Thermoregulation with Age: Restoration of β_3-Adrenergic Responsiveness in Brown Adipose Tissue by Cold Exposure (43983)

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Abstract. **The β_3-adrenergic–stimulated thermogenic response in brown adipose tissue (BAT) is impaired in senescent rats, whereas cold-induced thermogenesis is not. To determine if cold exposure can restore β_3-adrenergic receptor responsiveness in senescent rats, we examined BAT mitochondrial GDP binding in young and old rats, and UCP mRNA levels in young rats following stimulation by the β_3-adrenergic agonist CGP-12177 with and without prior cold exposure. F-344 male rats were maintained at thermoneutrality or exposed to 8°C for 48 hr, followed by a 24-hr period of rewarming before administration of 0.75 mg/kg CGP-12177 or vehicle solution. During the rewarming period, GDP binding remained elevated but UCP mRNA levels with a half-life of 11 hr returned to levels observed in the thermoneutral controls. In young rats, both cold exposure and administration of the β_3-adrenergic agonist to thermoneutral controls increased GDP binding 2-fold and UCP mRNA levels 4-fold. However, in cold-exposed young rats, there was no further increase with β_3-agonist treatment. In senescent control rats, CGP-12177 did not increase GDP binding, but cold exposure did. However, in cold-exposed old rats, the β_3-agonist was now able to increase GDP binding. The induction of UCP mRNA by CGP-12177 was also investigated and found to be 25% less in senescent compared with young rats. These observations indicate that cold exposure restores the impaired β_3-adrenergic signal transduction in BAT from senescent rats. One possibility is that cold exposure induces the synthesis of one or more components in the β_3-adrenergic pathway in senescent rats.** [P.S.E.B.M. 1996, Vol 211]

The ability to regulate body temperature in response to cold diminishes with senescence (1–3). Nonshivering thermogenesis in brown adipose tissue (BAT) is an important contributor of the heat necessary to maintain body temperature (4). Thermogenesis in BAT is mediated by norepinephrine activation of adenylyl cyclase through sympathetically innervated β_3-adrenergic receptors (5, 6).

We previously reported that thermogenesis, as indicated by the increase in whole body oxygen consumption, body temperature, and GDP binding to the mitochondrial uncoupling protein (UCP) was decreased or absent in senescent rats following stimulation with a β_3-adrenergic agonist (7). This suggested that the β_3-adrenergic pathway in BAT is impaired in aged rats. Recently, we reported that thermogenesis in BAT stimulated by forskolin, a direct activator of adenylyl cyclase, was unchanged between young and old rats (8). Collectively, these data suggest that the β_3-adrenergic receptor or coupling of this receptor to adenylyl cyclase may be impaired with senescence.

Surprisingly, we recently reported that the increase in mitochondrial GDP binding and the induction of UCP mRNA were unchanged between young and old rats following cold exposure, despite a hypothermia in the senescent animals (1). These studies indi-

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cate that the hypothermia in senescent rats is due to factors other than thermogenesis in interscapular BAT. Moreover, these studies suggest that the cold-stimulated thermogenesis in BAT may be mediated by a receptor other than the β_3-adrenergic receptor in senescent rats. Another possibility is that cold exposure upregulates one or more components of the β_3-adrenergic receptor pathway such that responsiveness is restored in the senescent rats. To investigate further the latter possibility, we examined BAT mitochondrial GDP binding in young and old F-344 male rats and UCP mRNA levels in young rats following stimulation by the β_3-adrenergic agonist CGP-12177 with and without prior cold exposure.

Materials and Methods

Animals. Male F-344 NNia rats of 6 and 24 months of age were obtained from Harlan Sprague-Dawley (Indianapolis, IN) under contract with the National Institute on Aging. Upon arrival, rats were examined and remained in quarantine for 1 week. Animals were cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals*. Rats were housed individually in microisolated cages and maintained on Purina Rat Chow *ad libitum* with a 12:12-hr light:dark cycle (0600 to 1800 hr). Experiments were begun 60–90 min after the beginning of the light cycle. Food and water were provided during the overnight periods of cold exposure, and bedding material was kept at an absolute minimum. Ambient temperature was 26°C.

