Hypertension in Transgenic Mice With Brain-Selective Overexpression of the α_{2B}-Adrenoceptor

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BACKGROUND

Previous studies have shown that the presynaptic α_{2B}-adrenoceptor subtype in the central nervous system has a sympathoexcitatory function and its activation leads to a hyperadrenergic hypertensive state. The purpose of this project was to develop a novel hyperadrenergic model, a transgenic (TG) mouse model with brain-selective overexpression of the α_{2B}-adrenergic receptor (α_{2B}-AR).

METHODS

We used Southern blot analysis to confirm transgene, real-time PCR to assess gene expression, western Blot analysis and immunohistology to assess protein expression and localization in brain areas. Indirect blood pressure (BP) and heart rate were recorded.

RESULTS

In TG mice there was a 1.8-fold increase in α_{2B}-AR protein expression compared to wild-type (WT) mice. Immunostaining of brain sections revealed that concentration of α_{2B}-AR was much more pronounced in TG than in WT mice. Systolic BP at 8 weeks of age was significantly elevated in TG 130 ± 6 mm Hg, compared with WT control nontransgenic littermates of the same age 107 ± 7 mm Hg, ($P < 0.05$), indicating that the TG mice had indeed developed hypertension.

CONCLUSIONS

We have therefore documented that overexpression of the α_{2B}-AR gene leads to increased production of α_{2B}-AR protein in brain regions known to regulate central sympathetic outflow, thus resulting in sustained BP elevation. This is a unique model of experimental hypertension driven purely by overexpression of the α_{2B}-AR that would result in an overactive sympathetic system and would be suitable for testing the pharmacologic properties of potential therapeutic agents.

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The contribution of the sympathetic nervous system to cardiovascular regulation is mostly exerted through the function of presynaptic α_2-adrenergic receptors (α_2-ARs) located in various areas of the central nervous system. Three α_2-AR subtypes have been cloned and characterized[1]: The α_{2A}-, α_{2B}- and α_{2C}-AR. Earlier studies have shown that pharmacologic α_2-AR agonists, such as clonidine, suppress the sympathetic nervous system and lower the blood pressure (BP),[2] whereas antagonists, such as yohimbine, have the opposite effect. However, pharmacologic probes have varying degrees of selectivity for various subtypes and cannot differentiate subtype-specific functions. The development of genetically engineered mice with deleted gene to each subtype permitted a better definition of each subtype's functions, including their role in BP regulation, as well as other functions, ranging from analgesia and sedation to behavioral aspects (motion, cognition, affect) and metabolic functions (thermoregulation, insulin sensitivity, lipolysis).[3–13] Regarding BP regulation, studies by other investigators,[9–13] as well as our own studies using either α_2-AR subtype gene knockout mice[14–16] or gene treatment with an antisense-DNA sequence targeting a specific subtype's gene in rats,[17–19] led us to conclude that the α_{2A}-AR has a sympathoinhibitory function and is the predominant presynaptic α_2-AR subtype in the central nervous system. On the other hand, the α_{2B}-AR has a sympathoexcitatory action and its activation (e.g., by the sodium ion) leads to a hyperadrenergic hypertensive state. Indeed, animals with deleted or inactivated α_{2A}-AR gene tend to have higher baseline BPs and a more pronounced hypertensive response to salt-loading than their wild-type (WT) counterparts.[14–16] In contrast, mice with deleted α_{2B}-AR gene are unable to raise their BP in response to acute or chronic sodium overload[14,16] and rats with salt-induced hypertension respond to antisense-DNA against the α_{2B}-AR gene with immediate and protracted BP lowering.[17–19]

Following-up on these findings, we have now successfully developed a novel transgenic (TG) mouse model with brain-selective overexpression of the α_{2B}-AR obtained by addition of extra copies of the α_{2B}-AR gene. It should be noted that although there are several experimental animal models of renin–angiotensin-dependent hypertension, this is the first experimental model of presumed inherent sympathetically driven hypertension. It could, therefore, become a unique

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resource for studying the consequences of a chronic hyperadrenergic state and its reversal by pharmacologic interventions.

