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Novel method for localized, functional sympathetic nervous system denervation of peripheral tissue using guanethidine

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Abstract

A simple technique for local chemical sympathectomy of peripheral tissues is described using guanethidine. Multiple microinjections of guanethidine were made into inguinal or epididymal white adipose tissue (IWAT and EWAT) pads or spleens of hamsters. Guanethidine virtually abolished the sympathetic innervation of both EWAT and IWAT, as measured by the absence of significant norepinephrine (NE) tissue content two weeks later and as suggested by the two-fold increase in IWAT mass characteristic of surgically induced WAT denervation. These measures were not affected in the contralateral pads given equivolumetric injections of saline. Guanethidine injections into the spleen lead to a functional sympathectomy, as indicated by significant depletions of NE content. Because guanethidine treatment did not decrease body mass, induce ptosis, or spread to closely associated adjacent tissue (contralateral EWAT pad), no chemical-induced malaise or global sympathetic denervation was suggested. Guanethidine was more effective than two other local sympathectomy treatments, injections of the sympathetic neurotoxin anti-dopamine-β-hydroxylase saporin or surgical denervation, in decreasing IWAT NE content and increasing IWAT pad mass. Collectively, these results suggest that locally applied, chemical sympathectomy with guanethidine provides an effective, restricted method for sympathectomizing WAT, spleen and likely other peripheral tissues.

Keywords: Saporin; Sympathectomy; Adipose tissue; Lipolysis; Spleen; Siberian hamsters axotomy; Norepinephrine

1. Introduction

The sympathetic nervous system (SNS) plays a major role in the regulation of many important physiological functions (Loewy and Spyer, 1990) including a critical role in the 'fight-or-flight' response (Cannon, 1927). Sympathectomy, defined as the selective destruction of postganglionic sympathetic neurons using norepinephrine (NE) as their primary neurotransmitter (Picklo, 1997), has been accomplished using a variety of chemical and surgical methods (for review see, (Picklo, 1997)). The oldest and most commonly used sympathectomy technique is surgical denervation of the tissue of interest. For example, the seminal report of sympathectomy in a non-human animal model was accomplished through the surgical removal of the entire sympathetic chain in cats (Cannon, 1929). Partial or total sympathetic chain removal, or severing of the postganglionic neural fiber bundles, has been used extensively for a wide range of sympathetic tissue denervations and animal species (for review see, (Picklo, 1997)). In the present report, however, we will focus on the functional sympathetic denervation of adipose tissues (for review see, (Bartness and Bamshad, 1998; Bartness et al., 2001) as an example of the chemical sympathectomy using our novel technique described below.

Each of the previously available sympathectomy methods has advantages and disadvantages. The major advantages of surgical sympathectomy are that it is relatively complete and produces nearly irreversible destruction of the sympathetic innervation of the tissue of

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interest with the possible exception of sprouting by any missed nerves (Picklo, 1997). It has the major disadvantages of being non-selective in that parasympathetic and/or sensory nerves are severed because they are indistinguishable at the light microscopic level and it is often a slow and tedious surgery.

Due to the limitations of the surgical denervation method, several techniques have been developed using neurotoxins (either immunotoxins or other chemical toxins) that specifically target sympathetic nerves, leaving parasympathetic and sensory nerves intact and offering the possibility of local application. One such neurotoxin is 6-hydroxy-dopamine (6-OHDA), a toxin selective for catecholaminergic neurons (for review see, (Picklo, 1997)). 6-OHDA has the advantages of selectivity and of rapid application time, however, there are some serious disadvantages to this technique in that significant recovery of NE content can occur after a few days or weeks (e.g. in brown fat (Thureson-Klein et al., 1976)). Moreover, 6-OHDA typically is used to produce a global sympathectomy with consequent sympathetic denervation extending beyond the tissue of interest. Therefore, interpretation of the results of studies using systemic 6-OHDA is often difficult at best. Another approach is the application of immunolesioning, where antibodies conjugated to toxins selectively kill neurons that express the antigen. For example, the sympathetic toxin, anti-dopamine β-hydroxylase-saporin (DBH-SAP) has the advantages of being highly selective, rapidly administered, and is relatively permanent (reviewed in (Picklo, 1997; Wiley and Kline, 2000)) resulting in complete noradrenergic sympathectomy (Picklo et al., 1994). There are disadvantages here too. For example, from a practical perspective, DBH-SAP can be cost prohibitive for some laboratories (i.e. hundreds of dollars per animal) and typically is administered systemically; thus, in terms of the latter, the chemical sympathetic denervations produced by DBH-SAP are not restricted to the specific tissue of interest.

