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Differential regulation of β_3-adrenoceptors in gut and adipose tissue of genetically obese (ob/ob) C57BL/6J-mice

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1 Levels of β_3-adrenoceptor (AR) mRNA were compared using reverse transcription-polymerase chain reaction (RT–PCR) in white adipose tissue (WAT), brown adipose tissue (BAT), ileum and colon from genetically obese (ob/ob) and lean (+/+) C57BL/6J mice. Functional responses to the β_3-AR agonist CL 316243 were also characterized in ileal longitudinal smooth muscle from obese and lean mice.

2 β_3-AR mRNA levels were significantly higher in WAT (100 ± 16%) and BAT (100 ± 13%) from lean compared to WAT (21.0 ± 0.9%; $n=4$; $P<0.005$) and BAT (14.1 ± 2.2%; $n=5$; $P<0.01$) from obese mice. In contrast, β_3-mRNA levels were not significantly different in ileum (100 ± 15%) and colon (100 ± 22%) from lean mice, compared to ileum (78 ± 13%; $n=4$; $P=0.31$) or colon (82 ± 15%; $n=4$; $P=0.52$) from obese mice.

3 Concentration-response curves to CL 316243 did not differ significantly in slope or position in ileal longitudinal smooth muscle from obese or lean mice. pEC_{50} (± s.e.mean) values were not significantly different ($P=0.59$) between obese (7.90 ± 0.13, $n=7$) and lean (7.77 ± 0.20, $n=7$) mice.

4 pK_B values for the β_1-AR and β_2-AR selective antagonist propranolol or the β_3-AR selective antagonist SR 58894 against relaxations to CL 316243 were similar in ileum of genetically obese (propranolol 6.31 ± 0.22 and 6.13 ± 0.12; SR 58894 8.22 ± 0.06) and lean mice (propranolol 6.40 ± 0.08 and 6.60 ± 0.13; SR 58894 8.27 ± 0.12) and were consistent with values previously found at β_3-AR.

5 Treatment of lean C57BL/6J mice with dexamethasone (1 mg kg^{-1}, i.p.) significantly reduced β_3-AR mRNA levels after 4 h in WAT (100 ± 6.1 to 41.4 ± 4.3; $n=16-18$; $P<0.0001$) and BAT (100 ± 8.0 to 35.1 ± 5.8; $n=17$; $P<0.0001$), but caused no change in ileum (100 ± 6.1 to 101 ± 17; $n=10-11$; $P=0.95$) or colon (100 ± 11 to 101 ± 11; $n=11$; $P=0.94$). β_3-mRNA levels in ileum and colon also did not change significantly when examined over 24 h or after the administration of a higher dose of dexamethasone (5 mg kg^{-1}).

6 In summary, β_3-AR mRNA levels were considerably lower in WAT and BAT of obese compared to lean mice whereas the levels in ileum and colon were not significantly different. The similar β_3-mRNA levels in ileum of obese and lean mice were associated with indistinguishable responses of carbachol-contracted ileum to a β_3-agonist and similar affinity for β-antagonists. Administration of glucocorticoids to lean mice reduced β_3-AR mRNA levels in WAT and BAT but not in ileum or colon. These studies show that in mice, β_3-ARs are differentially regulated in ileum and colon compared to adipose tissues.

Keywords: β_3-Adrenoceptors; obesity; regulation; glucocorticoids; CL 316243; SR 58894; adipose tissue; gut

Introduction

The β_3-adrenoceptor (AR) is resistant to short-term desensitization (Nantel *et al*., 1993) and to blockade by conventional β-AR antagonists such as (−)-propranolol (see Arch & Kaumann, 1993), is specifically activated by agonists such as BRL 37344 and CL 316243 and blocked by antagonists such as SR 58894 and SR 59230 (Manara *et al*., 1995a). β_3-AR mediate lipolysis in white adipose tissue (WAT) where they have been identified by functional (Harms *et al*., 1977; Hollenga *et al*., 1990; Lafontan & Berlan, 1993), and molecular approaches (Emorine *et al*., 1989; Granneman *et al*., 1991; Muzzin *et al*., 1991; Nahmias *et al*., 1991). In brown adipose tissue (BAT) where stimulation of β_3-ARs causes thermogenesis and increased oxygen consumption (Arch *et al*., 1984), the receptors can be detected by receptor binding (Sillence *et al*., 1993; Muzzin *et al*., 1994) and β_3-mRNA is present (Granneman *et al*., 1991; Muzzin *et al*., 1991). The β_3-AR also has a widespread distribution in the gastrointestinal tract. Relaxation responses with β_3-AR properties are found in many different gastrointestinal tissues from a variety of species (for review see Manara *et al*., 1995b) including guinea-pig ileum (Grassby & Broadley, 1984; Bond & Clark, 1988; Growcott *et al*., 1993b) and gastric fundus (Coleman *et al*., 1987), rat ileum (Growcott *et al*., 1993a), and gastric fundus (McLaughlin & MacDonald, 1990a; 1991), jejunum (MacDonald *et al*., 1991; 1994), colon (McLaughlin & MacDonald, 1990b) and oesophagus (De Boer *et al*., 1993), and human taenia coli (McLaughlin *et al*., 1988; 1991; Roberts *et al*., 1997; Kelly *et al*., 1997) and colonic circular muscle (De Ponti *et al*., 1996). Recent binding studies in rat ileum (Roberts *et al*., 1995) and human colon (Summers *et al*., 1995; 1996; Roberts *et al*., 1997) have demonstrated binding sites for [^{125}I]-cyanopindolol with appropriate properties for a β_3-AR. mRNA coding for the β_3-AR has been detected in rat ileum (Granneman *et al*., 1991; Evans *et al*., 1996), colon (Bensaid *et al*., 1993; Evans *et al*., 1996) and in human ileum and colon (Granneman *et al*., 1993; Krief *et al*., 1993; Summers *et al*., 1996; Roberts *et al*., 1997).