METHODS

Generation of TG mice and animal husbandry. TG production was performed by the TG Core Facility at Boston University School of Medicine (http://www.bumc.bu.edu/transgenic). All TG mice used in this study are on FVB genetic background, therefore age and gender-matched (male) WT FVB mice were used as controls. All mice were fed standard mouse chow and water *ad libitum*. The transgene construct is composed of the human platelet-derived growth factor-β chain promoter (PDGF-β) (provided by Dr. T. Collins, Harvard Medical School, Cambridge, MA) for brain-specific expression,[20] followed by the mouse α_{2B}-AR complementary DNA (provided by Dr. B. Kobilka, Stanford University, Standford, CA) and a human growth hormone polyadenylation signal (hGH-polyA). The PDGF-β promoter is known to target neurons in the cortex, hippocampus, hypothalamus, and cerebellum.[20] The PDGF-β promoter and α_{2B}-AR DNA fragments were cloned into the multicloning site of the pCMV-MCS (Invitrogen, Carlsbad, CA) vector already containing the hGH-polyA. The complete construct was further confirmed by DNA sequencing. To release the construct and prepare for microinjection into one-cell embryos, the vector was sequentially digested with *Xba*I and *Rsr*II (New England BioLabs, Beverly, MA), separated on an agarose gel and the transgene was purified using a gel extraction kit (Qiagen, Valencia, CA). We followed DNA construction and purification approaches as well as TG production and screening protocols, which were successfully demonstrated at our TG center.[21,22] To screen for the presence of the transgene in potential founder mice, genomic DNA was isolated from mouse tail clippings, as described in previous publications.[14–16] TG mice were identified via PCR, using primers specific to the α_{2B}-AR/hGH-polyA overlap (α_{2B}-AR-TG): forward, 5′-ATCAGTCCAGGACCAGAAGAAG-3′ and reverse, 5′-TAATCCCAGCAATTGGGAG-3′ (**Figure 1a**). Three founder lines TG10, TG 13, and TG16 were obtained. The line TG13 was used for further experiments.

Southern blot analysis of tail DNA. Southern blotting to identify founders was performed as described elsewhere.[21,22] Genomic DNA was digested with *Xba*I and *Bsa*I (New England BioLabs, Beverly, MA) and the blot was probed with a probe shown in **Figure 1b**. The expected size of the transgene is 3.35 kilobase.

Total RNA extraction and gene expression analysis. Total RNA was isolated from the brains using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). This was followed by DNA-free DNase treatment and removal of contaminating DNA from the RNA preparation (Ambion, Austin, TX). The cDNA was generated from 1 μg of RNA by random hexamers using an RNA-PCR kit (Perkin-Elmer, Branchburg, NJ) according to the manufacturer's instructions. To determine whether α_{2B}-AR was overexpressed in the brain of TG mice we used reverse transcriptase PCR (RT-PCR) with α_{2B}-AR-TG primers (as described above) or α_{2B}-AR primers: forward 5′-CAACGAGCTGCTGGGCTATT-3′ and reverse 5′-TGTCCAGACTGATGGCACACA-3′. RT-PCR results were confirmed with quantitative real-time RT-PCR (Q-RT-PCR), was performed with the ABI prism 7900HT Sequence Detection System using an iTaq SYBR Green-based protocol (Applied Biosystems, Branchburg, NJ). The α_{2B}-AR transcripts were producing a 115 base pair size fragment. Glyceraldehyde phosphate dehydrogenase primers: forward 5′-TGCACCACCAACTGCTGAG-3′ and reverse 5′-GGATGCAGGGATGATGTTC-3′ were used to produce a 172-base pair fragment. All reactions were run in triplicate. After initial denaturation at 95 °C for 3 min, the cDNA products were amplified for 40 cycles consisting of denaturation at 95 °C for 15 s, and annealing and extension were performed in a single step at 60 °C for 45 s. The SDS 2.1 software generated standard curves from tenfold serial cDNA dilutions, and threshold cycle (C_t) was normalized for each standard curve. The slopes were between −3.19 and −3.57, and we chose −3.33, which corresponds to 100% efficiency of the PCR reaction. The copy numbers for all samples were normalized with the data obtained from glyceraldehyde phosphate dehydrogenase endogenous controls.

Western blot analysis. Brains were homogenized in lysis buffer (1× phosphate-buffered saline (PBS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) complete with protease inhibitor cocktail (Sigma, St.Louis, MO). The homogenates were clarified by centrifugation, and the pellets were discarded. The supernatants were collected and protein concentrations were determined by the Lowry method (Bio-Rad, Hercules, CA).

