Cold exposure regulates the norepinephrine uptake transporter in rat brown adipose tissue

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King, Victoria L., Linda P. Dwoskin, and Lisa A. Cassis. Cold exposure regulates the norepinephrine uptake transporter in rat brown adipose tissue. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R143–R151, 1999.—The neuronal uptake of norepinephrine (NE) in sympathetically innervated tissues is mediated by a high-affinity NE uptake transporter (NET). Rat interscapular brown adipose tissue (ISBAT) is densely innervated by the sympathetic nervous system for the control of cold- and diet-induced thermogenesis. To determine if cold exposure regulates the NET, kinetic parameters for [3H]NE uptake and [3H]nisoxetine (Nis) binding were determined in ISBAT from 7-day cold-exposed (CE) and control rats. Uptake of [3H]NE in ISBAT slices was of high affinity (1.6 µM). After 7 days of cold exposure the affinity for [3H]NE uptake was not altered; however, the uptake capacity was decreased (38%) in ISBAT slices from CE rats. Kinetic parameters for [3H]Nis binding demonstrated a single high-affinity site in ISBAT from CE and control rats with similar affinity. The density of [3H]Nis sites in ISBAT was decreased (38%) following cold exposure. A time course (2 h–7 days) for cold exposure demonstrated downregulation of [3H]Nis binding density by day 3, which remained through day 7. The affinity for [3H]Nis binding was transiently decreased at 2 h of cold exposure. Similarly, ISBAT NE content was decreased at 2 h of cold exposure. Pair feeding CE rats to food intake of controls normalized plasma NE content; however, [3H]Nis binding density in ISBAT remained decreased in pair-fed rats. These results demonstrate that ISBAT NET is downregulated following cold exposure. Reductions in ISBAT NE content precede alterations in NET density; however, plasma NE content is not related to regulation of the NET.

Interscapular brown adipose tissue; nisoxetine

THE NEURONAL UPTAKE of norepinephrine (NE) in sympathetically innervated tissues is mediated by a high-affinity (range 0.2–0.4 µM) NE transporter (NET) located on presynaptic sympathetic terminals in the central and peripheral nervous system (17). The NET regulates synaptic NE concentrations in sympathetically innervated tissues and serves as a conservatory mechanism for recycling neurotransmitter. Previous investigators have demonstrated regulation of the NET in response to agents modulating NE substrate content. Specifically, following reperfusion treatment and depletion of catecholamine stores, decreases were observed in steady-state mRNA levels for NET in adrenal medulla and locus coeruleus (10, 11). Moreover, reperfusion treatment resulted in a decrease in the number of NET binding sites in cerebral cortex and locus coeruleus (10, 11, 21). In vivo treatment with monoamine oxidase inhibitors, which increased tissue NE content, resulted in an increase in the number of NET sites in cerebral cortex (21). Short- and long-term inhibition of NET by in vivo desipramine treatment resulted in an increase in mRNA expression for the NET in locus coeruleus (33). Collectively, results from previous studies suggest that treatment with compounds that increase NE content positively regulate mRNA expression and protein density of the NET; conversely, treatment with compounds that deplete NE stores negatively regulate the NET.

The activity of the sympathetic nervous system is inversely related to environmental temperature, with an increase in sympathetic activity following cold exposure. Initial increases in sympathetic nerve activity following cold exposure (≤1 wk) result in generalized vasoconstriction and skeletal muscle shivering to maintain body temperature (20). During prolonged cold exposure, increases in sympathetic drive result in the long-term maintenance of body temperature through nonshivering thermogenesis. The organ most strongly stimulated by sympathetic activity following chronic cold exposure is brown adipose tissue. Rat interscapular brown adipose tissue (ISBAT) is a well-defined anatomic tissue, receiving bilateral sympathetic innervation via five intercostal nerves with innervation to the vasculature and individual brown adipocytes (13). Sympathetic innervation of brown adipose tissue is comparable to other densely innervated peripheral tissues (30). Activation of the sympathetic nervous system enables heat production in ISBAT through nonshivering thermogenesis (14). After different periods of cold exposure, an increase in the firing rate of sympathetic nerves to ISBAT results in catecholamine-mediated stimulation of brown adipocyte lipolysis, providing the fatty acid fuel for nonshivering thermogenesis (19). Evidence supporting an increase in sympathetic activity in ISBAT following cold exposure includes increased tyrosine hydroxylase activity (32), increased NE turnover (23, 39), and increased in vivo responsiveness to NE (19). Together, results from previous studies demonstrate that cold exposure represents a state of enhanced sympathetic neurotransmission to ISBAT to maintain body temperature through nonshivering thermogenesis.

