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2 **Identification of a novel 12-nucleotide insertion polymorphism in the promoter region of**
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4 **ADRA2B : full linkage with the 9-nucleotide deletion in the coding region and influence on**
5
6 **transcriptional activity**
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Abstract

The α_{2B} -adrenoceptor (α_{2B} -AR) mediates vasoconstriction and a common polymorphism (+901 Ins/Del), located in the coding region of the human α_{2B} -AR gene (ADRA2B), has been demonstrated to affect receptor function *in vitro*. In this study, we have identified a novel polymorphism corresponding to the insertion of 12 nucleotides (GGGACGGCCCTG) at position -4825 relative to the start codon (-4825 del/ins) in the far upstream region of the ADRA2B promoter. The genotyping of 71 unrelated Finnish individuals showed that the -4825 ins polymorphism is common and in complete linkage with the Del polymorphism at position +901 and a G/C substitution at position -98. Transfection of various cell lines with luciferase constructs containing a 5.5 kb fragment of the ADRA2B promoter region indicated that the -4825 ins polymorphism resulted in a large reduction of transcriptional activity. Electrophoretic mobility shift assays with oligonucleotide probes corresponding to the two ADRA2B alleles demonstrated that the region where the -4825 del/ins variation occurs binds nuclear proteins and that the 12 nucleotide insertion affects the pattern of bound transcription factors. Altogether, the present findings show that the previously identified +901 Del polymorphism is linked with a variation in the ADRA2B promoter that affects transcriptional activity *in vitro*. The molecular mechanisms underlying this effect are still unclear but a possible impact of the -4825 ins polymorphism on α_{2B} -AR expression would merit to be examined *in vivo* as a diminution of promoter activity may limit the functional consequences of the +901 Del polymorphism.

Keywords: adrenoceptor; ADRA2B promoter; polymorphism; transcription; haplotype.

1. Introduction

The α_2 -adrenoceptors (α_2 -ARs) are Gi/Go protein coupled receptors that are involved in the regulation of many physiological functions including central control of sympathetic tone, feedback auto-inhibition of neurotransmitter release from noradrenergic nerve endings, platelet aggregation and vasoconstriction. Based on divergent pharmacological properties and molecular cloning, α_2 -ARs consist in three subtypes (α_{2A} -, α_{2B} - and α_{2C} -AR) encoded by distinct intronless genes. Employment of mutation screening methods in the study of cohorts of individuals from various ethnic backgrounds has shown that all three human α_2 -AR genes are polymorphic and have variations in their third intracellular loops that modify either their biochemical properties or their coupling efficacy to cognate G-proteins [1]. Notably, a common polymorphism in the gene encoding the α_{2B} -AR subtype (ADRA2B) has been identified [2]. This polymorphism, which corresponds to a 9-nucleotide deletion in the coding block (+901 Del), results in the removal of three glutamic acid residues in an acidic motif located in the third cytoplasmic loop of the receptor. Examination of the functional characteristics of the α_{2B} -AR variant in heterologous cell systems showed that the deletion does not affect ligand binding or G-protein coupling, but results in decreased GRK-mediated phosphorylation and attenuated receptor desensitization [3]. Hence the Del α_{2B} -AR variant is expected to exhibit enhanced function. Clinical trials and epidemiologic studies investigating the consequences of the +901 Del variation on vascular responses to α_2 -agonist administration [4] or its possible association with cardiovascular diseases [5] have, however, yielded inconsistent results. Examination of a 5.8 kb genomic fragment comprising 2.2 kb upstream and 2.3 kb downstream from the coding sequence demonstrated the existence of several single nucleotide polymorphisms (SNPs) in different regions of the ADRA2B gene [6-8]. Most of these SNPs are rare, but nucleotide variations at positions -98, +1182 and +1776 are common and in linkage disequilibrium with the +901 Ins/Del