Polyclonal rabbit antibody, raised against α_{2B}-AR (Genex Biosciences, Hayward, CA) was utilized for Western blot analysis. Proteins (25 μg protein/lane) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the separated proteins were electro-transferred onto nitrocellulose membranes, which were blocked with 5% (wt/vol) nonfat dried milk for 1 h at room temperature. Nitrocellulose blots were washed in PBS-Tween 20 (0.05%) primary rabbit anti-α_{2B}-AR antibody (1:5,000) for 16 h at 4 °C. The membranes were then sequentially incubated with the secondary antibody horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (Santa Cruz, Santa Cruz, CA). Antibody binding was visualized by the Enhanced Chemiluminescence Detection System (Amersham, Piscataway, NJ). All blots were stripped with western blot stripping buffer for 1 h (Pierce, Rockford, IL) and then incubated with anti-rabbit glyceraldehyde phosphate dehydrogenase antibodies (Santa Cruz, Santa Cruz, CA).

Tissue preparation. For histology purposes, the mice were anesthetized with intraperitoneal pentobarbital at 50 mg/kg and then rapidly perfused transcardially with 4% paraformaldehyde in PBS buffer. The brains were removed and postfixed in the same fixative overnight at 4 °C, and then placed in 30%

sucrose in PBS buffer overnight at 4 °C. Thereafter, the tissues were frozen on dry ice in support medium (OCT; Bayer, Elkhart, IN). Brains were serially cut at 30 µm with a cryostat. Brain sections were kept in PBS buffer at 4 °C until use.

Immunohistochemistry. Immunohistochemistry was performed on free-floating tissue sections according to standard procedures. Briefly, sections were pretreated with 3% H_2O_2 in order to quench peroxidase activity, and incubated with 10% normal goat serum to block nonspecific binding. Subsequently, they were incubated overnight at 4 °C with rabbit polyclonal anti-α_{2B}-AR antibodies (1:1,500). Primary antibodies were recognized by anti-rabbit immunoglobulin G secondary antibodies (Vector Laboratories, Burlingame, CA) for 90 min. Then sections were incubated with ABC reagent (Vector Laboratories, Burlingame, CA) for 60 min. The immunoreactions were visualized by incubation of sections in DAB substrate for 2 min. For each staining, two images covering the area of interest were captured from three to four sections per animal using identical parameters.

BP measurements. Systolic BP and heart rate were indirectly measured in awake animals using a noninvasive computerized tail-cuff system (BP-2000 Visitech Systems, Apex, NC) as described in detail in our previous publications.[14–16] Daily measurements were taken, each session comparing 22 separate readings. The endpoint tail-cuff BP for each week was calculated by averaging measurements of the 5-day readings. BPs, were recorded during the 8th and 9th week of age, after which the animals were killed and their brains were removed for messenger RNA and protein expression analysis.

Statistics. All data are expressed as mean ± s.e. Student's *t*-test for paired and unpaired data was used as appropriate. Differences at $P < 0.05$ were considered significant.

RESULTS

Identification of TG mice

TG mice overexpressing mouse α_{2B}-AR in the brain were developed to examine the effect of brain-specific expression of the gene *in vivo*. For this study, the mouse α_{2B}-AR cDNA and the brain-specific PDGF-β promoter were used. A schematic representation of the transgene is shown in **Figure 1a**. Three founder lines TG10, TG13, and TG16 were generated, which allowed us to examine transgene effects independent of integration site. Positive TG founders (heterozygous +/– mice) were mated with WT (–/–) mice to produce F1 generation positive mice. These F1 mice were bred to produce F2 homozygote (+/+) TG mice. Southern blot analysis revealed the presence of the transgene in heterozygous and homozygous animals but not in WT mice in the F2 stage. A representative screen is shown in **Figure 1b** for founder TG13, which was further studied.

Expression of the α_{2B}-AR transgene messenger RNA

RT-PCR using α_{2B}-AR-TG- and α_{2B}-AR-specific primers identified transgene expression. Quantitative real-time PCR assays

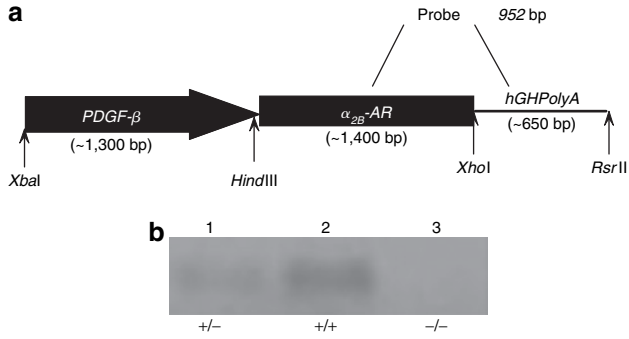

Figure 1 | The mouse α_2-adrenergic receptor (α_{2B}-AR) transgene and Southern blotting. (**a**) Schematic representation of the mouse α_{2B}-AR transgene. (**b**) Southern blot analysis to identify transgenic offspring. Lane 1: (+/–) heterozygote transgenic (TG), lane 2: (+/+) homozygote TG mice, lane 3: (–/–) wild-type mice.