In this study ISBAT was used as a model system for examination of substrate-mediated regulation of the NET. A variety of evidence demonstrates cold-induced elevations in sympathetic neurotransmission of ISBAT; however, the consequences of sympathetic activation on

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the NET have not been extensively examined. We hypothesized that increases in sympathetic neurotransmission following cold exposure positively regulate the ISBAT NET. In the present study the kinetic parameters for [3H]NE uptake in ISBAT slices from control and cold-exposed (CE) rats were defined. Moreover, radioligand binding analysis of the NET was characterized in ISBAT membranes from control rats using the selective inhibitor [3H]nisoxetine (Nis). Time courses examined [3H]Nis binding in ISBAT membranes from control and CE rats (2 h-7 days of cold exposure). Additionally, the effect of cold-induced increases in food intake on the kinetic parameters for [3H]Nis binding were determined.

**METHODS**

Animals. Male Sprague-Dawley rats (250–300 g; Harlan Sprague Dawley, Cumberland, IN) were used in all experiments. On arrival, rats were housed at a constant temperature of 24°C with a 12:12-h light-dark schedule and ad libitum access to food and water. For the reseprine study control rats received a single injection of reseprine (2.5 mg/kg ip; Sigma Chemicals, St. Louis, MO) 4 h before examination of [3H]NE uptake in ISBAT slices. For cold exposure studies rats were housed in individual cages for up to 7 days in an animal hibernarium maintained at a constant temperature of 4°C. Control rats were moved to individual cages for the duration of time corresponding to cold exposure. For the time course study body weight was measured before moving rats to the hibernarium and on the final day of the study. The experimental design assigned one rat from each group (control, 2 h, and 1, 3, 5, and 7 days) to be studied each day for 5 consecutive days. For the pair-feeding study, body weight, food intake, and water intake were measured in control and CE rats (7 days) every day at 10 AM. CE rats were pair fed based on the average food intake of control rats over the consecutive 24-h period; water intake was not limited. All rats were killed by decapitation.

Kinetic analysis of [3H]NE uptake in ISBAT slices. ISBAT was removed from rats, and 500 µm ISBAT slices (24 slices 10–15 mg) were made from vertically oriented adipose tissue sequentially across the entire fat pad using a McIlwain tissue chopper (7). ISBAT slices were preincubated for 30 min at 37°C in a Dubnoff metabolic shaking incubator in oxygenated (95% O₂-5% CO₂) Krebs bicarbonate buffer (40 ml) containing (in mM) 108 NaCl, 5.5 dextrose, 14.9 NaHCO₃, 0.11 ascorbic acid, 4.7 KCl, 1.18 KH₂PO₄, 1.2 MgSO₄, 1.3 CaCl₂, and 0.004 EDTA, pH 7.4. Pargyline (4 µM, Sigma) and metanephrine HCl (1 µM, Sigma) were added to the Krebs buffer to inhibit monoamine oxidase and extraneuronal uptake of NE, respectively. For measurement of [3H]NE uptake kinetics, individual ISBAT slices were transferred to conical vials containing buffer (400 µl) with a fixed concentration of L-[3H]NE (10.5 Ci/mmol specific activity; New England Nuclear, Boston, MA) 6 (nM) and increasing concentrations of unlabeled NE (10 nM-6 µM, 8 points; Sigma) in duplicate and incubated for 10 min at 37°C. Nonspecific uptake was determined by incubating ISBAT slices in duplicate with [3H]NE (6 nM) in the presence of the neuronal uptake inhibitor, nomifensine maleate (10 µM; RBI, Natick, MA). The incubation was terminated via vacuum filtration over glass-fiber filters (no. 30; Schleicher & Schuell, Keene, NH) using a Millipore 12-well manifold followed by washing filters three times with 10 ml of ice-cold buffer. ISBAT slices retained on filters were removed, weighed, and solubilized in 500 µl TS-2 (Research Products, Mt. Prospect, IL) by incubation at 50°C overnight. Scintillation cocktail (10 ml, 3a70B; Research Products) was added to the solubilized slices, and tissue [3H] was measured by liquid scintillation spectrometry using a Packard Tri-Carb Liquid Scintillation Analyzer with an efficiency for [H] counting of 65%. Saturation isotherms for specific [3H]NE uptake in ISBAT slices were constructed using GraphPad Prism 2 software, and the affinity (Kₘ) and maximal velocity (Vₘₐₓ) were derived using nonlinear regression analysis.