Finally, another sympathectomy agent that has a long history of use is guanethidine, the sympathectomizing agent used in the present experiments. Guanethidine has several effects on peripheral sympathetic neurons including blockade of neural transmission, depletion of neuronal NE stores, and blockade of reuptake of NE into the neurons collectively creating a functional sympathectomy (Burnstock et al., 1971; Heath et al., 1972). This functional denervation by guanethidine in adult rodents is considered permanent (Burnstock et al., 1971; Johnson et al., 1976; Evans et al., 1979), with no evidence of reinnervation in a variety of tissues as long as 63 weeks after the initial treatment (Evans et al., 1979). Typically, guanethidine, as for 6-OHDA, is given systemically, e.g. (Burnstock et al., 1971; Johnson et al., 1976), thus limiting conclusions to issues of global sympathectomy rather than tissue-specific effects. Here we report a novel technique for permanent, localized, functional sympathectomy of peripheral tissue in rodents via local injection of guanethidine (specifically into white adipose tissue depots and into spleen). Not only is this technique simple, rapid and inexpensive compared with other types of sympathectomy, but also in the example illustrated here, it is more effective in abolishing NE content than surgical denervation or DBH-SAP.

2. Materials and methods

2.1. Animals and housing conditions

Adult (>60 days of age) Siberian hamsters (*Phodopus sungorus*) ($n=70$) were obtained from our breeding colony at Georgia State University. This colony was originally derived from stock animals supplied by Dr Bruce Goldman (University of Connecticut). Hamsters were weaned at 21 days of age and housed with same sex siblings. Two weeks before the start of the experiments, animals were housed individually in polypropylene cages ($27.8\times7.5\times13.0$ cm) in colony rooms with a 24 h LD 16-h light:8-h dark cycle (lights on 03:00 h EST). Temperature was kept constant at 20°C and relative humidity was maintained at $50\pm5\%$. Food (Purina Rat Chow 5001) and tap water were available ad libitum throughout the experiment.

2.2. Experiment 1: chemical denervation of IWAT with guanethidine

In Experiment 1, hamsters received unilateral sympathetic chemical denervations of the left IWAT pad using guanethidine sulfate (Sigma Chemical, St. Louis, MO). The fur was removed around the hindquarters of each animal and the area was wiped with a 95% ethanol-soaked gauze pad. An incision was made dorsally on the skin from a point near the tail and lateral to the spinal column. The incision continued rostrally along the dorsum adjacent to the spinal column to a point immediately rostral to the hindimb, then laterally and ventrally to a point ~2 cm from the ventral midline. Finally, the incision extended caudally to a point near the tail. Care was taken with the depth of the incision to avoid cutting the underlying vasculature and musculature. The full extent of each IWAT pad was visualized, and a series of microinjections (2 μl per injection) of guanethidine sulfate (Sigma Chemical, St. Louis, MO) dissolved in 0.9% NaCl were made evenly throughout the pad using a 10 μl Hamilton syringe. Hamsters in Group 1 ($n=8$) received 10 injections of guanethidine (5 μg/μl); hamsters in Group 2 received 20 injections of guanethidine (5 μg/μl); hamsters in Group 3 received 10 injections of guanethidine (10 μg/μl);