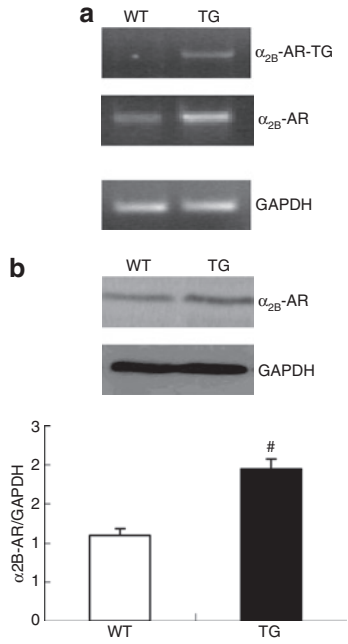

Figure 2 | Gene expression and protein expression analysis: (**a**) Representative reverse transcriptase PCR on RNA extracted from whole brain shows increased expression of α_{2B}-AR-TG and α_2-adrenergic receptor (α_{2B}-AR) bands in transgenic (TG) mice compared to wild-type (WT) mice, whereas glyceraldehyde phosphate dehydrogenase (GAPDH) bands are similar. (**b**) Representative western blot analysis of protein extracts from whole brains shows increased amount of α_{2B}-AR protein in TG compared to WT mice, whereas GAPDH bands are similar. Densitometry of the western blots normalized with GAPDH shows that the differences are statistically significant, #$P < 0.05$.

were used to confirm the expression of α_{2B}-AR transgene in whole brains of TG and WT mice. Real-time PCR using α_{2B}-AR specific primers, amplifying the 115 base pair PCR product revealed ~40-fold more α_{2B}-AR expression in the whole brains of TG animals than in the brains of WT animals. Representative RT-PCR is shown in **Figure 2a**. This demonstrates upregulated expression of transcripts encoding the α_{2B}-AR transgene compared to control WT mice.

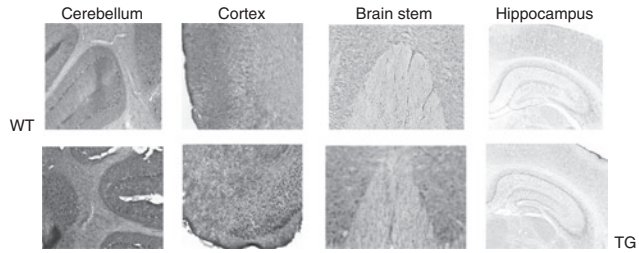

Figure 3 | Immunohistology of mouse brain sections (cerebellum, brainstem, hippocampus, and cortex) with α_2-adrenergic receptor (α_{2B}-AR) antibodies showing increased concentrations of immunostaining in the sections of transgenic (TG) compared to corresponding sections from wild-type (WT) mice.

Table 1 | Blood pressure and heart rate of α_{2B}-AR transgenic vs. wild-type mice

	8 Weeks of age WT	8 Weeks of age TG	9 Weeks of age WT	9 Weeks of age TG
Systolic blood pressure (mm Hg)	107 ± 7	130 ± 6*	108 ± 7	131 ± 12*
Heart rate (beats/min)	692 ± 46	696 ± 23	690 ± 40	665 ± 51
N	8	8	8	8

α_{2B}-AR, α_2-adrenergic receptor; TG, transgenic mice; WT, wild-type mice.

Kidney, heart, aorta, and liver tissues from TG mice were checked for α_{2B}-AR transgene expression using the same technology. No TG-specific bands were detected in any of the tissues (data not shown).

Brain α_{2B}-AR protein expression in mice

Western blot analysis of brain protein homogenates using α_{2B}-AR polyclonal antibody revealed α_{2B}-AR overexpression in the TG mouse brain at levels ~1.8-fold higher than endogenous α_{2B}-AR levels in brains of WT mice (**Figure 2b**). Immunoblot analysis of dissected regions of the brain showed that in cerebellum, brainstem, (which includes, the medullary regions that are crucial for BP control), hippocampus and cortex from TG mice, α_{2B}-AR expression was significantly increased compared to expression in corresponding brain regions of WT mice (data not shown).