Kinetic analysis of [3H]Nis binding in ISBAT membranes. ISBAT was removed from rats, placed in 5 ml ice-cold buffer (in mM, 50 Tris, 120 NaCl, 5 KCl, pH 7.4), and homogenized three times for 10 s with a polytron (Kinematica). Homogenates were diluted to a total volume of 30 ml with the ice-cold membrane buffer and centrifuged in a supraspeed centrifuge (Sorvall RC28S; F-28/36 rotor) at 1,100 g for 15 min at 4°C. The supernatant was resuspended with 30 ml of ice-cold buffer and centrifuged at 40,000 g for 10 min at 4°C. The centrifugation procedure was repeated, and the final membrane pellet (1–2.5 mg protein/ml buffer) was resuspended in ice-cold binding buffer (in mM, 50 Tris, 300 NaCl, 5 KCl, pH 7.4). Protein concentration was determined spectrophotometrically using Coomassie blue dye with bovine serum albumin as the standard (2).

Saturation binding isotherms were performed by incubating duplicate aliquots of ISBAT membrane (70 µg) with increasing concentrations of N-[methyl-3H]Nis (5.9 Ci/mmol specific activity; New England Nuclear: 0.1–20 nM, 8 points, 50 µl) and binding buffer (250 µl final volume) for 30 min at 22°C. Nonspecific binding was determined at each concentration of [3H]Nis by the addition of mazindol (2 µM; RBI) and fluoxetine (1 µM; Lilly, Indianapolis, IN) to eliminate NE, dopamine, and serotonin uptake ligand binding sites (34, 35). Binding was terminated by filtration over preoaked (0.3% polyethyleneimine) glass microfiber filters (no. 32; Schleicher & Schuell) using a Brandel cell harvester. Radioactivity retained on the filters was measured by liquid scintillation spectrometry. Saturation isotherms for specific [3H]Nis binding to ISBAT membranes were constructed using GraphPad Prism 2 software. For determination of dissociation constant (Kₐ) and maximal binding density (Bₘₐₓ), data were analyzed by nonlinear regression analysis using LIGAND.

Measurement of plasma and ISBAT catecholamine content. The HPLC system consisted of a System Gold model 116 pump (Beckman, Fullerton, CA), a model 7725 injection valve fitted with a 50-µl sample loop (Rheodyne, Cotati, CA), a Coulochem model 5100A electrochemical detector (ESA, Bedford, MA), a model 5011 analytic cell (ESA), and a catecholamine HR-80 reverse-phase column (ESA, Bedford, TX). The mobile phase consisted of (in mM) 70 citric acid, 11 NaCl, and 2.5% (vol/vol) methanol, pH 4.0. The HPLC system was equilibrated with mobile phase for 12 h before use, and separation was achieved at a flow rate of 1.0 ml/min.

Free catecholamines were extracted from ISBAT or plasma as follows: 50–60 mg ISBAT was homogenized on ice in 1 ml of 0.4 N perchloric acid containing 400 pg of the internal standard [dihydropyrenylamine hydrobromide (DHBA), Sigma] and 200 µl of a 20-mol solution of 0.5% EDTA with 500 µg sodium metabisulfate. Homogenized samples were centrifuged at 12,365 g for 10 min, and the supernatant was transferred to a tube containing 25 mg of activated alumina...
(Bioanalytical Systems, West Lafayette, IN). For plasma extraction, 500 µl of plasma were added to 0.4 N Tris (containing inhibitors and internal standard) before addition of 25 mg of activated alumina. On addition of activated alumina the pH was adjusted to 8.6 by the addition of 3 M Tris·HCl buffer (pH 10.9). The samples were vortexed for 10 min, interrupting every 2 min to allow the alumina to settle. The alumina mixture was washed three times with 3 ml of a 1:1 dilution of Tris·HCl buffer (1.5 M Tris·HCl, 0.5 mM EDTA, and 0.4 mM sodium metabisulfite, pH 8.7) and water. The supernatant was removed and the alumina slurry was transferred to microfilter tubes (0.45 µm nylon; Alltech, Deerfield, IL) and centrifuged in a tabletop centrifuge at 3,500 rpm for 1 min. The supernatant from this centrifugation was discarded. Catecholamines were eluted from the alumina utilizing a two-step procedure consisting of the addition of 100 µl of 0.15 N perchloric acid, vortexing for 10 s, and centrifugation at 3,500 rpm for 1 min (total elution volume of 200 µl). The eluent (50 µl) was injected onto the HPLC for catecholamine analysis. A set of catecholamine standards (50–900 pg NE and epinephrine) were used to determine plasma catecholamine content with the DHBA standards (50–900 pg NE and epinephrine) were used to determine plasma catecholamine content with the DHBA content in extracts used to correct for recovery.