hamsters in Group 4 received 20 injections of guanethidine (10 μg/μl). All animals received unilateral guanethidine injections in the left IWAT pad whereas the right IWAT pad was injected with equivolumetric injections of saline vehicle alone and served as within-animal controls. An additional control group of animals received injections of saline in both IWAT pads to control for the possible spread of guanethidine to the contralateral control pad. Every effort was made to cover the full extent of the pad and the needle was held at each injection site for ~30 s to minimize back-flow. Following the injections, IWAT pads were irrigated with saline and the skin was closed with surgical staples. Nitrofurazone antibacterial powder was applied to the skin surface to prevent infection and animals were returned to the colony room. Animals were allowed to recover from surgery for 2 weeks. At this time, animals were overdosed with sodium pentobarbital, IWAT, retroperitoneal WAT (RWAT), and epididymal WAT (EWAT) pads were dissected, weighed and snap frozen immediately in liquid nitrogen and stored at −80°C for subsequent HPLC analysis.

2.3. Experiment 2: comparison of guanethidine, DBH-saporin and surgical sympathectomies

In Experiment 2, the effectiveness of guanethidine-induced sympathectomy was compared with two other methods of SNS denervation. Specifically, guanethidine treatment was compared with surgical denervation (axotomy), chemical denervation using the neurotoxin DBH-SAP or with no treatment. The first group of hamsters ($n = 6$) received surgical denervations of the left IWAT pad as described previously (Youngstrom and Bartness, 1998). Briefly, hamsters were anesthetized with pentobarbital sodium (~50 mg/kg), the fur was removed from the selected hindquarter of the animal and the area was wiped with 95% ethanol-soaked gauze. An incision was exactly as described above for the guanethidine injections in Experiment 1. The IWAT pad was separated from the abdominal wall and overlying skin by blunt dissection keeping intact the major blood vessels leading into or through the pad. Nerves identified at 4× magnification as terminating in the pad were cut in two or more locations. Throughout the surgery, the pad was kept moist with 0.9% NaCl-soaked gauze. The sham surgery was as above except the skin was spread so that the lateral edges of the target IWAT could be seen. The edges of the fat pad were then lifted with forceps taking care to avoid stretching or tearing of the pad and associated nerves and blood vessels. After surgery, the sham or denervated pad was put back against the outside abdominal wall and was rinsed with 0.9% NaCl. The incision was closed with wound clips and nitrofurazone powder was applied to the incision.

Another group of animals ($n = 6$) received chemical sympathetic denervations of the left IWAT pad with guanethidine (20 injections of 10 μg/μl). This specific guanethidine protocol was chosen based on the results of Experiment 1 showing that this was the most effective regimen for functionally denervating IWAT.

The two remaining experimental groups of animals received 10 microinjections (2 μl per injection) of anti-dopamine β-hydroxylase-saporin (DBH-SAP; Chemicon, Temecula, CA) at either 0.65 μg/μl ($n = 6$) or 0.325 μg/μl ($n = 6$) dissolved in 0.9% NaCl. DBH-SAP microinjections were performed exactly as described above for guanethidine. An additional control group ($n = 6$) received sham surgeries in which the nerves innervating the IWAT pads were visualized but not cut; these animals served as a control for surgical denervation. All animals were killed and WAT was removed as described in Experiment 1.

2.4. Experiment 3: chemical denervation of EWAT and spleen using guanethidine

In Experiment 3, the effectiveness of guanethidine-induced sympathectomy technique was examined in another WAT pad, epididymal WAT (EWAT), as well as a non-adipose tissue, the spleen. The goal of this experiment was to determine if chemical denervation of IWAT described above was effective in other, more medially located tissues within the peritoneal cavity (e.g. EWAT), as well as non-adipose tissues (e.g. spleen). Specifically, one group of hamsters received injections of guanethidine (20 injections of 10 μg/μl) in the left EWAT and control injections of saline in the right EWAT pad ($n = 6$) as described above for IWAT, whereas the remaining animals ($n = 6$) received injections of saline in both EWAT pads. Another group of hamsters received injections of guanethidine (10 injections of 10 μg/μl) into the spleen ($n = 6$), whereas the remaining, control animals received injections of saline ($n = 6$). All animals were killed and WAT and spleens was removed as described in Experiment 1.