Experimental Design. All experiments were performed on conscious unanesthetized rats during the light phase of the light:dark cycle that corresponded to the normal sleeping period. The central experiment consisted of a two-phase design. Twelve young and twelve old rats were exposed to the cold (8°C) for 2 days followed by a 24-hr rewarming period at 26°C. One of the senescent rats died. An additional 12 young and 12 old rats were maintained at 26°C. One-half of the rats in the control and experimental groups of each age were then administered 0.75 mg/kg CGP-12177 and sacrificed 1 hr later.

Isolation of IBAT Mitochondria. Rats were sacrificed by cervical dislocation under 50 mg/kg pentobarbital anesthetic. The circulatory system was perfused with 20 ml of cold saline and interscapular BAT (IBAT) excised and trimmed of visible white fat. Mitochondria for use in the GDP binding assay were isolated as described previously (9). The final pellet was resuspended in 100 m*M* sucrose, 1.2 m*M* K-EDTA, 12.2 m*M* choline Cl, and 40 m*M* K-TES, pH 7.1. Mitochondria were used immediately in the GDP binding assay.

GDP Binding Assay. The density of binding sites was determined from a multipoint Scatchard analysis of [^{3}H]GDP binding (specific activity adjusted to 1.012 Ci/mmole; Dupont NEN, Boston, MA) as previously described (9). Briefly, protein (50 μg) and 0.02–3.0 μ*M* [^{3}H]GDP were incubated both with and without 1.5 m*M* unlabeled GDP for 15 min at 37°C in a total volume of 250 μl of the buffer used for final suspension with the addition of 0.1 mg/ml fatty acid-free BSA and 2 μ*M* rotenone. The reaction was terminated by dilution with buffer, and the bound [^{3}H]GDP separated from free by filtration over glass fiber filters (Whatman GF/B, Clifton, NJ). Specific binding was determined from the difference in binding with and without 1.5 m*M* unlabeled GDP.

UCP mRNA. Total cellular RNA and UCP mRNA were determined by extraction of minced and sonicated IBAT tissue by modification of a previously described procedure (1). For extraction, 100 mg of tissue were sonicated in 1 ml of 4 *M* guanidine thiocyanate, 25 m*M* Na citrate, pH 7.0. One milliliter of phenol, 50 μl of 10% Sarkosyl, 100 μl 2 *M* Na acetate, pH 5.0, and 200 μl of chloroform:isoamyl alcohol (49:1) were added to the sonicated IBAT, and this preparation was mixed vigorously, incubated 15 min on ice, centrifuged, and the aqueous phase retained. The RNA was twice precipitated with an equal volume of isopropanol, washed with 70% ethanol, suspended in 0.5% SDS, and heated to 65°C for 10 min. After centrifugation, the supernatant was harvested. The integrity of the isolated RNA was verified using agarose gels (1%) stained with ethidium bromide. The RNA was quantified by spectrophotometric absorption at 260 μm using multiple dilutions of each sample. For measurement of UCP mRNA levels, several concentrations of serially diluted RNA samples were immobilized on nylon membranes (Gene Screen; Dupont NEN, Boston, MA) using a slot blot apparatus (BioRad, Richmond, CA). The membranes were baked at 80°C for 2 hr. The baked membranes were prehybridized using 25 m*M* potassium phosphate, 750 m*M* NaCl, 75 m*M* Na citrate, 5× Denhardt's solution, 50 μg/ml denatured salmon sperm DNA, and 50% formamide. After incubation for 14–16 hr at 42°C, the membranes were hybridized with a ^{32}P random prime-labeled cDNA probe in the prehybridization buffer with the addition of 10% dextran sulfate (1). The cDNA clone for UCP was kindly provided by Dr. Leslie Kozak from the Jackson Laboratory (Bar Harbor, ME) (10) and verified by Northern analysis as previously described (4). After hybridization for 14–16 hr at 42°C, the membranes were washed and exposed to x-ray film (Kodak X-AR, Rochester, NY) for 96 hr at −70°C using intensifying screens. Optical density per microgram total cellular RNA was calculated by comparison with internal laboratory standards of IBAT UCP mRNA present on each nylon membrane.