1
2 polymorphism. Furthermore, the examination of variation frequency in different ethnic
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4 populations indicated that nine of the most common polymorphisms are organized in four major
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6 haplotypes, together representing 95 % of both African American and Caucasian populations [8].
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8
9 The significance of the different haplotypes for receptor expression is so far unknown. In the
10
11 present work, we characterized a novel polymorphism located 4.8 kb upstream from the
12
13 ADRA2B coding region (-4825 del/ins). This 12 nucleotide insertion polymorphism is common
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15 and, in the population studied in this work, in complete linkage with the previously described
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17 +901 Del polymorphism. The functional consequences of this variation were investigated by
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19 using reporter gene and electrophoretic mobility shift assays.
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2. Materials and methods

2.1. Materials

The BAC clone # RP11–139J6 was obtained from the BACPAC Resource Center (Oakland, CA, USA). The expression vector containing the MZF-1 transcription factor (CB6–MZF-1) and the corresponding empty control vector (CB6) were generously donated by Dr R. Hromas (Indiana University Cancer Center, Indianapolis, IN, USA). The pNF- κ B–Luc reporter vector was from Stratagene (La Jolla, CA, USA). Culture medium and fetal calf serum were purchased from Invitrogen (Life Technologies, Rockville, MD, USA). Restriction and DNA-modifying enzymes were from New England Biolabs (Ozyme, Saint-Quentin-en-Yvelines, France) and plasmids were purified using the Qiafilter Maxiprep kit (Qiagen, Courtaboeuf, France). Oligonucleotides were from Eurogentec (Seraing, Belgium), human recombinant TNF α and all other compounds were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Genotyping

Genotyping was performed on samples collected from 71 unrelated, young and healthy Finnish men. The collection of DNA and the genotyping was approved by the Ethics Committee of the Southwestern Health Care District, Finland. All subjects gave their written informed consent. The genotyping method used to identify the +901 Del polymorphism has been described elsewhere [9]. The -4825 ins polymorphism was determined on genomic DNA isolated from whole blood, using the sense primer 5'-ACGTGTAGAGGAAGAGGAAGG₃' and the antisense primer 5'-AATGTCTGGAA-TACAGGGAGG₃'. This set generates amplicons of 192 bp for the

1
2 deletion and of 204 bp for the insertion allele. Moreover, digestion with *Hae* III yields fragments
3
4 of 168 and 24 bp for the deletion allele and of 118, 62 bp and 24 bp for the insertion allele. The
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6 PCR products or the fragments resulting from *Hae* III digestion were separated in 2.5 % agarose
7
8 gel and stained with ethidium bromide, and alleles were identified based on their different
9
10 electrophoretic mobility.
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13 14 15 16 2.3. DNA sequencing

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18 Sequencing was performed by the dideoxy chain termination method using the BigDye®
19
20 Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an
21
22 automated DNA sequencer (ABI 3100, Perkin Elmer Applied Biosystems). DNA samples were
23
24 sequenced on both strands, using the same primers as those used for genotyping.
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31 32 33 2.4. Luciferase constructs