2.5. HPLC determination of catecholamine content

SNS denervation of IWAT, EWAT, and spleen was verified by measuring NE content in IWAT using reverse-phase HPLC with electrochemical detection according to the method of Youngstrom and Bartness (1998) after Mefford (Mefford, 1981). Briefly, tissue was thawed, weighed and minced. A 250 mg sample was added to 1 ml of 0.3 M perchloric acid in microcentrifuge tubes and 10 μl of dihydroxybenzoacetic acid (DHBA) was added to each sample and served as an internal standard. Tissue was further minced and then sonicated for 5 min on ice (5× for each sample). Catecholamines were extracted from the remaining infrana-

tant using alumina (200 mg per sample). The extracted samples were assayed using an ESA (Chelmsford, MA) HPLC system with electrochemical detection (guard cell, +35 mV; cell 1, +10 mV; cell 2, −30 mV). The mobile phase was Cat-A-Phase II purchased from a commercial supplier (ESA, Inc). Standard solutions were prepared at concentrations of 5.0, 3.3 and 1.65 ng/ml from commercially supplied standard kits (ESA, Inc) and were run at the beginning, in the middle and at the end of each set of unknowns. NE content in the samples were expressed as ng/g tissue.

3. Statistical analyses

Differences in body mass between experimental groups were determined using a one-way between-groups analysis of variance (ANOVA; Sigma Stat, Jandel Scientific, San Rafael, CA). Differences in WAT pad and spleen masses and NE content between the groups were assessed via a two-way mixed model ANOVA (type of sympathectomy x WAT pad or spleen). Post hoc comparisons between means were conducted using Tukey-HSD tests when the overall ANOVA revealed a significant difference. In all cases differences between group means were considered statistically significant at $P < 0.05$. Exact probabilities and test values have been omitted for simplification and clarity of the presentation of the results.

4. Results

4.1. Experiment 1: local guanethidine injections were effective in creating functional sympathectomies in IWAT

NE content was significantly reduced and IWAT pad mass was greater in animals treated with either 5 μg/μl guanethidine (20 injections) or 10 μg/μl guanethidine (10 or 20 injections) compared with the within-animal control pads or animals within the control group ($P < 0.05$, Fig. 1). Hamsters receiving unilateral injections of 10 μg/μl guanethidine (20 injections) also had significantly reduced NE content and increased IWAT pad mass compared with all of the other guanethidine-treated IWAT pads ($P < 0.05$, Fig. 1). Neither NE content nor IWAT pad mass was affected by 5 μg/μl guanethidine (10 injections). There were no differences in pad mass or NE content between left or right EWAT or RWAT pads in either guanethidine-treated or control animals (Table 1). There were no significant differences in body mass among any of the experimental groups nor was ptosis observed.