Statistical Analysis. Differences with cold expo-

sure and age were determined by two-way analysis of variance (ANOVA) followed by Scheffe's post hoc analysis to determine differences between individual means.

Results

Rats were cold exposed at 8°C for 48 hr in an attempt to upregulate the β_3-adrenergic stimulated thermogenic pathway prior to administration of CGP-12177. The temperature of 8°C was chosen because we have previously shown that at this temperature the older rats are able to maintain homeothermy (1). In contrast, at a lower temperature of 4°C, the senescent rats became progressively more hypothermic (1). The acclimation time of 48 hr was chosen based on initial investigations of both 48-hr and 1-week cold exposure. The objective was to find a period of cold exposure that increased GDP binding to mitochondrial membranes and that was not reversed by a short rewarming time. It was preferable to administer the CGP-12177 to rats that were rewarmed to thermoneutrality (26°C) to minimize endogenous stimulation of BAT in order to better compare with the control animals. In young rats, a 48-hr cold exposure increased mitochondrial GDP binding by 2-fold compared with rats maintained at thermoneutrality (26°C). This increased level of binding was mostly sustained during a 36-hr rewarming period (Fig. 1). Similarly, in senescent rats, a 48-hr cold exposure increased the GDP binding 2-fold with only a slight decrease during the 36-hr period of rewarming (Fig. 1). In contrast, a 1-week cold exposure increased the GDP binding by 4-fold in young rats that was maintained for 24 hr after rewarming but decreased between 24 and 36 hr (Fig. 1). Although the 1-week cold exposure manifested a greater increase in thermogenic capacity, the partial reversal with rewarming rendered this protocol less desirable. The 48-hr acclimation period was examined further by assessing UCP mRNA levels in young and old rats. The 48-hr cold exposure increased UCP mRNA levels 4- to 5-fold (Fig. 2). During the rewarming period, UCP mRNA levels decreased to control values by 24–36 hr of rewarming (Fig. 2). A similar pattern of decrease of mRNA levels with rewarming was observed in the senescent rats (Fig. 2). The UCP mRNA half-life in both young and old rat was calculated to be 11 hr. These data indicate that following a 48-hr cold exposure and a 24-hr rewarming, UCP mRNA levels are not significantly different from that of rats maintained at thermoneutrality, thus this protocol was chosen for further studies.

Six-month-old rats were chosen for the young group to minimize the weight differences between young and old rats. The F-344 rats demonstrated only a modest weight gain between 6 and 24 months of age (Table I). The amount of interscapular BAT recovered was somewhat less in the senescent compared with the young rats (Table I). Another important and perhaps more relevant parameter is the mitochondrial protein per gram of BAT. This parameter increased following 48 hr of cold exposure at 8°C, indicating recruitment of new mitochondria protein during cold exposure has occurred (Table I).