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37 The luciferase constructs used in this study were generated as follows: the *Eco*RI-*Not*I restriction
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39 fragment (corresponding to nucleotides -5505/-603 relative to the start codon) of the ADRA2B
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41 gene isolated from a human fetal genomic DNA library (Stratagene, Catalog n° 961200) [6] was
42
43 first subcloned into the pBluescript®II KS+ vector (pKS, Stratagene). The resulting construct,
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45 pKS/ADRA2B-5505/-603[del], served as a template to insert the sequence GGGACGGCCCTG
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47 at position -4825 using the QuikChange®II XL mutagenesis kit (Stratagene) and
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49 5'-ACCTGGTAATTCTTTATTGTGGGGACGGCCCTGAACCTTGCAGGACATTTGG₃' and
50
51 5'-CCAAATGTCCTGCAAGGTTCAAGGCGTCCCCACAATAAAGAATTACCAGGT₃' as
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53 sense and antisense primers. This procedure led to the pKS/ADRA2B-5505/-603[ins] construct.
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2 The construction of pGL3-derived vectors (Promega, Madison, WI, USA) containing the -
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4 1874/+3 region of the ADRA2B gene with a G (pGL3/ADRA2B-1874/+3[G]) or a C
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6 (pGL3/ADRA2B-1874/+3[C]) at position -98 was previously described [6]. The generation of a
7
8 pGL3–Basic vector, where an *EcoRI* site was created in the MCS by conversion of the *SmaI*
9
10 sequence CCCGGG to CCCATCGAATTC, was also described earlier [10]. The *EcoRI-SacII*
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12 fragment of pKS/ADRA2B-5505/-603[del] (nucleotides -5505/-1034) was ligated to the *SacII-*
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14 *SalI* fragment of pGL3/ADRA2B-1834/+3[G] (containing the -1034/+3 region of ADRA2B and
15
16 the luciferase gene) and the *SalI-EcoRI* of the pGL3-Basic (corresponding to vector backbone),
17
18 to generate the pGL3/ADRA2B-5505/+3[del/G] construct. In a similar way, the ligation of the
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20 *EcoRI-SacII* fragment of pKS/ADRA2B-5505/-603[ins] with the *SacII-SalI* fragment of
21
22 pGL3/ADRA2B-1834/+3[C] and the *SalI-EcoRI* fragment of pGL3-Basic led to
23
24 pGL3/ADRA2B-5505/+3[ins/C], which harbors the 12-nucleotide insertion at position -4825 and
25
26 possesses a C at position -98. The construction of pGL3/ADRA2B-3160/+3[G],
27
28 pGL3/ADRA2B-1034/+3[G] and pGL3/ADRA2B-1034/+3[C] construct has already been
29
30 described [6]; pCMV– β Gal was created by replacing the luciferase gene by a CMV promoter
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32 and a β -galactosidase reporter gene in a pGL3–Basic vector backbone.

33 2.5. Cell transfection and measurement of reporter gene activity

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47 The MCF-7 (human mammary adenocarcinoma) cell line was cultured in HEPES-buffered
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49 DMEM/F12 medium supplemented with 10 % fetal calf serum and 2 μ g/ml insulin. Non-
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51 transformed MCF-10A cells (human mammary epithelial) were cultured in the same medium
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53 with the addition of 20 ng/ml EGF and 50 nM hydrocortisone. HeLa (human cervical
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55 adenocarcinoma), HEK 293 (human embryonic kidney) and BHK-21 (baby hamster kidney) cell
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2 lines were cultured in DMEM supplemented with 10 % fetal calf serum. MCF-7 and MCF-10A
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4 cells were respectively transfected using Lipofectamine 2000 (Invitrogen) and jetPEI (Polyplus-
5
6 transfection, Illkirch, France). HeLa, HEK 293 and BHK-21 cells were transfected using the
7
8 FuGene-6 Transfection Reagent (Roche Diagnostics, Meylan, France). Briefly, cells were seeded
9
10 at appropriated density in 12-well plates and transfected with 0.8 μg of luciferase construct plus
11
12 0.2 μg of pCMV- βGal . All cell types were harvested 30 h post-transfection, disrupted in passive
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14 lysis buffer (Promega) and reporter gene activities were measured using the luciferase assay
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16 reagent (Promega) and *o*-nitrophenyl β -D-galactopyranoside as substrate. Luciferase activity was
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18 normalized for variations in transfection efficiency using β -galactosidase activity. Results are
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20 expressed as fold increases with regard to the promoterless vector pGL3-Basic.
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28 *2.6. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)*