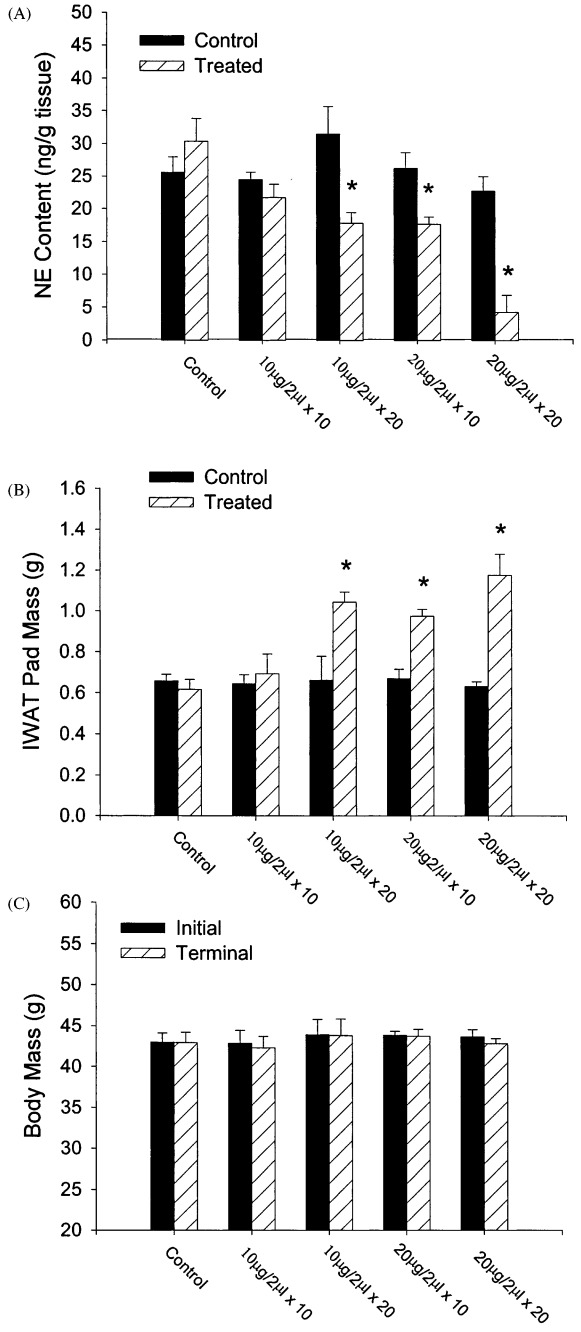

Fig. 1. (A) Mean (±S.E.M.) norepinephrine (NE) content (ng/g tissue) in control hamsters and hamsters receiving 10 or 20 injections of guanethidine (at 5 or 10 μg/μl). The solid black bars represent within-animal control IWAT pads injected with saline, whereas the stippled bars represent the contralateral pads injected with guanethidine. (B) Mean (±S.E.M.) IWAT pad mass (g) in control hamsters and hamsters receiving 10 or 20 injections of guanethidine (at 5 or 10 μg/μl). As above, the solid black bars represent within-animal control IWAT pads injected with saline, whereas the stippled bars represent the contralateral pads injected with guanethidine. (C) Mean (±S.E.M.) body mass (g) in control hamsters and hamsters receiving 10 or 20 injections of guanethidine (at 5 or 10 μg/μl). The solid black bars represent initial body masses whereas the stippled bars represent terminal body masses. Significant differences between the means are indicated by an asterisk (*).

4.2. Experiment 2: local guanethidine injections were most effective in creating functional sympathectomies of IWAT compared with axotomy and immunolesioning

NE content was significantly reduced and IWAT pad mass increased in both surgically denervated and guanethidine-treated IWAT pads compared with their respective within-animal controls pads ($P < 0.05$, Fig. 2). NE content, but not IWAT pad mass, was significantly lower in guanethidine-treated IWAT pads compared with surgically denervated pads ($P < 0.05$, Fig. 2). DBH-SAP did not significantly affect IWAT pad mass or NE content compared with the contralateral, within-animal control pad. There were no significant differences in body mass among any of the experimental groups, although there was a non-significant rend towards reduced body mass in DBH-SAP treated animals (Fig. 2).