In a number of animal models of obesity such as the fa/fa Zucker rat (Revelli *et al*., 1992) and the C57BL/6J (ob/ob) mouse (Collins *et al*., 1994), β_3-AR mRNA and function in WAT and BAT are both markedly reduced. In the case of the ob/ob mouse the obese genotype is not directly responsible for the decrease in β_3-AR function but may be associated with

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high circulating levels of glucocorticoids (Collins *et al*., 1994), which have been shown to down-regulate β_3-AR in 3T3-F442A adipocytes *in vitro* (Feve *et al*., 1992).

This study uses RT–PCR and functional techniques to determine if the down-regulation of β_3-ARs seen in the adipose tissue of genetically obese mice as compared to their lean counterparts is also seen in the ileum and colon, and to determine if glucocorticoids are capable of down-regulating β_3-AR mRNA in adipose and gut tissues *in vivo*. The study demonstrates that expression and function of the β_3-AR are differentially regulated in mouse adipose and gut tissue.

Methods

Tissue collection for molecular studies

Genetically obese (ob/ob) and lean (+/+) 12–13 week old male C57BL/6J mice were obtained from Animal Services, Monash University (Clayton, Vic., Australia). Homozygosity of the (ob/ob) mice was tested by staff at Animal Services in March, 1997. Mice were anaesthetized with 80% CO_2/20% O_2 and killed by cervical dislocation. To investigate the effect of glucocorticoids, lean C57BL/6J mice were injected (i.p.) with 1.3 mg kg^{-1} or 6.5 mg kg^{-1} dexamethasone 21-phosphate (disodium salt) (equivalent to 1 and 5 mg kg^{-1} base) in 0.13 ml or an equivalent volume of vehicle (sterilized distilled water) and killed after 4 or 24 h. White adipose tissue from epididymal fat pads, interscapular brown adipose tissue and ileal and colonic smooth muscle were dissected, frozen in liquid nitrogen and stored at −70°C. Ileum and colon were carefully dissected free of surrounding adipose tissue, cut open and pinned out, and the mucosa removed by gentle scraping with an edge of a sterile microscope slide. The resulting smooth muscle was washed in Krebs-Henseleit solution and dried before immersion in liquid nitrogen.

RNA extractions

Frozen tissue was ground to a fine powder in a stainless steel mortar and pestle pre-cooled in liquid nitrogen. Total RNA was extracted by homogenizing in Trizol. To avoid cross-contamination, the homogenizer probe was dismantled and washed thoroughly between each sample. The yield and quality of the RNA were assessed by measuring absorbance at 260 and 280 nm, and by electrophoresis on 1.2% agarose gels. Total RNA from colon and ileum was treated with DNase to remove any contaminating genomic DNA. The reaction mix contained 20 μg RNA, 100 mM sodium acetate (pH 7.0), 5 mM $MgSO_4$, 5 mM dithiothreitol, 36 u RNasin (Promega), and 10 u DNase I (Pharmacia) in a total volume of 40 μl. Following digestion at 37°C for 30 min, the solution was diluted to 400 μl with H_2O and extracted with an equal volume of phenol: chloroform (1:1). The RNA was precipitated with 1.0 ml of ethanol and 40 μl of 2 M sodium acetate. The yield and quality of DNase-treated RNA were determined as above.

Reverse transcription/PCR

cDNAs were synthesized by reverse transcription of 1.0 μg of each total RNA using oligo $(dT)_{15}$ as a primer. The RNA in a volume of 7.5 μl was heated to 70°C for 5 min then placed on ice for 2 min before the addition of reaction mix containing 1×RT buffer (supplied by Promega), 1 mM dNTPs, 5 mM $MgCl_2$, 18 u RNasin (Promega), 20 u AMV reverse transcriptase (Promega) and 50 μg ml^{-1} oligo$(dT)_{15}$ in a volume of 12.5 μl. Following brief centrifugation, the reactions were incubated at 42°C for 45 min, then at 95°C for 5 min. The completed reverse transcription reactions were stored at −20°C and used for PCR without further treatment.

PCR amplification was carried out on cDNA equivalent to 100 ng of starting RNA, using the following oligonucleotide primers: mouse β_3-AR (1) (forward, 5′ ATCATGAGC-CAGTGGTGGCGTGTAG 3′ and reverse, 5′ GCGAT-GAAAACTCCGCTGGGAACTA 3′), β_3-AR(2) (forward, 5′ TCTAGTTCCCAGCGGAGTTTTCATCG 3′ and reverse, 5′ CGCGCACCTTCATAGCCATCAAACC 3′), transferrin receptor (forward, 5′ CAAATGGTAACTTAGACCCAGTG-GAGTCTC 3′ and reverse, 5′ GATTGTTTGCACAGGTA-TATTAGGCAACCC 3′), actin (forward, 5′ ATCCTGCGTC-TGGACCTGGCTG 3′ and reverse, 5′ CCTGCTTGCT-GATCCACATCTGCTG 3′), and leptin (forward, 5′ GATG-ACACCAAAACCCTCATCAAG 3′ and reverse, 5′ GCCAC-CACCTCTGTGGAGTAG 3′) synthesized by Gibco-BRL (Gaithersburg, U.S.A.). The β_3-AR(2) and actin primers are intron-spanning, and were used to demonstrate the absence of any contaminating genomic DNA. Reverse primers were labelled before the PCR in a reaction mix containing 120 pmol of oligonucleotide, 70 μCi [γ-^{33}P]-ATP, 1× One-Phor-All Plus buffer (Pharmacia) and 20 u T4 polynucleotide kinase (Pharmacia) in a volume of 40 μl. Following incubation at 37°C for 30 min, reactions were diluted to 100 μl with H_2O and heated at 90°C for 2 min. The labelled primers were separated from unincorporated nucleotide by centrifugation through Chroma-spin 10 columns (Clontech), according to the manufacturer's instructions. PCR mixes contained 1 u of Taq polymerase (Life Technologies), the buffer supplied (20 mM Tris-HCl (pH 8.4) and 50 mM KCl), 200 μM dNTPs, 2 mM Mg-acetate, 2.5 pmol of forward primer, 2.5 pmol of labelled reverse primer and cDNA in a volume of 10 μl. For each set of tissues (eg. all samples of ileum), a single reaction mix containing all components except the cDNA was prepared for the entire PCR experiment and placed in aliquots to minimize variation between samples. Each PCR experiment included a negative control consisting of an RT reaction containing no added RNA. The PCR reactions were carried out in an FTS-1 capillary thermal sequencer (Corbett Research, Lidcombe, New South Wales, Australia). Following initial heating of samples at 95°C for 2 min, each cycle of amplification consisted of 30 s at 95°C, 30 s at 64°C, and 30 s at 72°C. For each set of primers, the log (PCR product) versus cycle number (20 to 36) was plotted, and a cycle number chosen within the linear portion of the graph (Figure 1a). Twenty cycles were used for measurement of leptin mRNA in WAT. Following amplification, PCR products were electrophoresed on 1.3% agarose gels and transferred onto Hybond N^+ membranes by Southern blotting in 0.4 M NaOH/1 M NaCl. The membranes were rinsed for 5 min in 0.5 M Tris-HCl (pH 7.5)/1 M NaCl, then in 2×SSC (1× =0.15 M NaCl/15 mM sodium citrate), and air-dried. Membranes were apposed directly to phosphorimager plates for 7 h, and scanned using a Molecular Dynamics SI phosphorimager.