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33 MCF-7 cells were stimulated or not for 15 min with 20 ng/ml TNF α and were harvested in ice-
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35 cold PBS. Cell pellets were solubilized for 5 min at 4°C in EMSA I buffer (50 mM Tris-HCl, 10
36
37 mM KCl, 1 mM EDTA, 0.2 % NP-40, 10 % glycerol, pH=7.9) containing 100 mM NaF, 2 mM
38
39 Na₃VO₄ and protease and phosphatase inhibitor cocktails (Roche Diagnostics). Lysates were then
40
41 centrifuged at 6500 g for 3 min and pelleted nuclei further incubated for 20 min at 4°C in EMSA
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43 II buffer (20 mM Hepes, 400 mM NaCl, 10 mM KCl, 1 mM EDTA, 20 % glycerol, pH=7.9)
44
45 containing protease and phosphatase inhibitors. Nuclear extracts were centrifuged at 14,000 g for
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47 10 min and supernatants were adjusted to a protein concentration of 1 mg/ml before being used
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49 for EMSA. Assays were performed in a 12 μl total volume of binding buffer (20 mM Hepes, 70
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51 mM NaCl, 2 mM dithiothreitol, 4 % Ficoll, 0.01 % NP-40, 100 $\mu\text{g}/\text{ml}$ bovine serum albumin,
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53 pH=7.5) containing 1 μg of poly(dI-dC) and 1 ng of [³²P]-labeled double-stranded probe
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2 (approximately 100,000 cpm ng⁻¹). The binding reaction was initiated by adding 5 µg of nuclear
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4 extract, containing or not a 50 fold excess of unlabeled double-stranded competitor. Mixtures
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6 were then incubated for 20 min at room temperature prior to analysis on a non-denaturing 5 %
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8 polyacrylamide gel. The gel was then dried under vacuum and subjected to autoradiography.
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10 11 12 13 14 *2.7. Data analysis*

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18 Data were analyzed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA)
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20 using either two-tailed paired t-test or one-way ANOVA followed by Turkey's test.
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3. Results

3.1. Identification and frequency of -4825 ins polymorphism

In the frame of previous work aiming to investigate the functional characteristics of the promoter region of the gene encoding α_{2B} -AR [6], the screening of a human fetal brain genomic DNA library has allowed us to isolate a 10 kb *EcoRI-KpnI* fragment containing 5.5 kb of the 5'-flanking region. Sequencing indicated that the isolated fragment corresponds to the long form of α_{2B} -AR, i.e. the allele without deletion at position +901 in the coding region. The complete sequence of this region (GenBank accession number AF005900) was compared to that of a BAC clone (RP11-139J6) obtained in the frame of the human genome program (accession number AC092603), which corresponds to the short form of α_{2B} -AR (i.e. with the 9-nucleotide deletion at position +901). Besides the already reported SNPs at position -98 (-98 G/C) and +1182 (+1182 C/A), which are known to be in strict linkage with the +901 Del polymorphism [6], alignment of the two sequences showed a major divergence in a region far upstream from the start codon. As seen in Fig. 1A, this variation consists of the insertion of 12 nucleotides (GGGACGGCCCTG) at position -4825, and was thus termed -4825 ins. The two sequences were also checked for other SNPs previously described in the promoter and coding regions [6-8]. Both sequences were identical at the 19 positions (-2142, -1787, -1661, -1410, -1396, -1237, -1100, -687, -587, -576, -524, -523, -514, -429, -338, +36, +120 +632 and +1135) where rare SNPs have been identified. Thus, according to previously proposed nomenclature [8], our genomic sequence corresponds to haplotype 1 organization, whereas the sequence of the BAC corresponds to haplotype 3. The respective frequencies of these two major haplotypes have been reported to be 65 and 15 % in African Americans and 52 and 37 % in Caucasians [8].

1
2 The next step of our study was therefore to determine the frequency of the -4825 ins variation in
3 a Caucasian population. Of the 71 Finnish unrelated subjects who were genotyped, 25 were -
4 4825 del/del (35 %), 17 were -4825 ins/ins (24 %) and 29 were of the heterozygous genotype (41
5
6 4825 del/del (35 %), 17 were -4825 ins/ins (24 %) and 29 were of the heterozygous genotype (41
7
8 %), indicating that this newly discovered polymorphism is common. As illustrated in Fig. 1B, the
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10 -4825 ins polymorphism was found to be in complete linkage with the +901 Del polymorphism.
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12 All carriers of the heterozygous genotype at position -4825 are heterozygous at position +901.
13
14 Furthermore, all individuals carrying the -4825 del/del genotype are +901 Ins/Ins and vice versa.
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16 Given that the +901 Ins/Del polymorphism is strictly associated with the -98 G/C transversion
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18 [6], the -4825 ins genotype is also associated with the presence of a C at position -98 and
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20 conversely.
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28 *3.2. Consequence on transcriptional activity of ADRA2B promoter*