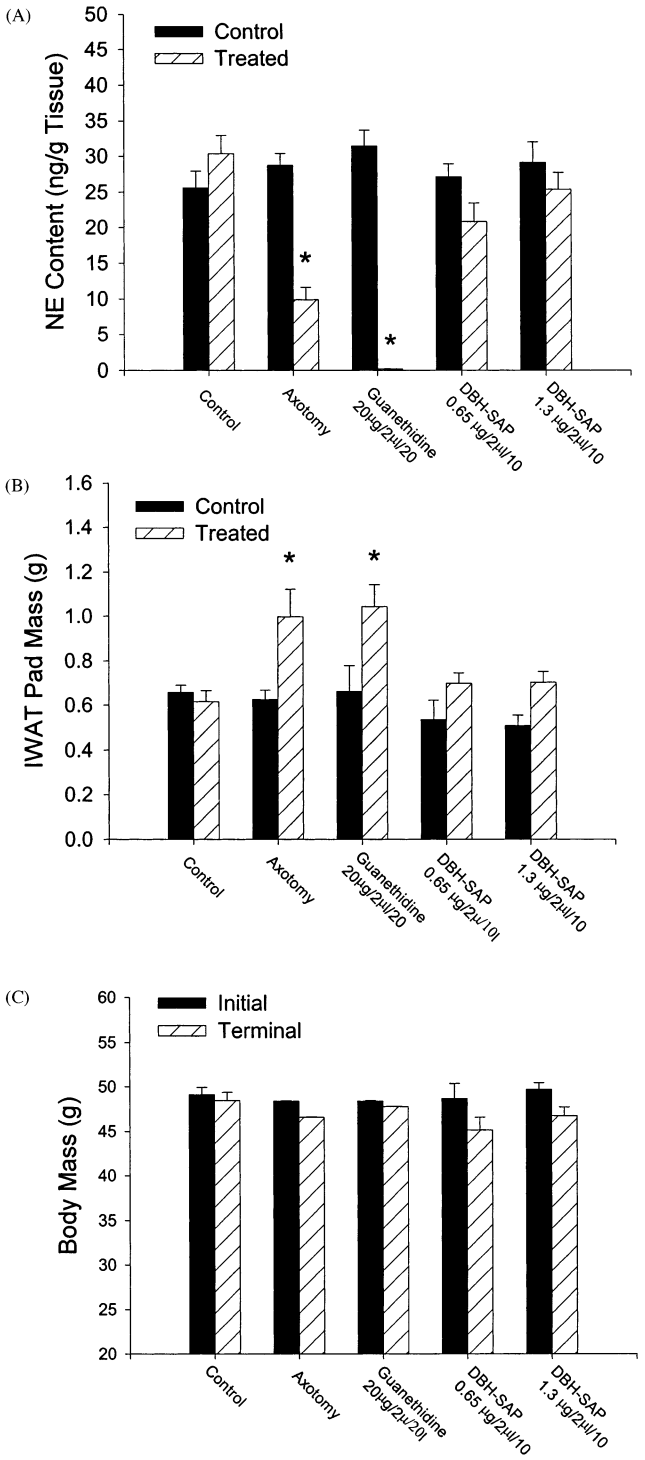

Fig. 2.

4.3. Experiment 3: local guanethidine injections were effective in creating functional sympathectomies in both EWAT and spleen

NE content was significantly reduced and EWAT pad mass was greater in guanethidine-treated EWAT compared with within-animal control pads or control animals injected with saline ($P < 0.05$, Fig. 3). Spleen NE content also was significantly reduced and spleen mass increased in guanethidine-treated hamsters compared with control animals ($P < 0.05$, Fig. 4). There were no significant differences in body mass among any of the experimental groups.

5. Summary and future applications

We describe a simple and rapid technique for the effective, localized chemical sympathectomy of peripheral tissue using guanethidine. This technique also is selective in that, unlike surgical denervation, it is specific to the SNS nerves innervating peripheral tissue (Johnson et al., 1976; Johnson and O'Brien, 1976), leaving parasympathetic nerves intact (if they exist [e.g. WAT has no parasympathetic innervation (Bart-

Fig. 2. (A) Mean (±S.E.M.) norepinephrine (NE) content (ng/g tissue) in control hamsters, hamsters receiving surgical denervation of IWAT (Axotomy), chemical denervation of IWAT with guanethidine or chemical denervations with DBH-saporin (DBH-SAP) at 0.325 or 0.65 µg/µl. The solid black bars represent within-animal control IWAT pads injected with saline, whereas the stippled bars represent the experimentally treated pads. (B) Mean (±S.E.M.) IWAT pad mass (g) in control hamsters, hamsters receiving surgical denervation of IWAT (Axotomy), chemical denervation of IWAT with guanethidine or chemical denervations with DBH-saporin (DBH-SAP) at 0.325 or 0.65 µg/µl. As above, the solid black bars represent within-animal control IWAT pads injected with saline, whereas the stippled bars represent the contralateral pads injected with guanethidine. (C) Mean (±S.E.M.) body mass (g) in control hamsters, hamsters receiving surgical denervation of IWAT (Axotomy), chemical denervation of IWAT with guanethidine or chemical denervations with DBH-saporin (DHB-SAP) at 0.325 or 0.65 µg/µl. The solid black bars represent initial body masses, whereas the stippled bars represent terminal body masses. Significant differences between the means are indicated by an asterisk (*).