Organ bath studies

Genetically obese (ob/ob) or lean (+/+) 12–13 week old male C57BL/6J mice were anaesthetized by 80%CO_2/20%O_2 and killed by cervical dislocation. Approximately 12 cm of ileum proximal to the ileo-caecal junction was removed and its contents flushed out with Krebs Henseleit (KH) solution (composition in mM: NaCl 118.4, KCl 4.7, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 2.5)

containing ascorbic acid (0.1 mM) and EDTA (0.04 mM). Segments (approximately 2 cm long) were suspended in KH solution in jacketed organ baths at 37°C and bubbled with 95% O_2/5% CO_2, pH 7.4. Changes in length were measured with Ugo Basile isotonic transducers (resting tension 5 mN) and recorded by a MacLab system connected to a Macintosh IIci computer. Tissues were equilibrated for 20 min in the presence or absence of antagonist (1 μM propranolol, 10 μM propranolol or 32 nM SR 58894), precontracted with a submaximal concentration of 10 μM carbachol (80% of the maximum response) and allowed to equilibrate for 10 min. Cumulative concentration-response curves to the β_3-AR selective agonist CL 316243 (0.1 nM to 3 μM) were conducted using 0.5 log unit increments administered at 8–10 min intervals. At the end of each concentration-response curve, tissues were maximally relaxed with 50 μM papaverine and responses expressed as a percentage of the papaverine response. Paired tissues were used with one concentration-response curve performed in each tissue, either in the absence (control) or presence of antagonist. Non-linear regression was used to fit sigmoid concentration-response curves to the data (GraphPad Prism version 2.0) and to determine pEC_{50} values. Antagonist pK_B values were calculated by the method of Furchgott (1972).

$$pK_B = \log(\text{Concentration ratio} - 1) - \log[\text{Antagonist}]$$

Statistics

Results are expressed in the text as mean±s.e.mean. The statistical significance of differences between groups was assessed by Student's unpaired *t* test (two tailed). Probability (*P*) values equal to or less than 0.05 were considered significant. When there was heterogeneous variance between groups, Welch's correction was applied (assumes two normally distributed populations with different variance). Iterative curve fitting and calculation of pEC_{50} was performed using GraphPad Prism version 2.0.

Drugs and other reagents

CL 316243 ((**R,R**)-5-[2-[[2-3-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (disodium salt)) and SR 58894 (3-(2-allylphenoxy)-1-[(1S)-1,2,3,4 tetra-hydronaphth-1-ylamino]-(2S)-2-propanol hydrochloride) were gifts from Wyeth-Ayerst and Sanofi-Midi, respectively. Carbachol, (−)-propranolol, papaverine, reserpine and dex-amethasone-21-phosphate were from Sigma (St. Louis, U.S.A.). Oligo $d(T)_{15}$ and other oligonucleotide primers were from Bresatec (Adelaide, S.A.). Taq DNA polymerase and 10×PCR buffer were from GibcoBRL (Gaithersburg, U.S.A.). RNasin, AMV reverse transcriptase and 10×RT buffer were from Promega (Madison, U.S.A.), and DNase, T4 polynucleo-tide kinase and 'One for All-Plus' buffer were from Amrad-Pharmacia (Sydney, N.S.W.).

Results

Determination of mRNA levels in mouse tissues by semi-quantitative PCR

A series of experiments were performed to validate the RT–PCR technique used to compare β_3-AR mRNA levels in tissues from lean and obese mice and lean mice treated with dexamethasone. In Figure 1a, the $\log_{10}$ PCR product is plotted against the number of PCR cycles for RNA extracted