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33 To determine the functional consequence of 5'-flanking region variations, luciferase constructs
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35 were generated and assayed in transformed (MCF-7) and non-transformed (MCF-10A) human
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37 mammary epithelial cells which spontaneously express the α_{2B} -AR [11]. In the two cell types
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39 (Fig. 2), the construct containing the 5.5 kb of 5'-flanking region with the 12-nucleotide deletion
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41 at position -4825 and a G at -98 (pGL3/ADRA2B-5505/+3[del/G]) exhibited fairly similar
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43 activity as the previously used construct containing a shorter fragment (pGL3/ADRA2B-
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45 3160/+3[G]) [6], indicating that the region spanning from -5505 to -3160 does not contain major
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47 repressor or enhancer element. In agreement with previous results obtained in a different cellular
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49 context [6], the shorter constructs pGL3/ADRA2B-1034/+3 containing a G or a C at position -98
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51 exhibited similar activity. On the other hand, the comparison of the activity of pGL3/ADRA2B-
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53 5505/+3[del/G] with that of pGL3/ADRA2B-5505/+3[ins/C] indicated that the 12-nucleotide
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2 insertion and the G to C transversion resulted in a large reduction of transcriptional activity (84
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4 % and 85 % decreases in MCF-7 and MCF-10A cells, respectively). The difference between the
5
6 two constructs was repeatedly found with 4 independent plasmid DNA preparations so that the
7
8 observed change is not attributable to differences in DNA quality. Similar differences in activity
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10 were also observed in other cell-lines (HEK 293, HeLa, BHK-21) that do not express α_{2B} -AR
11
12 (Table 2).
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15
16 *In silico* analysis of the consequences of the -4825 ins polymorphism on potential *cis*-acting
17
18 elements predicted that the insertion of GGGACGGCCCTG does not disrupt consensus
19
20 sequences for any known transcription factor, but creates putative binding sites for NF- κ B
21
22 (TGGGGACGGCCCTGA) and MZF-1 (TGGGGAC). Since members of the NF- κ B family have
23
24 the capacity to form homo- or hetero-dimers and can behave either as activators or repressors of
25
26 transcription [12], the effect of TNF α treatment was tested. As expected, the treatment of HEK
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28 293 cells for 16 h with human TNF α (20 ng/ml) caused a 30-fold increase in the activity of the
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30 pNF- κ B-Luc construct, but induced a similar decrease in the activity of pGL3/ADRA2B-
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32 5505/+3[del/G] and pGL3/ADRA2B-5505/+3[ins/C] (-77 and -78 % respectively; data not
33
34 shown), indicating that the 12-nucleotide insertion does not influence the response to this
35
36 cytokine. MZF-1 is a bi-functional transcriptional regulator, acting as an activator in cells of
37
38 hematopoietic origin and as a repressor in other cell types [13]. A role for MZF-1 in the
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40 repression of -4825ins/-98C allele appears unlikely because impairment of the transcriptional
41
42 activity of pGL3/ADRA2B-5505/+3[ins/C] was observed in HEK 293 or MCF-7 cells, which do
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44 not express this transcription factor [14, 15]. In further support with this view, co-transfection of
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46 these two cell-lines with a constant amount of each luciferase construct and increasing amounts
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48 of CB6-MZF-1 did not reveal any effect of this transcription factor on promoter activity (data
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50 not shown).
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3.2. Impact on the formation of DNA–nuclear protein complexes