ness and Bamshad, 1998)]). Regarding sensory innervation, guanethidine most likely spares sensory innervation based on studies in other tissues where guanethidine either did not change or increased the sensory-associated neuropeptides substance P and calcitonin gene-related peptide (Benarroch et al., 1994; Cherruau et al., 1999). Thus, interpretation of the results of this restricted sympathectomy is simplified because of the local nature of the manipulation and its selectivity. In addition, the problems of blood flow disruption after surgical denervation due to the inadvertent surgical trauma that inevitably results from damage to the inextricable intertwining of innervation and blood vessels is minimized or avoided by the local application of guanethidine.

Chemical sympathetic denervation using guanethidine, 6-OHDA or DBH-SAP is typically performed via systemic injections of these compounds, often repeatedly within the same animal, to ultimately yield a global sympathectomy. Although these treatments result in relatively complete, but not permanent sympathetic ablation (with respect to 6-OHDA only), the global destruction of the SNS by both substances may lead to non-specific physiological and behavioral effects unrelated to denervation of the intended target tissue. The inability to restrict these global sympathectomies makes the interpretation of the results of such experiments nearly impossible. Local application of guanethidine, however, avoids these issues of global sympathectomy.

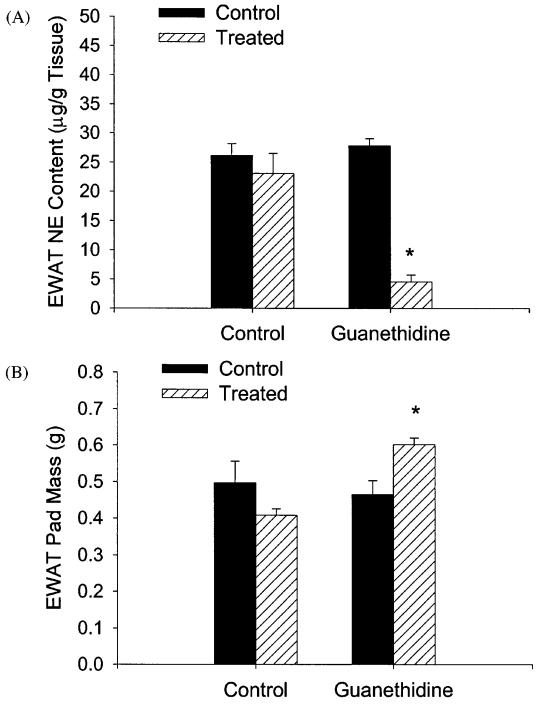

Fig. 3. (A) Mean (±S.E.M.) norepinephrine (NE) content (ng/g tissue) and (B) EWAT mass (g) in control hamsters and hamsters receiving injections of guanethidine (20 μg/2 μl) into the left EWAT pad. As above, the solid black bars represent within-animal control IWAT pads injected with saline, whereas the stippled bars represent the contralateral pads injected with guanethidine. Significant differences between the means are indicated by an asterisk (*).

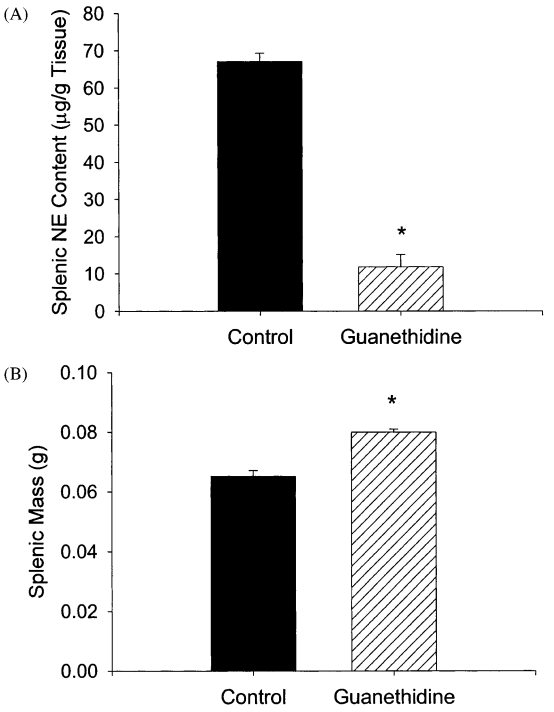

Fig. 4. (A) Mean (±S.E.M.) norepinephrine (NE) (ng/g tissue) and (B) splenic mass (g) in control hamsters and hamsters receiving injections of guanethidine (20 μg/2 μl) into the spleen (10 injections). Significant differences between the means are indicated by an asterisk (*).