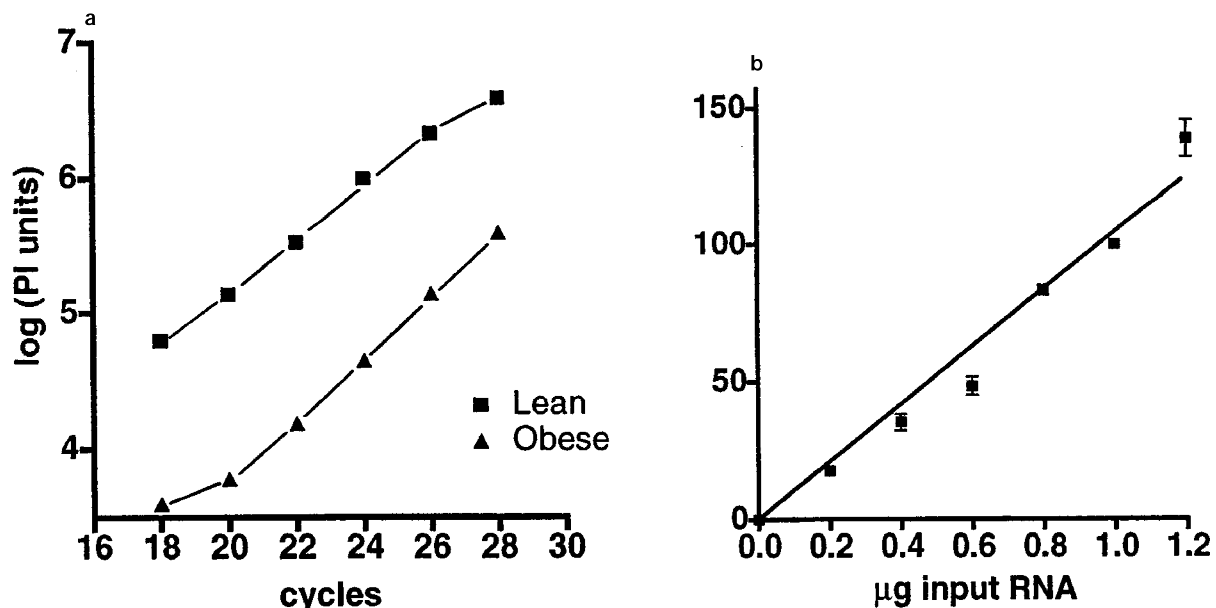

Figure 1 (a) Product/cycle relationship for β_3-AR PCR performed on WAT cDNA from lean and obese mice. The cDNA was produced by reverse transcription of 1 μg total RNA, and one tenth used for PCR with β_3-AR(2) primers. Note that the log (PI) units (a measure of product) to cycle number is linear up to 26 cycles beyond which the amount of product starts to plateau. (b) Relationship between the amount of cDNA product formed and the amount of starting RNA. A series of samples containing in total 1.2 μg of RNA were made by mixing different portions of WAT RNA from lean mice (containing β_3-AR mRNA) with soleus muscle RNA (containing no detectable β_3-AR mRNA), and each sample reverse transcribed. One tenth of the resulting cDNA was amplified by PCR in triplicate using β_3-AR(2) and actin primers, and the β_3-AR product corrected for actin. The relationship between PCR product formed and amount of starting WAT RNA was linear ($r^2 = 0.98$) for a range of RNA between 0.2 and 1.2 μg.

from WAT. The amount of PCR product corresponds to the starting level of mRNA at PCR cycle numbers where the amount of product is increasing exponentially, as shown by a linear relationship between $\log_{10}$ PCR product and PCR cycle number. For both lean and obese mice, there was a linear increase in $\log_{10}$ PCR product up to 26 cycles. Based on similar experiments for the other tissues (data not shown) we chose 22 cycles for WAT and BAT, and 30 cycles for ileum and colon, where levels of β_3-AR mRNA were substantially lower. Actin PCR was done at 16 cycles for all tissues. Next, we made samples of RNA (1.2 μg) consisting of increasing amounts of WAT RNA from lean mice (containing β_3-AR mRNA) mixed with decreasing amounts of RNA from soleus muscle (with no detectable β_3-AR mRNA). After reverse transcription of each sample, one tenth of the cDNA was amplified by 22 cycles of PCR. Figure 1b shows that the amount of cDNA product expressed as a ratio of actin was linear ($r^2 = 0.98$) over a range of starting WAT RNA from 200 ng – 1.2 μg. In all subsequent experiments, comparisons of β_3-AR mRNA between lean and obese or control and treated animals were conducted under identical conditions for a given tissue. This included the use of common reaction mixes for RT and PCR, the same amount of starting RNA, the same number of PCR cycles, and electrophoresis on the same gel.

β_3-AR mRNA levels in WAT, BAT, ileum and colon of lean and obese mice

There were clear differences between β_3-AR mRNA levels in adipose tissue from lean and obese mice. Figure 2 shows a Southern blot of the β_3-AR mRNA PCR products from WAT, BAT, ileum and colon. It can be seen that the β_3-mRNA levels in WAT and BAT from 4 obese mice were markedly less than in adipose tissues from lean mice, whereas the levels in ileum and colon of both strains of mice were similar. The relative levels of β_3-AR mRNA was measured by analysing band intensities using a Molecular Dynamics phosphorimager and ImageQuaNT software, and correcting levels of β_3-AR mRNA for the level of internal standard mRNA (transferrin receptor or actin) determined in the same cDNA sample. The levels of β_3-AR mRNA were significantly lower ($P < 0.01$) in BAT of obese mice (14.1 ± 2.2%, $n = 4$) than in those from lean mice (100 ± 16%, $n = 4$) with a 7.1 fold difference in the means (Figure 3). The difference in β_3-AR mRNA in WAT of obese (21.0 ± 0.9%, $n = 5$) and lean (100 ± 13%, $n = 5$) mice was also significantly different ($P < 0.005$) with a 4.8 fold difference in the means.

In contrast to the adipose tissues, levels of β_3-AR mRNA in ileum of obese (78 ± 13%, $n = 4$) and lean (100 ± 15%, $n = 4$) mice were not significantly different ($P = 0.31$). β_3-AR mRNA levels in colon were similar to those in ileum and again showed no significant differences ($P = 0.52$) between obese (82 ± 15%, $n = 4$) and lean (100 ± 22%, $n = 4$) animals.

The effects of dexamethasone treatment on levels of β_3-adrenoceptor mRNA

In order to test whether the β_3-AR mRNA in adipose and gut tissues is regulated by corticosteroids, lean C57BL/6J mice were treated with dexamethasone (1.3 mg kg^{-1} equivalent to 1 mg kg^{-1} base). Analysis of lean mouse tissues 4 h after dexamethasone treatment demonstrated that β_3-AR mRNA in WAT and BAT was reduced substantially, but