As a first attempt to identify DNA-binding proteins on the short and the long alleles, EMSA were performed using relevant double stranded oligonucleotides (Table 1). As illustrated in Fig 3, the ADRA2B-del and ADRA2B-ins probes gave rise to different retardation profiles after incubation with nuclear extracts prepared from MCF7 cells. A single retarded band (termed A) was observed with the ADRA2B-del probe. The formation of complex A was prevented by the addition of an excess of the corresponding unlabeled oligonucleotide (homologous competition), but was hugely increased in the presence of the NF- κ B oligonucleotide. On the other hand, the ADRA2B-ins probe gave rise to several retarded bands, two of which were specific as they were strongly inhibited by the addition of homologous competitor. The upper band, which exhibits the same relative mobility but much stronger signal intensity than that found with the ADRA2B-del probe, likely corresponds to the same complex as band A and was therefore termed A'. As expected, the formation of complex A' was blunted by homologous competition. However, in clear contrast with that observed for complex A, its intensity was unaffected by the addition of NF- κ B competitor. Another major difference between the ins- and the del-probe is the presence of another complex exhibiting lower molecular weight (termed B) whose formation is totally prevented by the addition of NF- κ B probe. Finally, as shown by experiments with the NF- κ B probe (right panel), none of the formed complexes corresponded to the classical p50-p65 NF- κ B dimer, which exhibited lower mobility and was only observed when nuclear extracts prepared from TNF-treated MCF7 cells were used.

4. Discussion

Experiments carried out on heterologous cell systems have demonstrated that the deletion of 9 nucleotides encoding 3 glutamic acid residues located in the third intracellular loop of the α_{2B} -AR attenuates receptor phosphorylation/desensitization [3] and may therefore result in enhanced receptor function *in vivo*. Several epidemiological studies reported a possible link between the +901 Del polymorphism and an augmented risk of cardiovascular diseases [9, 16, 17]. The existence and nature of such associations are, however, unclear, as inconsistent results have been reported and because the genetic associations have not necessarily reflected changes in receptor-mediated responses [4, 18]. The +901 Del polymorphism has, however, been associated with exaggerated coronary constriction in response to adrenaline infusion [19]. In another trial, homozygous carriers of the Del allele exhibited augmented peripheral vasoconstriction within the first minutes following dexmedetomidine administration, but the enhanced response was not sustained [20]. On the other hand, measurement of dorsal hand vein diameter demonstrated no contribution of the +901 Del polymorphism to inter-individual variability in venoconstriction responses to dexmedetomidine or azeperole infusion [21, 22]. In agreement with this conclusion, a study of the hemodynamic effects of yohimbine showed that this α_{2B} -AR variation is not a major determinant of variability in blood pressure responses to α_2 -antagonists [7]. The failure to demonstrate an impact of this polymorphism on the constrictive responses to α_2 -agonists may be due to the presence of all three α_2 -AR subtypes in the vasculature; therefore, the specific contribution of α_{2B} -AR is extremely difficult to evaluate with subtype non-selective agonists. Alternatively, the lack of clear functional consequences *in vivo* might be due to yet unknown variations in non-coding regions of ADRA2B that would attenuate the consequences of the +901 Del polymorphism.