Statistics. Results from all studies are presented as means ± SE. For kinetic parameters derived from [3H]NE uptake studies in ISBAT slices from CE and control rats, a Student's unpaired t-test was used to test for significant difference. For tissue NE content and kinetic parameters derived from [3H]Nis binding in the CE time course study, a one-way ANOVA (time as a between group factor) was used followed by Tukey's multiple comparison test for post hoc analysis of time effect. For plasma and tissue NE content and kinetic parameters derived from [3H]Nis binding in ISBAT membranes from pair-feeding studies, a two-way ANOVA (CE, pair-feeding as between group factors) was used followed by Tukey's multiple comparison test for post hoc analysis of treatment effect. Statistical significance was accepted at P < 0.05.

RESULTS

Characterization of [3H]NE uptake and [3H]Nis binding in control rat ISBAT. Preliminary experiments established that [3H]NE uptake in vertically oriented slices of ISBAT across the entire pad was of similar magnitude, indicating sufficient homogeneity of innervation for uniform analysis of neuronal uptake in ISBAT slices (data not shown). Nonspecific uptake of [3H]NE in ISBAT slices represented <24% of total [3H]NE uptake. Initial experiments demonstrated that the time course for specific [3H]NE uptake in ISBAT slices from control and CE rats was linear over the 90-min time course examined (data not shown). An incubation time of 10 min was chosen from the linear part of the curve representative of the initial velocity and used in subsequent [3H]NE uptake experiments to determine saturation kinetics of the ISBAT NET.

Saturation isotherms for [3H]NE (6 nM) uptake in ISBAT slices were performed to determine the affinity (Km) and capacity (Vmax) for the NET in control rats. [3H]NE uptake in ISBAT slices was of high affinity (Km 1.62 ± 0.35 µM) and low capacity (Vmax 392 ± 46 fmol·mg wet wt tissue−1·min−1; Fig. 1 and Table 1). Previous investigators have demonstrated that accurate examination of the initial velocity for neuronal uptake requires elimination of vesicular stores of catecholamines (1). To determine the effect of vesicular catecholamine stores on the kinetic analysis of [3H]NE uptake, control rats were treated with reserpine 4 h before removal of ISBAT. This treatment regimen for reserpine has been previously demonstrated to result in significant depletion of endogenous catecholamine stores in tail arteries from spontaneously hypertensive and Wistar-Kyoto rats (8). Reserpine pretreatment did not alter the kinetic parameters for [3H]NE uptake in ISBAT slices (Fig. 2 and Table 1).

For radioligand binding studies, the selective noradrenergic inhibitor [3H]Nis was used as a ligand for the NET binding site (35). Initial experiments determined [3H]Nis (5 nM) binding over a range of ISBAT membrane protein concentrations 60 min at 22°C (Fig. 3A). [3H]Nis binding was linear over the examined range of ISBAT membrane protein concentrations. Subsequent experiments were performed using 70 µg of ISBAT membrane protein at each concentration of [3H]Nis. To determine the time course for equilibrium binding, ISBAT membrane protein was incubated with a fixed concentration (5 nM) of [3H]Nis at 22°C for various times (Fig. 3B). Specific [3H]Nis binding plateaued at 10 min and remained unchanged over 60 min.

![Figure 1. Saturation isotherm for specific [3H]noradrenaline (NE) uptake in interscapular brown adipose tissue (ISBAT) slices from control and cold-exposed (CE) rats. ISBAT slices from control and CE rats were prepared as described in METHODS for examination of [3H]NE uptake. Nonspecific uptake was determined by incubation of slices from control and CE rats in presence of nonisofensine. The affinity (Km) for [3H]NE uptake was not different between ISBAT slices from control and CE rats. Maximal velocity (Vmax) was significantly (P < 0.05) decreased in ISBAT slices from CE rats compared with control. Data are means ± SE of control and CE rats (n = 10 rats/group).](image)