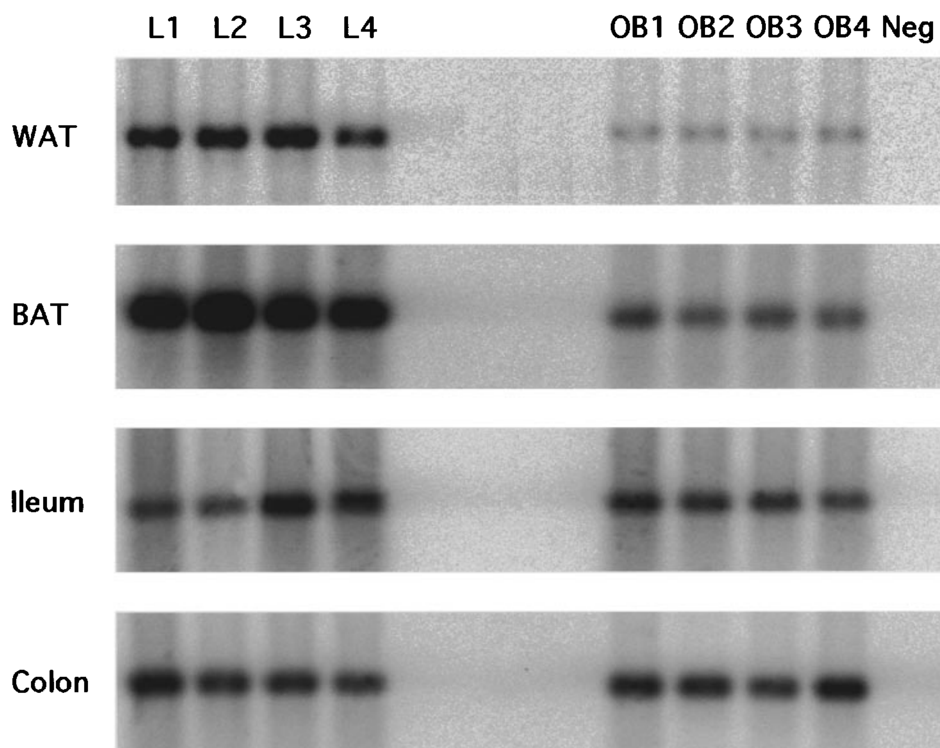

Figure 2 Southern blots of β_3-AR mRNA in lean and obese C57B1/6J mice. The figure shows sets of PCR products from tissues of individual mice (L1 to L4, OB1 to OB4). Cycle numbers were 22 for WAT and BAT, and 30 for colon and ileum smooth muscle, at which stage the PCR gave sufficient product for detection, but remained in the exponential phase of amplification.

there was no significant change in ileum or colon (Figure 4). WAT β_3-AR mRNA was reduced from 100±6.1% (n=16) to 41.4±4.3% (n=18; P<0.0001) and the levels of BAT β_3-AR mRNA were reduced from 100±8.0% to 35.1±5.8% (n=17; P<0.0001). In contrast to the marked reductions in β_3-AR mRNA levels in WAT and BAT caused by the dexamethasone treatment, β_3-mRNA levels in ileum from control mice were 100±6.1% (n=10) and in treated mice 101±17% (n=11; P=0.95). Likewise in colon, levels in control mice were 100±11% (n=11) and in treated mice 101±11% (n=11; P=0.94).

One explanation for this finding is that β_3-AR mRNA has a longer half-life in gut than in adipose tissues, resulting in delayed effects of decreasing gene transcription. We therefore measured β_3-AR mRNA in all 4 tissues 24 h after dexamethasone treatment (Figure 4). Interestingly, the levels of β_3-AR mRNA in WAT and BAT were no longer reduced but increased in WAT from 100±11.3% (n=5) in control to 142±7.9% (n=5; P<0.02) in treated mice and in BAT from 100±6.1% (n=6) to 127±7.6% (n=6; P<0.02), respectively. Levels in ileum and colon had not changed significantly in the control and treated groups, being 100±10% and 108±12% (n=5; P=0.63) in ileum and 100±5.6% and 86.4±11.4% (n=6; P=0.31) in colon. To test whether the effects of dexamethasone persist up to 24 h, we measured leptin mRNA in WAT at both the 4 h and 24 h time points, as expression of the leptin gene is known to be modulated by glucocorticoids (de Vos *et al.*, 1995). At 4 h there was a substantial increase in leptin mRNA from 100±23% (n=6) in control to 238±35% (n=6; P<0.01) in treated mice. This increase was maintained at 24 h, with leptin mRNA levels of 100±12% (n=5) in control and 172±21% (n=5; P<0.02) in dexamethasone-treated mice. The difference between leptin mRNA levels in the treated mice at 4 and 24 h was not significant (P=0.152).

Dexamethasone was also tested at a higher dose level (5 mg kg^{-1}) to examine whether regulation of β_3-AR mRNA in gut tisuses was less sensitive than that in adipose tissues. Figure 5 shows that dexamethasone at 5 mg kg^{-1} had a significantly larger effect on β_3-AR mRNA levels in WAT and BAT but still failed to reduce levels significantly in ileum or colon. Levels of β_3-mRNA were reduced to 24.0±4.5% (n=6; P<0.0001) in WAT, 18.5±2.8% (n=4; P<0.0001) in BAT, but remained at 87.1±12.2% (n=5; P=0.31) in ileum and 74.1±11.4% (n=4; P=0.23) in colon.

Functional studies: maintenance of carbachol contraction in ileum

A concentration of carbachol that caused a submaximal (~80%) contraction was used and the level of contraction was well maintained over the whole of the experimental period (Figure 6a). However, contractions to carbachol in genetically obese mice were not as well maintained as those in lean mice and over an extended time period this was apparent (Figure 6b). In order to examine whether the poor

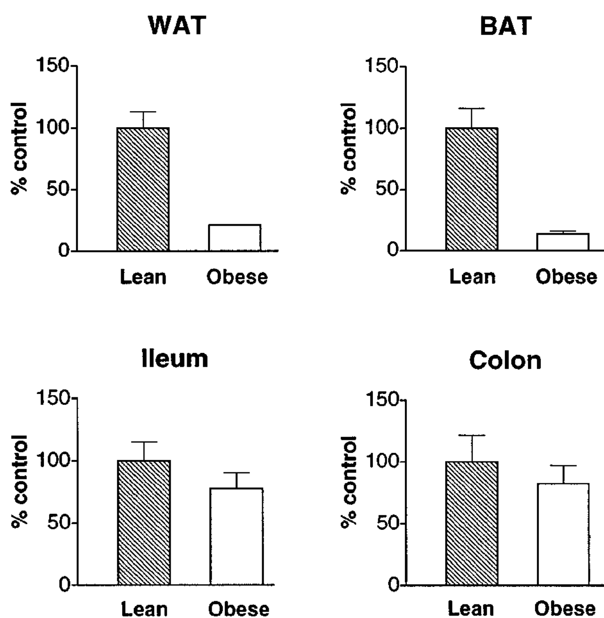

Figure 3 Relative levels of β_3-AR mRNA in lean and obese C57B1/6J mice. PCR experiments using either β_3-AR(1) or transferrin receptor primers were carried out separately. Cycle numbers for amplification of β_3-AR cDNA were 22 for BAT and WAT, and 30 for colon and ileum smooth muscle. Transferrin receptor PCR products were measured after 24 cycles for all tissues. PCR products were quantified by phosphorimaging and values for β_3-AR product from each individual sample corrected for the signal obtained for the corresponding transferrin receptor product. Each value was then converted to a % of the mean from the lean animals. Columns show mean±s.e.mean (n=4).

