipocytes. Thus our previous demonstrations of the increased FCN after IWAT surgical denervation in Siberian hamsters (5, 49, 55) and increased FCN after retroperitoneal WAT surgical denervation in laboratory rats by others (12) were likely due to increases in white adipocyte proliferation caused by destruction of its sympathetic innervation.

To our knowledge, this is only the second use of the BrdU technique for measuring fat cell proliferation in vivo, the first being the seminal study by the Himms-Hagen and Cinti groups (28), where it was used to show that treatment of mice with a β3-adrenoceptor agonist stimulated a subpopulation of white adipocytes to transdifferentiate into brown adipocytes rather than occurring as a result of proliferation. Before this technique, in vivo assessments of fat cell proliferation was achieved using the [3H]thymidine method (e.g., Refs. 10, 20, 35, 43). The present method using BrdU+AD-3 immunohistochemical labeling with light microscopic quantification has a number of benefits over the [3H]thymidine approach. Beyond the nonisotopic advantage of BrdU, dual-labeling with AD-3 not only permits detection of proliferating cells, as also does [3H]thymidine, but identifies the cells as adipocytes, whereas, with the former, adipocytes are only detected if the cells

![Fig. 6. 6-Hydroxy-dopamine (6OHDA) denervation. A: IWAT pad mass. B: BrdU-ir positive staining. *P < 0.05. C: TH-ir and CGRP-ir positive staining. *P < 0.05. 6OHDA injected (sympathetic denervated) vs. vehicle. Values are means ± SE.](http://ajpregu.physiology.org/)

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accumulate enough lipid to be separable by buoyancy. The remaining [¹H]thymidine labeled cells in the S-V compartment cannot be distinguished as proliferated adipocyte precursors, because this fraction contains supportive and vascular components (for review, see Ref. 1). In addition, the present approach also can yield anatomical features of the tissue, such as the existence and location of the proliferating zones (9) and its innervation, which are both accomplished here.

Unlike previous studies, surgical denervation did not result in significantly increased WAT mass (5, 13, 15, 49, 55); however, there was a short postsurgical period in the present experiment (i.e., 10 days), whereas in the previous studies this period was considerably longer (10–12 wk). Thus, with a longer postaxotomy time period combined with a positive energy balance, the filling of the proliferated cells with lipid likely would result in increased WAT mass for the surgically or sympathetically chemically denervated (6OHDA-injected) adipose tissue compared with the corresponding innervated WAT.

The functions of the sensory innervation of WAT have not been explicitly defined. The results of the present study show little or no impact of sensory innervation on fat cell proliferation, with the capsaicin-treated IWAT showing equivalent numbers of BrdU+AD-3-ir adipocytes to that of vehicle-injected WAT. These data are consistent with our laboratory’s previous work (49) showing capsaicin-induced sensory denervation increases fat cell size, but not FCN, with the former effect perhaps suggesting sensory nerve feedback in inhibiting the stimulatory role of increased sympathetic neural drive on WAT lipolysis. Capsaicin sensory denervation of WAT also stimulates increases in the mass of noninjected WAT (48), reminiscent of the compensatory increases in nonexcised WAT masses after surgical partial lipectomy (for review, see Ref. 41). These latter data seem to suggest that the inability of WAT to communicate with the brain via its sensory nerves is responsible to a loss of body fat triggering responses to increase adiposity. Therefore, it may be that the sensory innervation of WAT conveys the level of body fat to the brain, although considerable additional work is needed to determine whether this notion is correct and to identify what is being sensed.

The present study does not address the mechanisms underlying the increase in fat cell proliferation triggered by sympathetic nerve denervation. Clearly, the absence of stimulation of β-adrenoceptors by NE initiates the effect because NE is the principal postganglionic neurotransmitter of the SNS, and NE inhibits proliferation of adipocyte precursor cells in vitro (34). Moreover, pretreatment of adipocyte precursor cells with propranolol, a general β-adrenoceptor blocker, eliminates the subsequent NE inhibition of proliferation (34). Together, these data buttress the evidence for the role of sympathetic nerve-released NE in the control of white adipocyte proliferation.

Collectively, the data from the present experiments reinforce the view that the SNS innervation of WAT has an important function beyond the control of lipid mobilization (for review, see Refs. 2 and 4) and the control of FCN and, for the first time, demonstrate that this control is via changes in white adipocyte proliferation. This was made possible by administering BrdU in vivo to label proliferating adipocytes in a novel combination with an adipocyte membrane protein-specific antibody AD-3 (26, 36, 54) to yield immunohistochemical labeling of proliferating adipocytes using the unilateral WAT denervation model. Given that white adipocyte proliferation is a hallmark of obesity (17), this approach may prove valuable in understanding the mechanisms underlying this process.

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