Table 1. Kinetic parameters for [3H]NE uptake in ISBAT slices

<table>
<thead>
<tr>
<th>Rats</th>
<th>n</th>
<th>Km, µM</th>
<th>Vmax, fmol·mg wet wt tissue−1·min−1</th>
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<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>1.62 ± 0.35</td>
<td>392 ± 46</td>
</tr>
<tr>
<td>CE, 7 days</td>
<td>10</td>
<td>1.09 ± 0.19</td>
<td>245 ± 25*</td>
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<tr>
<td>Reserpine</td>
<td>5</td>
<td>1.80 ± 0.82</td>
<td>318 ± 82</td>
</tr>
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Values are means ± SE; n = no. of rats. NE, norepinephrine; ISBAT, interscapular brown adipose tissue; Km, affinity; Vmax, maximal velocity; CE, cold exposed. *Significantly different from control, P < 0.05.
From these data an incubation time of 30 min at 22°C was chosen for all saturation binding isotherms.

Regulation of \([ {3H} \text{NE}] \) uptake and \([ {3H} \text{Nis}] \) binding following 7 days of cold exposure.

Saturation isotherms for \([ {3H} \text{NE}] \) uptake in ISBAT slices were performed to determine the affinity \( (K_m) \) and capacity \( (V_{max}) \) for the NET following 7 days of cold exposure. The affinity for \([ {3H} \text{NE}] \) uptake was not different in ISBAT slices from control and CE rats (Table 1 and Fig. 1). However, the \( V_{max} \) for \([ {3H} \text{NE}] \) uptake was decreased (38%) in ISBAT slices from CE rats compared with control (Table 1 and Fig. 1).

To determine if decreases in the capacity for \([ {3H} \text{NE}] \) uptake in ISBAT slices from CE rats were the result of a diminished number of NET sites, saturation isotherms for \([ {3H} \text{Nis}] \) binding were performed in ISBAT membranes from control and 7-day CE rats. \([ {3H} \text{Nis}] \) bound with high affinity to a single class of binding sites (Hill coefficient 1.06 ± 0.05; 0.95–1.17 confidence interval) in ISBAT membranes from control and CE rats. The affinity of \([ {3H} \text{Nis}] \) binding in ISBAT was not altered in CE rats compared with control; however, the density (pmol/mg protein) of \([ {3H} \text{Nis}] \) binding sites was decreased (38%) in ISBAT membranes from CE rats compared with control (see Fig 5A).

Time course for cold-induced regulation of the NET.

Control rats gained weight over the 7-day time course (Table 2). Body weight decreased from 2 to 3 days of cold exposure, followed by a rebound in body weight of CE rats from 5 to 7 days (Table 2). ISBAT mass (expressed as a percentage of body weight) increased following cold exposure (Table 2). At 2 h of cold exposure ISBAT NE content decreased followed by a gradual return to levels not significantly different from control at 7 days of cold exposure (Fig. 4).

To determine the time course for cold-induced regulation of NET density, saturation isotherms for \([ {3H} \text{Nis}] \) binding were performed in ISBAT membranes from control and CE rats (2 h-7 days). After 2 h of cold exposure the \( K_d \) for \([ {3H} \text{Nis}] \) binding was increased in ISBAT membranes from CE rats compared with control (Table 3 and Fig. 5, A and B). On longer cold exposure (≥3 days) the \( K_d \) for \([ {3H} \text{Nis}] \) binding returned to levels not significantly different from control. The density of \([ {3H} \text{Nis}] \) binding decreased over time following cold exposure (Table 3 and Fig. 5, A and B). \([ {3H} \text{Nis}] \) binding density (pmol/mg protein) was significantly decreased compared with control at 3 days of cold exposure, with a

<table>
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<th>Table 2. Time course study weight change and ISBAT mass</th>
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<tr>
<td>Rats</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>CE, 2 h</td>
</tr>
<tr>
<td>CE, 24 h</td>
</tr>
<tr>
<td>CE, 3 days</td>
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<tr>
<td>CE, 5 days</td>
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<td>CE, 7 days</td>
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Values are means ± SE from control \(( n = 10) \) and CE \(( n = 5) \) rats. *Significantly different from control, \( P < 0.05 \).
maximum of 40% reduction in binding density at 7 days (Table 3 and Fig. 5, A and B). When normalized to total ISBAT protein content (pmol/mg protein), [3H]Nis binding density represents the specific activity of the NET protein. When [3H]Nis binding density was normalized to account for the increased mass of tissue following cold exposure (see Table 2), the absolute number (fmol/g tissue wet wt) of [3H]Nis binding sites in the entire ISBAT was significantly decreased over time following cold exposure (Table 3).