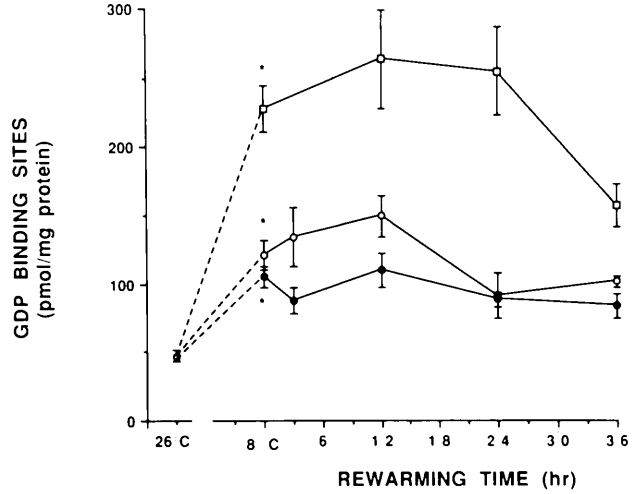

Figure 1. GDP binding to BAT mitochondrial membranes in young and senescent rats maintained at thermoneutrality (values indicated by 26°C), in young rats cold exposed for 1 week (values indicated by 8°C), in young rats cold exposed for 48 hr (8°C, ○), or in senescent rats cold exposed for 48 hr (8°C, ●). Following cold exposure, the rats were returned to 26°C for the times indicated. Data represents the mean ± SEM of four to six rats per group except in the 12, 24, and 36 hr rewarming after 1-week cold-exposed groups (□) where the data represent the average of two rats. *$P < 0.001$ for difference from corresponding thermoneutral control by Student's t test.

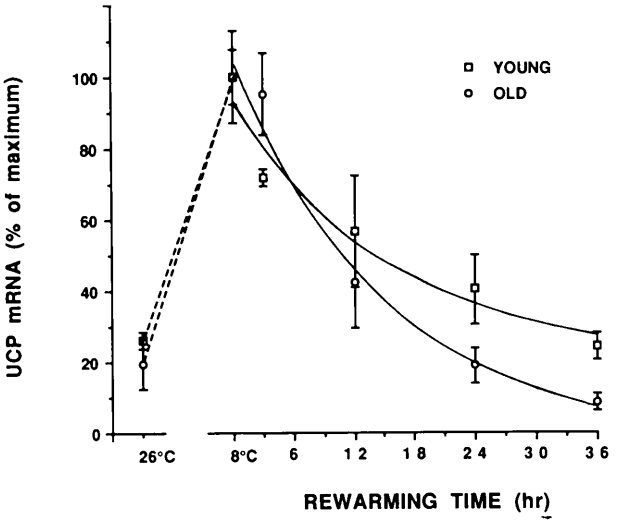

Figure 2. UCP mRNA levels from BAT of young and senescent rats maintained at thermoneutrality (values indicated by 26°C) or cold exposed for 48 hr (values indicated by 8°C). Following cold exposure, the rats were returned to 26°C for the times indicated. Data represents the mean ± SEM of three or four rats per group. $P < 0.001$ for difference from 8°C values and corresponding thermoneutral control by Student's t test.

Table I. Effects of Cold Exposure and Age on Body and Interscapular BAT Parameters

Parameters	Age			
	6 months		24 months	
	Thermoneutral	Rewarmed cold exposed	Thermoneutral	Rewarmed cold exposed
Body weight (g)	361 ± 12	381 ± 5	406 ± 12	421 ± 8
BAT weight (mg)	271 ± 17	295 ± 24	189 ± 23	245 ± 30
Mitochondria protein/BAT (mg/g)	6.25 ± 0.70	8.35 ± 0.67[a]	4.94 ± 0.8	10.05 ± 1.35[a]

Note. Data represents the mean ± SEM of 10–13 rats in each group.
[a] $P < 0.001$ for difference with cold exposure by two-way ANOVA. $P < 0.04$ (young) and $P < 0.003$ (old) for difference with cold exposure by Scheffe's post hoc analysis.

To evaluate the effects of cold exposure on the efficacy of CGP-12177 to stimulate GDP binding, rats were maintained at thermoneutrality or acclimated to 8°C for 48 hr, followed by a 24-hr period of rewarming. The rats were administered 0.75 mg/kg CGP-12177 or vehicle solution and sacrificed 1 hr later for assessment of GDP binding to mitochondrial membranes or UCP mRNA levels in BAT.

Young Rats. The β_3-adrenergic–specific agonist CGP-12177 increased the density of GDP binding by 65% in rats maintained at thermoneutrality (Table II). Similarly, with cold exposure, there was an 82% increase in GDP binding sites compared to the thermoneutral controls. However, when CGP-12177 was administered to cold-exposure rats, there was only a nonsignificant increase of 21% in GDP binding (Table II). In contrast to the number of GDP binding sites, the dissociation constant (K_d) for GDP binding was unchanged with cold exposure or CGP-12177 administration (Table II).