We found that locally applied guanethidine to IWAT, EWAT or spleen increased tissue mass (~ 100% for WAT and ~ 40% for spleen). Thus, it is difficult to argue that a lesion technique such as this, or others, produces its effects non-specifically (e.g. via tissue damage) when hypertrophy, not atrophy, is triggered. This increase in fat pad size in WAT is comparable to that we found previously in surgically denervated Siberian hamster IWAT (Youngstrom and Bartness, 1998), as well as in the present experiment, and to that of surgically denervated rat retroperitoneal WAT (Cousin et al., 1993). This increase in fat pad size is due to a marked doubling of fat cell number in the denervated pads (Cousin et al., 1993; Youngstrom and Bartness, 1998). These findings suggest that the SNS normally inhibits the growth of fat pads, not only through changes in fat cell size via inhibition of lipolysis (Cantu and Goodman, 1967; Bray and Nishizawa, 1978; Lazzarini and Wade, 1991), but also through an undetermined mechanism affecting fat cell number. Strong support for this notion also comes from the inhibition of fat cell proliferation by NE in an in vitro cell culture system (Jones et al., 1992). Therefore, it seems likely

that the increase in WAT pad size after guanethidine treatment is at least partially due to increases in fat cell number, rather than to increases in fat cell size caused by decreases in lipolysis, and triggered by sympathetic denervation of the tissue. The increase in spleen mass after sympathetic denervation is reminiscent of the response of WAT to sympathetic denervation, prompting us to speculate that the SNS activity may generally modulate cellular hyperplasia in a variety of tissues, although we did not measure cell number in this experiment.

We initially were reluctant to apply guanethidine to Siberian hamsters because it has been suggested that it does not work when administered systemically in Syrian hamsters (*Mesocricetus auratus*), dogs or rabbits, (Johnson et al., 1977). It may be, however, that local application of guanethidine might produce an effective sympathectomy in these species, given its extreme effectiveness in the hamster species used in the present experiment. Finally, we have found that locally applied guanethidine to IWAT of laboratory rats appears to be effective. For example, guanethidine (30 injections, 10 μg/μl guanethidine, 3 μl per injection) injected in rat IWAT increased IWAT pad mass by $\sim 25\%$ and reduced NE content of the pad by $\sim 30\%$ compared with within-animal control pads (G.E. Demas and T.J. Bartness, unpublished results). Although these results are not as impressive as the results reported here for Siberian hamsters, it is important to note that IWAT pads are 5–10 times larger in laboratory rats than in Siberian hamsters. Thus, we may have underestimated the increase in guanethidine (i.e. dose, injection number and volume) required to compensate for the size differences in IWAT. These results also emphasize the importance of performing pilot experiments to determine the exact treatment regime for both the tissue and animal species of interest.

In summary, we have presented a chemical sympathetic denervation technique using guanethidine that has the advantages of being simple, rapid, effective and localized compared with the global sympathectomy techniques. In addition, this method produces greater NE depletions than either surgical denervation or local injections of DBH-saporin, at least with the injection/dose parameter used here for the latter. It is conceivable that DBH-SAP may have become as effective or more effective than guanethidine if we increased the dose injected; however, we stopped testing its effectiveness when the cost per hamster exceeded $500.00 (this cost in contrast to pennies per hamster with guanethidine). Finally, we also envision that this technique would be useful to study the effects of sympathetic denervation of not only WAT pads and spleen, but also of other peripheral tissues that would tolerate multiple injections during application (e.g. liver, adrenal gland).

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