Effect of pair feeding on cold-induced regulation of NET. Food and water intake increased ~30% following 7 days of cold exposure (Fig. 6, A and B). Pair-fed rats maintained at 4°C were restricted to the average food intake (27.3 ± 0.9 g) of ambient temperature control rats. Pair-feeding of CE rats eliminated the increase in water intake (Fig. 6B). Body weight decreased in CE rats compared with controls, with pair-fed rats losing weight over the 7-day study (Fig. 6C).

When normalized to body weight, ISBAT mass increased (~43 and 38%, respectively) in CE and CE pair-fed rats compared with control; however, ISBAT tissue weight did not increase in pair-fed CE rats. Plasma NE content was increased in CE rats compared with control (control 4.10 ± 0.72, CE 8.90 ± 0.65 ng/ml, P < 0.05); however, plasma NE content in pair-fed CE rats (3.11 ± 0.78 ng/ml) was not different from control.

To determine the effect of cold-induced increases in food and water intake on the regulation of the NET, saturation isotherms for [3H]Nis binding were performed in ISBAT membranes prepared from control, CE, and pair-fed CE rats. The affinity for [3H]Nis binding was similar in ISBAT membranes from all three groups of rats (Table 4 and Fig. 7, A and B). [3H]Nis binding density (pmol/mg protein) was decreased in ISBAT from CE (37%) and pair-fed CE rats (40%) compared with control (Table 4 and Fig. 7, A and B).

Table 3. Time-dependent alterations in kinetic parameters for [3H]Nis binding in ISBAT membranes from CE and control rats

<table>
<thead>
<tr>
<th>Rats</th>
<th>Kd, nM</th>
<th>Bmax, pmol/mg protein</th>
<th>Bmax, fmol/g tissue wet wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.87 ± 0.17</td>
<td>1.74 ± 0.11</td>
<td>28.4 ± 1.9</td>
</tr>
<tr>
<td>CE, 2 h</td>
<td>4.18 ± 0.29*</td>
<td>1.87 ± 0.09</td>
<td>42.8 ± 9.9</td>
</tr>
<tr>
<td>CE, 24 h</td>
<td>3.13 ± 0.43</td>
<td>1.60 ± 0.19</td>
<td>33.7 ± 4.4</td>
</tr>
<tr>
<td>CE, 3 days</td>
<td>2.45 ± 0.32</td>
<td>1.11 ± 0.06*</td>
<td>17.6 ± 2.6*</td>
</tr>
<tr>
<td>CE, 5 days</td>
<td>2.54 ± 0.33t</td>
<td>1.02 ± 0.06*</td>
<td>23.4 ± 6.5*</td>
</tr>
<tr>
<td>CE, 7 days</td>
<td>2.12 ± 0.12t</td>
<td>1.04 ± 0.20*</td>
<td>20.5 ± 1.8*</td>
</tr>
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</table>

Values are means ± SE; n = 5 rats per group per time point. Nis, nisoxetine. *Significantly different from control, P < 0.05. †Signiﬁcantly different from CE, 2 h, P < 0.05.
DISCUSSION

Results from this study do not support the hypothesis that elevated sympathetic neurotransmission in ISBAT following cold exposure positively regulates the NET. In contrast, following 7 days of cold exposure at 4°C, the specific activity of the NET protein (by binding density) and the functional capacity of [3H]NE uptake were decreased in ISBAT. Decreases in uptake and binding density for the NET in ISBAT were evident despite elevated plasma NE content. Moreover, normalization of plasma NE content by pair feeding CE rats did not influence cold-induced downregulation of NET binding density. Results from time course studies demonstrated that reductions in ISBAT NE content preceded downregulation of the NET. Collectively, these results demonstrate that cold exposure downregulates the NET in ISBAT. Moreover, results from this study suggest that cold-induced downregulation of the ISBAT NET occurs subsequent to enhanced turnover of ISBAT sympathetic activity.

The description and initial characterization of the NET occurred in the early 1960s (16), with cloning of the transporter for NE uptake in 1991 (24). Since the initial description of the NET, research has addressed the kinetics of transport and definition of the substrate binding site (4). However, there have been relatively...
few studies specifically addressing regulation of the NET. Previous studies demonstrated that acute and chronic treatment with tricyclic antidepressants increased mRNA for the NET in rat locus coeruleus (33). Other regulators of the NET that have been examined in rats include reserpine (decrease in uptake sites and mRNA expression; 10, 21) and monoamine oxidase inhibitors (increase in uptake sites; 10). Collectively, results from previous studies suggest that the NET is positively regulated by NE substrate levels.