1
2 The main finding of the present work is the identification of a novel common 12-nucleotide
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4 deletion/insertion polymorphism in the distal upstream region of the ADRA2B gene (-4825
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6 del/ins) and its association with modified transcriptional activity. Based on the genotyping of a
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8 cohort of 71 unrelated Finnish individuals, the -4825 insertion variant is in complete linkage with
9
10 the deletion variant in the coding region (+901 Ins/Del). As the +901 Ins/Del variation is strictly
11
12 linked with the -98 G/C polymorphism [6], the -4825 del / -98 G / +901 Ins and the -4825 ins / -
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14 98 C / +901 Del alleles correspond to haplotypes 1 and 3 of the ADRA2B gene [8]. Transfection
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16 of different cell types, expressing spontaneously the α_{2B} -AR or not, demonstrated that the
17
18 promoter region containing the 12-nucleotide insertion at position -4825 and a C at position -98
19
20 exhibits strongly attenuated transcriptional activity. The mechanism accounting for impaired
21
22 activity remains to be clarified. As already suggested by our previous study in another cellular
23
24 context [6], the difference in transcriptional activity seems not to be due to the -98 G/C
25
26 substitution since luciferase constructs with a shorter promoter region and containing a G or C
27
28 exhibited identical transcription activity in MCF-10A and MCF-7 cells. The 12-nucleotide
29
30 insertion created a putative binding site for MZF-1; however involvement of this transcription
31
32 factor can be eliminated as the difference in activity between allele is found in cells lacking
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34 MZF-1 and because co-transfection of these cells with MZF-1 did not further affect activity.
35
36 Interestingly, the results of EMSA with oligonucleotide probes corresponding to the del or the
37
38 ins allele showed that the region of ADRA2B where the -4825 del/ins polymorphism occurs
39
40 binds nuclear proteins and that the insertion of GGGACGGCCCTG affected the pattern of bound
41
42 factors in at least two respects. First, both del and ins probes gave rise to complexes exhibiting
43
44 the same apparent mobility (A and A') which likely correspond to the same transcription
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46 factor(s), but clearly diverge in intensity and by the fact that their formation is differently
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48 affected in the presence of a NF- κ B competitor. Indeed, whereas the formation of complex A is
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2 surprisingly enhanced in the presence of NF- κ B competitor, that of complex A' is not. Given
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4 that the two alleles do not differ in their response to TNF α treatment, this divergence may only
5
6 have subtle functional consequences, if any. Moreover, neither A nor A' is expected to reflect
7
8 binding of a transcription factor belonging to the NF- κ B family, as these complexes were also
9
10 formed in the absence of TNF α treatment and were not blunted by the NF- κ B competitor.
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12 Second, the ins probe, but not del probe, was specifically recognized by an additional factor (B)
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14 whose binding was completely blunted in the presence of the NF- κ B competitor. Thus, complex
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16 B exhibited affinity for the NF- κ B consensus sequence and likely corresponds to a transcription
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18 factor which binds to the 12 nucleotide insertion. On the basis of its small molecular weight,
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20 complex B may be a factor devoid of transactivation domain and acting as a repressor, thus
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22 accounting for the impaired transcriptional activity of the -4825 ins allele.
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27 The potential impact of the difference in transcriptional activity observed *in vitro* remains to be
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29 investigated *in vivo*. Indeed, according to the haplotype organization, the -4825 ins and -98 G/C
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31 polymorphisms are in linkage with the +1182 C/A and +1776 C/A transversions, which are
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33 located in the ORF and the 3'-UTR, and may also affect gene expression. Because there is no
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35 known human tissue expressing only the α_{2B} -AR, and because no ligand truly selective for the
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37 α_{2B} -AR subtype is available, ADRA2B mRNA quantification appears currently the only way to
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39 approach the question of functional significance.
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44 In conclusion, the present work describes a novel deletion/insertion polymorphism in the distal
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46 promoter of the human ADRA2B gene (-4825 ins) that is in strict linkage with the +901 Del
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48 polymorphism in its coding region. Assessment of the strength of the linkage between the
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50 polymorphisms in the promoter and the coding region of ADRA2B in a non-Scandinavian
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52 population may also help to understand some of the discrepancies seen in the results of genetic-
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54 epidemiological studies of cardiovascular morbidity [4, 5]. The -4825 ins allele is associated
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2 with markedly reduced transcriptional activity. The functional impact of this novel promoter
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4 polymorphism on α_{2B} -AR expression will merit to be examined *in vivo*, as a decrease in receptor
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6 expression could in part compensate for the previously reported refractoriness of the mutated
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8 α_{2B} -AR to desensitization and would thus lessen its adverse consequences.
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Legends for figures

Fig. 1

Characterization of the -4825 del/ins polymorphism and association with +901 Ins/Del.

(A) Electrophoregrams of the -4825 del (upper panel) and -4825 ins (lower panel) alleles of the ADRA2B gene.

(B) PCR/RFLP-based genotyping for the -4825 del/ins (upper panel) and +901 Ins/Del (lower panel) polymorphisms.

Fig. 2

Transcriptional activity of luciferase constructs.