The levels of UCP mRNA increased by almost 3-fold in response to CGP-12177 administration in the rats maintained at thermoneutrality (Table II). As expected, there was no difference between the thermoneutral controls and the rewarmed cold-exposed controls. (Table II). However, it is surprising that the CGP-12177 administration to the cold-exposed rats did not significantly increase UCP mRNA levels. In this respect, these findings are similar to those for GDP binding following CGP-12177 administration in the cold-exposed rats (Table II).

Senescent Rats. In contrast to its effect in young rats, CGP-12177 did not increase the GDP binding in senescent rats maintained at thermoneutrality (Table III). However, with cold exposure, there was a 2-fold increase in GDP binding compared with the thermoneutral controls, which was similar to the increase observed following cold exposure in young rats (Tables II and III). Moreover, in contrast to the rats maintained at thermoneutrality, in cold-exposed senescent rats, CGP-12177 induced a significant increase in GDP binding of 35% (Table III). Similar to the results in young rats, there were no changes in the dissociation constant for GDP binding under any conditions investigated in the senescent rats (Table III).

The levels of UCP mRNA were not determined due to insufficient amounts of tissue recovered in the senescent rats. However, in a separate experiment, the CGP-12177 induction of UCP mRNA was examined in young and old rats. Similar to our previous report (1), the steady-state levels of UCP mRNA were not different between young and old control rats. Following administration of CGP-12177, the increase in mRNA was evident by 1.5 hr and appeared to ap-

Table II. Effects of Cold Exposure on GDP Binding in Response to CGP-12177 Administration in Young Rats

Parameters	Thermoneutral		Rewarmed cold exposed	
	Control	CGP-12177	Control	CGP-12177
GDP-binding sites (pmol/mg protein)	47.0 ± 4.3	88.3 ± 0.6[a]	85.6 ± 11.5[a]	104 ± 12.1
K_d (n*M*)	2.11 ± 0.16	1.94 ± 0.22	2.03 ± 0.33	1.84 ± 0.16
UCP mRNA (arbitrary OD units)	10.49 ± 2.92	29.17 ± 5.21[b]	9.74 ± 2.30	14.78 ± 3.26

Note. Values represent the mean ± SEM of five or six rats per group. Cold-exposed rats were kept at 8°C for 48 hr, returned to 26°C for 24 hr, administered CGP-12177 (0.75 mg/kg), and sacrificed 1 hr later.
[a] $P < 0.006$ (cold exposed) and $P = 0.03$ (CGP-12177) by two-way ANOVA. $P = 0.02$ for the difference between control and CGP-12177 in the thermoneutral group, and $P = 0.02$ for difference with cold exposure among control rats by Scheffe's post hoc analysis.
[b] $P < 0.007$ for difference with CGP-12177 by two-way ANOVA. $P = 0.02$ for difference from thermoneutral control by Scheffe's post hoc analysis.

Table III. Effects of Cold Exposure on GDP Binding in Response to CGP-12177 Administration in Senescent Rats

	Thermoneutral		Rewarmed cold exposed	
	Control	CGP-12177	Control	CGP-12177
GDP-binding sites (pmol/mg protein)	44.2 ± 4.1	45.3 ± 8.3	97.0 ± 10.2[a]	131 ± 9.9[a]
K_d (n*M*)	2.21 ± 0.23	2.02 ± 0.27	2.12 ± 0.31	2.36 ± 0.24

Note. Values represent the mean ± SEM of four to six rats per group. See Table II for description of cold exposure and CGP-12177 administration.
[a] $P < 0.001$ (cold exposed) and $P = 0.04$ (CGP 12177) by two-way ANOVA. $P < 0.001$ for difference with cold exposure among control rats, and $P < 0.04$ for difference with CGP-12177 administration among cold-exposed rats.

proaching a maximum by 5 hr postinjection (Fig. 3). The induction by CGP-12177 was significantly less in the older rats by 25% (Fig. 3).