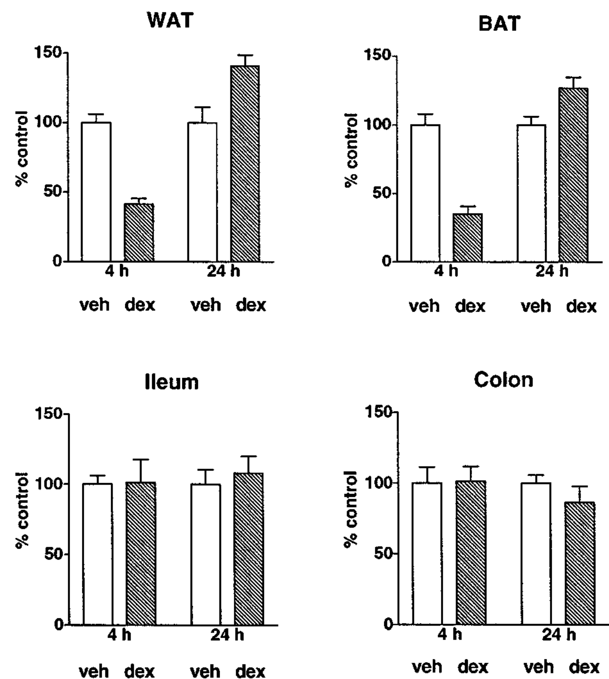

Figure 4 Time course of the effect of dexamethasone treatment on levels of β_3-AR mRNA in adipose and gut tissues. Lean C57BL/6J mice were injected (i.p.) with 1.3 mg kg^{-1} dexamethasone 21-phosphate (1 mg kg^{-1} base) or vehicle 4 or 24 h before collection of tissues. PCR experiments using either β_3-AR(2) or actin primers were carried out separately. Cycle numbers for amplification of β_3-AR cDNA were 22 for BAT and WAT, and 30 for colon and ileum smooth muscle. Actin PCR products were measured after 16 cycles for all tissues. For each tissue, values corrected for the level of actin product were converted to a % of the mean from vehicle-treated animals. Columns show mean±s.e.mean. Dexamethasone at 4 h caused a significant reduction in the β_3-AR mRNA levels in WAT and BAT (P<0.0001) but not in ileum or colon. At 24 h dexamethasone treatment increased β_3-AR mRNA levels in WAT and BAT but levels in ileum and colon showed no significant change.

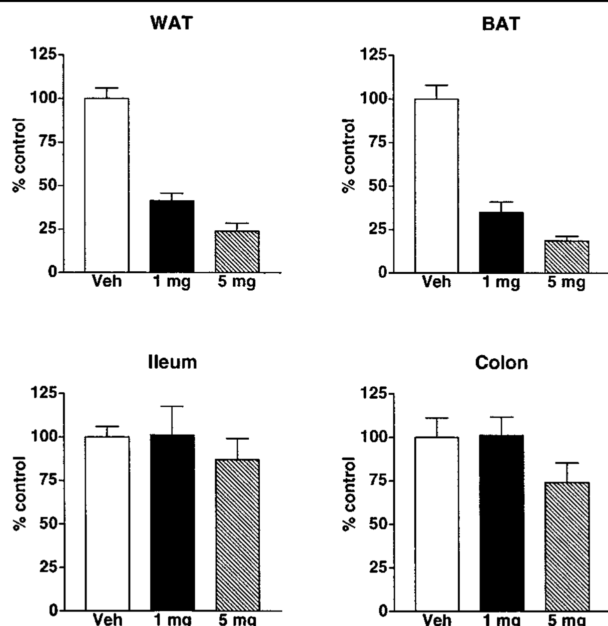

Figure 5 The effect of dexamethasone treatment on levels of β_3-AR mRNA in adipose and gut tissues. Lean C57BL/6J mice were injected (i.p.) with 1.3 or 6.5 mg kg^{-1} dexamethasone 21-phosphate (1 or 5 mg kg^{-1} base) or vehicle 4 h before collection of tissues. β_3-AR mRNA was measured by RT/PCR as described in Figure 4. For each tissue, values corrected for the level of actin product were converted to % of the mean from vehicle-treated animals. Columns show mean±s.e.mean. After 1 mg kg^{-1} dexamethasone there was a significant reduction ($P<0.0001$) in the β_3-mRNA levels in WAT and BAT but not in ileum or colon. After 5 mg kg^{-1} dexamethasone the effects in WAT and BAT were more marked but there was still no effect in ileum or colon.

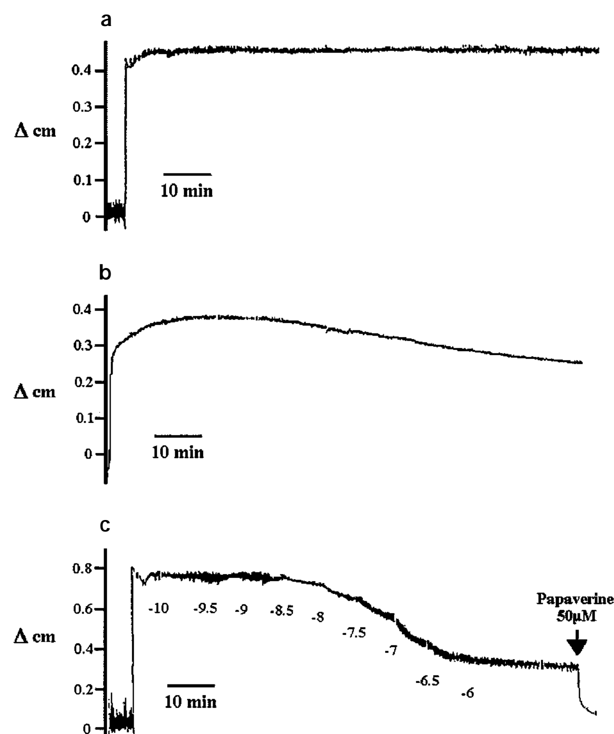

Figure 6 Representative traces showing maintenance of carbachol contraction (10 μM) in ileum from (a) lean mice, (b) obese mice and (c) a concentration-dependent relaxation to CL 316243 (log concentrations of CL 316243 shown) in lean mouse ileum contracted with 10 μM carbachol. At the end of the concentration-response curve to CL 316243 a maximal relaxation was induced by addition of papaverine (50 μM).