Distribution of α_{2B}-AR protein in mouse brain

Study of α_{2B}-AR immunoreactivity throughout the brain of control, WT mice revealed a widespread distribution of α_{2B}-AR. Immunostaining of α_{2B}-AR was much more pronounced in all brain areas of TG than corresponding brain areas of the WT mice, but cerebellum, brainstem, hippocampus, and cortex showed higher effect of immunostaining of the α_{2B}-AR as shown in **Figure 3**.

Hemodynamic measurements

To determine whether overexpression of α_{2B}-AR in the brain is associated with hypertension, BPs, and heart rates were recorded five times per week using a tail-cuff system. As shown in (**Table 1**), SBP was significantly elevated at 8 and 9 weeks of age in the TG ($n = 8$), by an average 23 and 21 mm Hg, respectively ($P < 0.05$), compared with control nontransgenic littermates of the same age ($n = 8$), indicating that the TG mice had indeed developed hypertension. However, there was no difference in heart rates between the two groups at either 8 or 9 weeks of age.

DISCUSSION

In previous studies with normotensive and hypertensive rats, we have mapped the localization of various α_2-AR subtypes in specific brain regions, including the cortex, hypothalamus, pons-medulla, and cerebellum.[23,24] The current report describes the creation of TG mice generated with the use of a PDGF-β promoter driving a mouse α_{2B}-AR cDNA construct. The PDGF-β promoter is known to target neurons in the cortex, hippocampus, hypothalamus and cerebellum of TG animals.[20] Accordingly, alterations in expression of α_2-AR subtypes in these areas would alter the equilibrium between sympathoinhibitory (α_{2A}-AR mediated) and sympathoexcitatory (α_{2B}-AR-mediated) influences, as mentioned earlier.

Presence of the transgene was confirmed by Southern blot analysis in homozygous animals and quantitative real-time RT-PCR using α_{2B}-AR-specific primers revealed that the gene was overexpressed by ~40-fold in whole brains of TG compared to WT mice. Western blot analysis of brain protein homogenates from TG mice indicated a ~1.8 fold increase in α_{2B}-AR protein expression compared to WT mice. Analysis of specific sections: cortex, brainstem, and cerebellum, indicated increase of α_{2B}-AR protein expression in TG mice compared to corresponding brain regions of WT mice. Immunostaining of the various brain sections also revealed that α_{2B}-AR expression in cortex, brainstem, and cerebellum was much more pronounced in TG than in WT mice. The TG mice, at 8 and 9 weeks of age, had significantly higher systolic BPs—by an average of 23 and 21 mm Hg, respectively—than their WT counterparts. We have, therefore, documented in these mice that overexpression of the α_{2B}-AR gene leads to increased production of α_{2B}-AR protein in brain regions known to regulate the central sympathetic outflow, resulting in sustained BP elevation. The lack of increase in heart rate could be explained by activation of baroreflexes in response to BP elevation.

This is the first experimental animal model of hypertension driven by presumed endogenous sympathetic overactivity, i.e., not induced by pharmacologic or dietary manipulations. Studies of the pathophysiology and complications of hypertension have mostly used either spontaneously hypertensive animals, where the mechanisms of hypertension are incompletely understood, or experimental models of angiotensin-induced hypertension that can be obtained by a variety of procedures causing renal ischemia, such as renal artery ligation or kidney wrapping. Recently, a TG mouse model with brain-selective overexpression of the AT_1 receptor of angiotensin II was proposed for investigation of the central nervous system-mediated mechanisms of angiotensin II contribution to cardiovascular

homeostasis.[25] It was suggested that this model could also serve for studies of the role of the sympathetic system in cardiovascular regulation[26] on the basis of the known interaction between the renin–angiotensin system and the sympathetic nervous system.[27] However, this relationship would be indirect and the results would be open to alternative interpretations.

In summary, this new TG mouse model is a unique model of experimental hypertension driven purely by overexpression of the α_{2B}-ARs, that would be expected to result in an overactive sympathetic system and we are currently in the process of defining its phenotype. On the basis of our clinical and experimental studies with interventions targeting the α_{2B}-ARs,[28] we would expect that overexpression of the α_{2B}-AR will cause the TG mice to develop higher baseline BP with aging, accentuated further by salt loading; to exhibit higher levels of catecholamines; earlier and more pronounced evidence of diastolic and possibly systolic cardiac dysfunction; increased susceptibility to cardiac hypertrophy and myocardial infarcts and possibly the development of renal insufficiency. Such findings would suggest that if a pharmacologic antagonist were developed with selective affinity for the α_{2B}-AR, it might be capable of preventing or reversing the adverse consequences of chronic sympathetic overactivity. This model would be uniquely suitable for testing the pharmacologic properties of potential therapeutic agents.

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