Discussion

Sympathetically activated thermogenesis in BAT declines with age (2, 7). The β_3-adrenergic receptor is the principal mediator of thermogenesis in the mature adipocyte (5), and β_3-adrenergic agonist stimulation of thermogenesis, including oxygen consumption, body temperature, and mitochondrial GDP binding, is impaired with age (7). In addition, although the specific subtype quantification of β_3-adrenergic receptors has not been determined in young compared with senescent rats, the total number of β-adrenergic receptors declines with age (11). In white adipose tissue, both β_1- and β_3-adrenergic receptor mRNA levels decrease with age (12). These observations have led us to suggest that the decline in β_3-adrenergic stimulation of BAT thermogenesis contributes to the impaired thermoregulatory capacity in older rats (7). However, our recent study indicated that the cold-induced increase

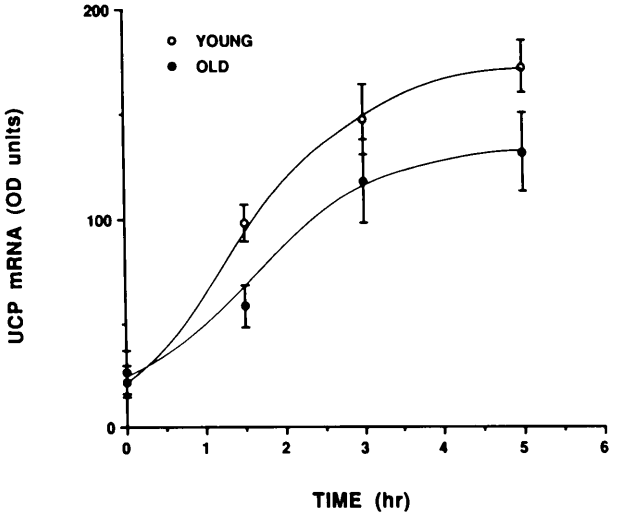

Figure 3. The time course induction of UCP mRNA following 0.75 m/kg CGP-12177 administration in young (○) and senescent (●) rats. Data represent the mean ± SEM of six to eight rats at each time point and age. $P < 0.003$ for difference with age by two-way ANOVA.

in BAT GDP binding and the cold-induced expression of UCP mRNA levels were unchanged in young and senescent rats (1). Collectively, these observations suggest that in senescent rats thermogenesis in BAT is mediated by other than the β_3-adrenergic receptors or that the process of cold exposure has restored the responsiveness of the β_3-adrenergic pathway in the older rats.

When animals are exposed to the cold, the capacity for thermogenesis in BAT increases (13). In cold-acclimated rats, the norepinephrine-stimulated whole body oxygen consumption and BAT mitochondrial GDP binding are greater than in rats adapted to thermoneutrality. This increase in total thermogenic capacity occurs even though desensitization to norepinephrine often is observed in isolated brown adipocytes (13). The increased capacity is partly due to recruitment of additional brown adipose tissue through cell proliferation. In addition, within cells there is an increase in the density of mitochondrial UCP. The cell proliferation, at least in young rats, appears to be mediated by β_1-adrenergic receptors, whereas the induction of UCP is mediated by β_3-adrenergic receptors (13). Thus, following cold exposure, β-adrenergic stimulation of GDP binding can increase due to both the recruitment of new BAT and to the synthesis of new UCP in existing BAT. Stimulation of the latter, however, is tempered by potential desensitization of β-adrenergic receptors due to elevated norepinephrine release.

The present report is concerned with the β_3-adrenergic stimulation of GDP binding and the β_3-adrenergic induction of UCP mRNA. The experimental design involved a 2-day cold exposure followed by a 24-hr rewarming period. This two-phase design was chosen (i) to allow sufficient time at 8°C for the potential upregulation of the thermogenic pathway and (ii) to examine the stimulation by exogeneously administered CGP-12177 with minimal interference from endogenous stimulation by norepinephrine. In the absence of a rewarming period, the cold-induced release of norepinephrine would compete with the administered CGP-12177. We demonstrated that in young