maintenance of contractions in obese mice had a sympathetic component, mice were either pretreated with reserpine (1 mg kg^{-1}, i.p., 18 h) or their isolated tissues were treated with propranolol (10 μM). Both treatments improved the maintenance of contraction to carbachol (not shown), indicating that this phenomenon has a sympathetic component.

Effect of the β_3-adrenoceptor agonist CL 316243 on carbachol contracted ileum

CL 316243 caused concentration-dependent relaxation of carbachol (10 μM) contracted ileum (Figure 6c). Concentration-response curves to CL 316243 in lean and obese mice (Figure 7) were not significantly different in slope or position ($P=0.59$), and pEC_{50} values were 7.77±0.19 ($n=7$) and 7.90±0.13 ($n=7$) in lean and obese mice, respectively.

Effect of β-AR antagonists on the relaxation to CL 316243

The β_1 and β_2-adrenoceptor antagonist propranolol and the β_3-AR selective antagonist SR 58894 were tested for their effect on the relaxation produced by CL 316243. Both antagonists caused parallel rightward shifts in the concentration-response curve to CL 316243. pK_B values were calculated at 1 and 10 μM propranolol and 32 nM SR 58894 (Table 1) in ileum from both lean and obese mice.

The pK_B values for propranolol (1 μM) were 6.31±0.22 and 6.40±0.08 and were not significantly different in ileum from

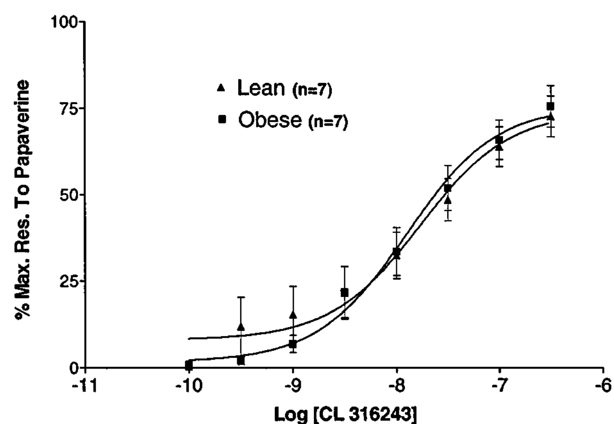

Figure 7 Concentration-response curves to the β_3-adrenoceptor agonist CL 316243 in ileum from genetically obese C57BL/6J (ob/ob) and lean C57BL/6J (+/+) mice. The concentration-response curves for ileum from obese and lean mice were not different in either slope or position ($P=0.59$). The pEC_{50} value for obese mice was 7.90±0.13 ($n=7$) and for lean mice 7.77±0.19 ($n=7$). Vertical lines show s.e.mean.

lean and obese mice ($P=0.73$). However, pK_B values for propranolol (10 μM) were 6.13±0.12 and 6.60±0.13 and were significantly different in the two groups ($P=0.022$). This difference may be due to the somewhat poorer maintenance of the contraction to carbachol in obese moue ileum as previously described. The pK_B values for SR 58894 (32 nM) were 8.22±0.06 and 8.27±0.12 and were not significantly different

Table 1 Comparison of pK_B values for β-adrenoceptor antagonists in mouse ileum

Antagonist	*Concentration*	*Obese*	*Lean*	P
Propranolol	1 μM	6.31 ± 0.22 (7)	6.40 ± 0.08 (6)	0.73
Propranolol	10 μM	6.13 ± 0.12 (7)	6.60 ± 0.13 (6)	0.022
SR58894	32 nM	8.22 ± 0.06 (5)	8.27 ± 0.12 (6)	0.74

pK_B values for (−)-propranolol (1 and 10 μM) and SR58894 (32 nM) were obtained in ileum from genetically obese C57BL/6J (ob/ob) and lean C57BL/6J (+/+) mice. Comparisons between values (*n* in parentheses) were made by Students *t* test.

in ileum of lean or obese mice ($P = 0.74$). The pK_B values obtained for propranolol and SR 58894 were within the range expected for an action at β_3-ARs.

Discussion

A number of studies have demonstrated substantial decreases in β_3-AR mRNA levels in adipose tissues from obese animals compared to their lean counterparts. These include the genetically obese fa/fa Zucker rat (Muzzin *et al*., 1991), ob/ob C57BL/6J mouse (Collins *et al*., 1994) and +/+ C57BL/6J mouse fed a high fat diet for 16 weeks (Collins *et al*., 1997). In both of the mouse models there is a concomitant decrease in β_3-AR function assessed by the effectiveness of β_3-AR stimulation of adenylate cyclase by β_3-agonists in membrane preparations from BAT and WAT (Collins *et al*., 1997). Our data confirm, using RT/PCR, that there is a substantial reduction in β_3-mRNA from WAT and BAT of ob/ob C57BL/6J mice.