The activity of the NET in brown adipose tissue has not been examined extensively. To kinetically characterize the uptake of [3H]NE in ISBAT several conditions pertaining to assessment of transporter activity were optimized, including examination of substrate uptake during the initial velocity of the transporter, inhibition of substrate metabolism during the experimental incubation, and removal of the influences of vesicular and extraneuronal uptake of substrate in the tissue preparation. Kinetic analysis of [3H]NE uptake in ISBAT slices demonstrated a single site transporter with an affinity in the range of that previously described for the NET (17, 24). The maximal functional capacity ($V_{\text{max}}$) for [3H]NE uptake in ISBAT slices was comparable to brain regions with dense noradrenergic innervation (9, 31). Moreover, results from this study are in agreement with previous studies reporting high catecholamine concentrations and extensive catecholamine histofluorescence in ISBAT as evidence for the density of sympathetic innervation (30).

Past findings have established that in animals chronically exposed to cold, shivering thermogenesis is replaced by nonshivering thermogenesis (15). Brown adipose tissue has been demonstrated to be a major site of nonshivering thermogenesis following cold exposure, accounting for an increase in heat production and resultant metabolic rate (29). Previous studies have demonstrated that the majority of the adaptive changes in essential components of ISBAT occur following 1 wk of cold exposure (26). However, as early as 1 h following cold exposure there is an increase in [3H]GDP binding to ISBAT mitochondria, indicative of increased thermogenic activity (22). Additional changes that have been observed in CE rats include increases in ISBAT weight (5, 6, 36), protein content (7), uncoupling protein (15), lipoprotein lipase (27), plasma NE content (25), and food intake (18). In agreement with previous studies, results from the present study demonstrate that cold exposure resulted in increased food intake, increased ISBAT mass, and elevated plasma NE content.

Previous estimations of turnover of NE as an index of sympathetic activity demonstrated a rapid activation of sympathetic activity within 4 h of cold exposure that was sustained for 10 days (38). In agreement with previous results, in the present study ISBAT NE content decreased following acute cold exposure, suggesting increased turnover of ISBAT catecholamines (26). Moreover, in agreement with previous studies (25), plasma NE content increased following 7 days of cold exposure. Collectively, these results demonstrate activation of the sympathetic nervous system following cold exposure.

Although it is well recognized that increases in sympathetic nervous system activity account for the majority of stimulated thermogenesis in response to cold, there have been few studies examining indexes of sympathetic neuroeffector function at the level of the sympathetic nerve terminal in brown adipose tissue. Moreover, the ramifications of cold-induced stimulation of sympathetic neurotransmission to ISBAT on regulation of the NET have not been extensively investigated. Previous studies in our laboratory have demonstrated a decrease in [3H]NE uptake in ISBAT slices following 7 days of cold exposure; however, kinetic analysis of the NET was not performed (7). Results from this study confirm and extend previous results, demonstrating a decrease in the maximum capacity for [3H]NE uptake in ISBAT slices following 7 days of cold exposure. However, these results do not support previous observations of substrate-mediated positive regulation of the ISBAT NET (10, 11, 21). Potential mechanisms contributing to a lack of substrate-induced positive regulation of the ISBAT NET in the present study include cold-induced alterations in a variety of humoral factors, differences between peripheral and central sympathetic neurons, and the degree of cold-induced activation of sympathetic neurotransmission.

The highly selective radioligand for the NET binding site, [3H]Nis, was used in these studies to determine if reductions in the functional capacity for [3H]NE uptake in ISBAT slices from CE rats were the result of a decreased density of NET binding sites. Previous investigators have demonstrated a 400- and 1,000-fold greater affinity of [3H]Nis for the NET compared with the dopamine and serotonin transporters, respectively (34, 37). Moreover, compounds that fail to inhibit the binding of [3H]Nis also have limited ability to inhibit [3H]NE uptake (34). Radioligand binding analysis using [3H]Nis in ISBAT membranes was saturable, protein-dependent, and demonstrated a single, high-affinity binding site with pharmacological characteristics similar to those previously identified (34). In agreement with the observed reductions (38%) in maximal functional capacity ($V_{\text{max}}$) for [3H]NE uptake, the density of [3H]Nis binding sites was reduced by 38% following 7 days of cold exposure.