rats: (i) the β_3-adrenergic agonist CGP-12177 increased GDP binding and UCP mRNA levels in rats maintained at thermoneutrality; (ii) 2 days of cold exposure increased the basal level of GDP almost 2-fold that was mostly sustained during the 24-hr rewarming period; (iii) 2 days of cold exposure increased UCP mRNA levels 4-fold and these levels nearly returned to control levels by the end of the 24-hr rewarming period; and (iv) in cold-exposed rats, there was no significant increase in either GDP binding or UCP mRNA levels with CGP-12177 stimulation. These observations are consistent with the above discussion of the effects of cold exposure on rats; that is, there is an activation of UCP, as evidenced by increased GDP binding and an elevation in the synthesis of UCP, further contributing to the increased density of GDP binding. β_3-adrenergic stimulation (by CGP-12177 administration) leads to activation of UCP as evidenced by an increase in GDP binding in the rats maintained at thermoneutrality. However, despite the elevated level of UCP in cold-exposed rats, further increases in GDP binding or induction of UCP mRNA in response to β_3-agonist stimulation are desensitized.

Some of the observations in senescent rats were distinctly different from these in young rats: (i) there was no β_3-adrenergic–stimulated increase in GDP binding in rats maintained at thermoneutrality; (ii) similar to its effects on young rats, cold exposure increased the basal level of GDP binding 2-fold; (iii) in contrast to young rats, in the cold-exposed senescent rats, the β_3-agonist significantly stimulated GDP binding; (iv) similar to the results in young rats, 2 days of cold exposure increased UCP mRNA levels 5-fold, and these levels returned to control levels during the 24-hr rewarming period. The half-life of UCP mRNA was calculated to be 11 hr for both young and old rats, and; (v) the induction of UCP mRNA by CGP-12177 over a 5-hr period was 25% less in the old compared with the young rats.

The salient observation from these studies is that in senescent rats without cold exposure, CGP-12177 failed to increase GDP binding, whereas following cold exposure CGP-12177 increased GDP binding by 35%. These observations indicate that cold exposure restores the impaired β_3-adrenergic signal transduction in BAT from senescent rats. An attractive hypothesis is that cold exposure induces the synthesis of one or more components in the β_3-adrenergic pathway. Supporting this hypothesis are reports from this (14) and Granneman's laboratory (15–17) indicating cold exposure increases post-receptor–stimulated adenylyl cyclase activity. A recent study by Granneman indicates cold exposure increases the steady-state mRNA levels of the specific adenylyl cyclase isoform, type III (17). We have previously reported that adenylyl cyclase activity, including forskolin-stimulated adenylyl cyclase activity, is diminished in BAT from senescent rats. If cold exposure also increases the synthesis of adenylyl cyclase type III in senescent rats, this may be contributing to cold-induced restoration of β_3-adrenergic signal transduction in older rats. However, our most recent report suggests that components of signal transduction pathway other than just adenylyl cyclase may be involved in the impaired β_3-adrenergic stimulation with age. We reported that thermogenesis, including oxygen consumption, BAT mitochondrial GDP binding, and induction of UCP mRNA, in response to forskolin was unchanged between young and senescent rats (8). Forskolin is a direct activator of adenylyl cyclase, suggesting the impaired β_3-adrenergic stimulation with age is due to components or events proximal to adenylyl cyclase. The most likely components are the β_3-adrenergic receptors or stimulatory G-protein (G_S). Cold exposure of senescent rats could either induce the synthesis of either of these components or increase receptor-G-protein coupling. There is some evidence from the heart that supports the latter possibility. The β_1-adrenergic receptor in heart tissue from senescent rats behaves as if it is desensitized in the basal state (18). If the β_3-adrenergic receptor in BAT from senescent rats is also desensitized (i.e., uncoupled from G_S and adenylyl cyclase) that would explain the lack of thermogenic stimulation by β_3-adrenergic agonists but full stimulation by forskolin. Cold exposure by some unknown fashion may recouple the receptors. Alternatively, there may be impaired or deficient numbers of β_3-adrenergic receptors or G_S protein in senescent rats, and cold acclimation could induce new synthesis of these components. In either case, cold exposure restores the β_3-adrenergic responsiveness in BAT from senescent rats.

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