It is well established that rat ileum (Granneman *et al*., 1991; Evans *et al*., 1996) and colon (Bensaid *et al*., 1993; Evans *et al*., 1996) express β_3-AR mRNA which is in good accord with many functional studies (Arch & Kaumann, 1993) and binding studies (Summers *et al*., 1995; Roberts *et al*., 1995), indicating the presence of a receptor with the pharmacological properties of a β_3-AR in these tissues. The present studies demonstrated that mouse ileum and colon also contain β_3-AR mRNA and interestingly, that the levels were not different in either ileum or colon between obese and lean mice. In order to examine whether the levels of mRNA reflected the levels of functional receptors, organ bath studies were carried out using mouse ileum longitudinal smooth muscle as a bioassay. Relaxation responses of carbachol-precontracted ileum to the selective β_3-AR agonist CL 316243 (Dolan *et al*., 1994) were obtained and characterized using the selective β_3-AR antagonist SR58894 (Manara *et al*., 1995a) and the β_1-/β_2-AR antagonist (−)-propranolol. Propranolol and SR 58894 caused rightward shifts of the concentration-response curves to CL 316243 with pK_B values that were not significantly different between lean and obese mice (Table 1), except at the higher concentration of propranolol (10 μM) where the difference, although significant, was not marked. The pK_B of approximately 6.5 calculated for propranolol was similar to the pK_B values obtained for propranolol at β_3-ARs (see Arch & Kaumann, 1993), but markedly different from the pK_B values of 8.0–8.5 expected at β_1- or β_2-ARs. In guinea-pig atria, guinea-pig trachea and rat colon, SR 58894 has been shown to antagonize β_1-, β_2- and β_3-ARs with pA_2 values of 6.88 ± 0.09, ≈ 6.4 and 8.06 ± 0.40, respectively (Manara *et al*., 1995a). In mouse ileum, SR 58894 antagonized the relaxation to CL 316243 with a pK_B value of 8.25. The pK_B values for both SR 58894 and propranolol indicate that the relaxation to CL 316243 in mouse ileum is β_3-AR-mediated. These results also indicate that β_3-AR function in longitudinal smooth muscle of ileum is not different in genetically obese and lean mice and is in accord with the similar levels of β_3-AR mRNA found in ileum from the two mouse strains.

It has been suggested that the low levels of β_3-AR mRNA in WAT and BAT of the obese mouse may result from high circulating levels of corticosteroids (Collins *et al*., 1994), which have been shown to be present together with increased circulating insulin in the ob/ob mouse (Bray & York, 1979). *In vitro* studies using mouse 3T3-F442A adipocytes have shown that corticosterone and insulin cause down-regulation of β_3-ARs (Feve *et al*., 1992; 1994; Granneman & Lahners, 1994). In accord with these findings, the low levels of β_3-AR mRNA in obese Zucker rats are restored towards normal by adrenalectomy (Okada *et al*., 1993). We therefore examined whether β_3-AR mRNA levels in WAT, BAT, ileum and colon of lean (+/+) mice are affected by administration of dexamethasone. It was found that β_3-AR mRNA was reduced in WAT and BAT 4 h after dexamethasone treatment, thus an increase in the circulating levels of glucocorticoids could be responsible for the observed reduction in levels of β_3-AR mRNA in the BAT and WAT of obese mice. However, when the levels of β_3-AR mRNA in WAT and BAT were examined 24 h after dexamethasone administration they had returned and now exceeded control. This may indicate desensitization of glucocorticoid receptors or a rebound phenomenon associated with the production of a protein transcription factor which has been described in BAT tissue cultures with prolonged exposure to β-agonists (Bengtsson *et al*., 1996). The continuous stimulation of glucocorticoid receptors by dexamethasone contrasts with the marked diurnal variation of plasma corticosteroid levels which show a 200 fold variation (Dallman *et al*., 1993). Under physiological conditions therefore the cycling of corticosteroid levels may prevent the production of the transcription factor and the escape of β_3-mRNA expression.

We found no effect of dexamethasone treatment on β_3-AR expression in smooth muscle from ileum or colon. Neither a higher dose nor longer exposure to dexamethasone produced any decline in β_3-AR mRNA in gut tissues. Two explanations that can be put forward to explain the absence of an effect are that the gut lacks glucocorticoid receptors or that the control of expression of β_3-AR in gut tissues differs from that in WAT and BAT. The first possibility is unlikely since in rat colon, mRNA coding for glucocorticoid receptors is found associated with all cells of the mucosa, the submucosa and the muscularis propria (Whorwood *et al*., 1993). In ileum, *in vitro* administration of dexamethasone produces dose-related effects on nerve-evoked contractions which are reversed by the glucocorticoid receptor antagonist RU-38486 (Persico *et al*., 1991). It is therefore unlikely that the lack of an effect of glucocorticoids on β_3-AR expression in gut tissues is due to a lack of

glucocorticoid receptors. Alternatively, control of the transcription of the β_3-AR gene may differ between gut and adipose tissues. There is evidence from the analysis of rat tissues that the major transcription start sites in both BAT and WAT are 161 nucleotides or more upstream from the ATG initiation codon, whereas in the gastric fundus the start sites are at -109 and to a lesser extent at -123 nucleotides upstream (Granneman & Lahners, 1994). Therefore, elements present in the promoter region may have different importance in modulating β_3-AR expression in adipose and gut tissues. In particular, there is a glucocorticoid response element (GRE) located in close proximity to an AP-1 site and numerous fat-specific elements in the promoter regions of the mouse and human β_3-AR genes. Van Spronsen *et al.* (1993) suggest that down-regulation of adipocyte β_3-AR expression by dexamethasone may be due to negative interactions of the glucocorticoid receptor with transcription factor binding to the AP-1 site. These interactions may be modified in ileum and colon smooth muscle if there are different start sites utilized in these tissues.

In conclusion, we have confirmed that WAT and BAT from genetically obese (ob/ob) mice contain low levels of β_3-AR mRNA compared with lean (+/+) controls. However, in contrast, ileum and colon from obese and lean mice contained levels of β_3-AR mRNA that were not significantly different. Furthermore, there was no significant difference in the response of the obese and lean mouse ileum to a β_3-AR agonist or in the affinity of β_3-AR selective or β_1-/β_2-AR selective antagonists. The administration of dexamethasone to lean mice caused dose-related down-regulation of β_3-AR mRNA in WAT and BAT but had no effect in ileum and colon, suggesting that this is a possible explanation for the difference between mRNA levels in the adipose and gastrointestinal tissues of lean and obese mice. However, the return of the WAT and BAT β_3-AR mRNA levels to above control levels 24 h after dexamethasone treatment remains to be explained. These findings provide evidence that control of β_3-AR expression in ileum and colon differs from that in adipose tissues.

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