Time-course studies demonstrated that cold-induced downregulation of [3H]Nis binding density occurred between 1 and 3 days of cold exposure and was preceded by reductions in ISBAT catecholamine content. To determine the role of tissue hypertrophy and hyperplasia in the observed regulation of the NET following cold exposure, [3H]Nis binding density was normalized by multiplying the density of [3H]Nis sites by the total tissue wet weight. This normalization allowed for calculation of the total number of NET binding sites in the entire ISBAT organ. Reductions in [3H]Nis binding density in response to cold exposure were evident by specific activity (pmol/mg protein) and absolute number (pmol/g wet wt). Moreover, correlation regression analysis of [3H]Nis binding density by ISBAT mass
was not significant ($r^2 = 0.029$). Collectively, these results demonstrate that reductions in $[^3H]Nis$ binding density following cold exposure are independent of tissue hypertrophy. Alternatively, the time course for cold-induced alterations in ISBAT NE content and $[^3H]Nis$ binding density suggests substrate-induced downregulation of the ISBAT NET following cold exposure.

Interestingly, in the present study the affinity for $[^3H]Nis$ binding in ISBAT decreased following acute (2 h) cold exposure. Reductions in the affinity for $[^3H]Nis$ binding following 2 h of cold exposure occurred independently of alterations in ISBAT NET density. These results demonstrate differential regulation of the NET following acute and chronic cold exposure, beginning with a transient reduction in binding affinity followed by downregulation of the number of NET sites on chronic cold exposure. Although results from the present study do not conclusively identify NE as the cold-induced modulator of the ISBAT NET, the time course for ISBAT NE content and regulation of $[^3H]Nis$ binding kinetic parameters ($K_d$, $B_{max}$) following cold exposure support further study of substrate-mediated regulation of the ISBAT NET.

It is generally accepted that elevations in food intake, as observed in the present study, result in stimulation of sympathetic nervous system activity (38). Conversely, previous studies have demonstrated that food restriction lowered turnover of NE in ISBAT (3, 39). Interestingly, in this study elevations in plasma NE content were normalized by pair feeding CE rats to food intake levels of controls. However, cold-induced reductions in $[^3H]Nis$ binding density remained evident despite restricted food intake. Previous studies have demonstrated that pair feeding CE rats to food intake levels of controls limited cold-induced hypertrophy of ISBAT (18). In agreement with these results, in the present study ISBAT tissue weight was not increased in CE rats that were pair fed. Importantly, reductions in ISBAT NET density remained evident in CE rats that were pair fed, demonstrating that reductions in ISBAT NET density were independent of changes in tissue mass. Collectively, these results demonstrate that reductions in the density of ISBAT NET sites are independent of cold-induced elevations in food intake, plasma NE content, and tissue mass.

In summary, results from this study demonstrate time-dependent regulation of the ISBAT NET following cold exposure. Acute cold exposure decreased the affinity of the ISBAT NET, whereas chronic cold exposure decreased the density of NET binding sites. Cold-induced alterations in the NET occurred independent of tissue mass, plasma NE content, and food intake. In contrast, cold-induced alterations in the ISBAT NET occurred coincident with (acute) or before (chronic) changes in ISBAT NE content. These results suggest that in the cold exposure model of heightened sympathetic neurotransmission, the functional activity of the ISBAT NET is reduced in a substrate-related manner, potentially contributing to the maintenance of stimulated thermogenesis.

**Perspectives**

Our results imply that the synaptic concentration of neurotransmitter substrate, i.e., NE, inversely regulates the density of the neuronal uptake transporter on peripheral sympathetic nerve terminals. In response to cold exposure and enhanced sympathetic drive, reductions in the density of uptake transporter sites on ISBAT sympathetic nerve terminals would limit removal of released NE, resulting in maximal stimulation at postsynaptic receptor sites. Importantly, the observed downregulation in NET density in brown adipose tissue would be anticipated to contribute to the maintenance of thermogenesis. We speculate that substrate-mediated regulation of the NET may extrapolate to other peripheral tissues innervated by the sympathetic nervous system. The broad implication of this finding is that pathophysiological states associated with altered synaptic concentrations of NE would exhibit substrate-dependent regulation of NET density. For example, in a disease state such as congestive heart failure with documented increases in sympathetic drive, we hypothesize that downregulation of NET density on cardiac sympathetic nerve terminals would potentially elevate synaptic NE concentrations and alter postsynaptic receptor responsiveness. Finally, we hypothesize that substrate-dependent regulation of the NET in peripheral sympathetic terminals may contribute to regional heterogeneity in peripheral sympathetic activation.

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