MCF-7 (left panel) and MCF-10A (right panel) cells were transfected with the indicated constructs as described in the “Materials and methods” section. Data are expressed as fold increases relative to the activity of the promoterless construct pGL3–Basic and presented as mean \pm S.E.M. from 4 to 7 independent experiments performed in duplicate. Data were analyzed using ANOVA followed by Tukey’s post test. Statistically significant differences between pGL3/ADRA2B-5505/+3[del/G] and pGL3/ADRA2B-5505/+3[ins/C] are indicated by asterisks (* $P < 0.01$; ** $P < 0.001$).

Fig. 3

Nuclear factor binding to the polymorphic region of the ADRA2B gene promoter.

EMSA were performed using MCF-7 nuclear extracts, with probes corresponding to both -4825 variants of the ADRA2B gene (ADRA2B-del and ADRA2B-ins; left panels) or to NF- κ B consensus sequence (right panel), as described in the “Materials and methods” section. Sequences of probes and competitors used are indicated in Table 1.

Table 1: Sequence of oligonucleotides used for EMSA

Probe	Sequence
ADRA2B-del (sense)	5' <u>G</u> TTTATTGTGAACCTTGCA ₃ '
ADRA2B-del (antisense)	5' <u>G</u> TGCAAGGTTTACAATAAAA ₃ '
ADRA2B-ins (sense)	5' <u>G</u> TTTATTGTGGGGACGGCCCTGAACCTTGCA ₃ '
ADRA2B-ins (antisense)	5' <u>G</u> TGCAAGGTTTCAAGGGCCGTCCCCACAATAAAA ₃ '
NF-κB (sense)	5' <u>GATC</u> TGGGGATTCCCCAT ₃ '
NF-κB (antisense)	5' <u>GATC</u> ATGGGGAATCCCCA ₃ '

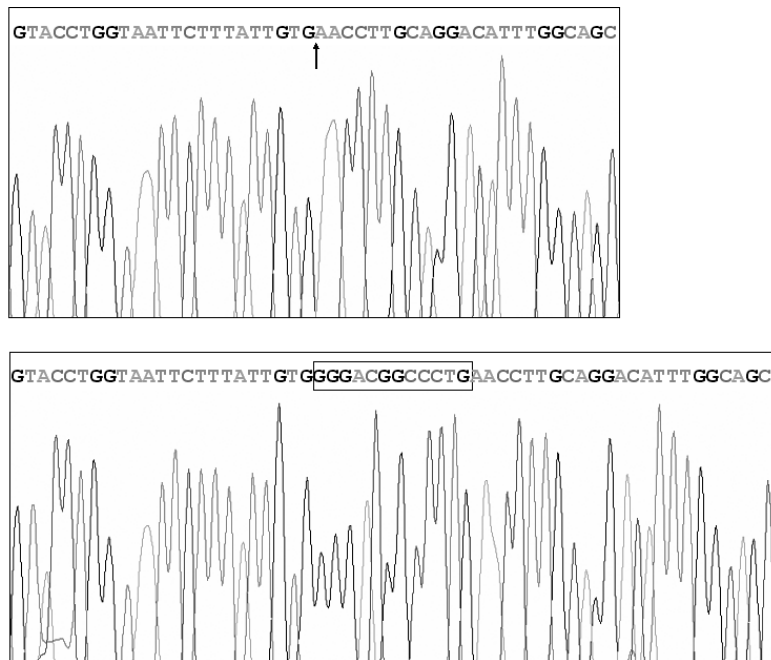
Table 2: Activity of ADRA2B promoter constructs in transfected cell lines

Luciferase construct	HEK 293	HeLa	BHK-21
pGL3/ADRA2B-5505/+3 [del/G]	10.2 ± 1.5	4.9 ± 0.7	5.5 ± 0.4
pGL3/ADRA2B-5505/+3 [ins/C]	3.3 ± 0.4 **	2.3 ± 0.4 *	1.9 ± 0.2 **

HEK 293, HeLa and BHK-21 cells were transfected with the indicated constructs as described in the Methods section. Data are expressed as fold increases of luciferase activity relative to the promoterless construct pGL3–Basic. Results are means ± S.E.M. from 5 to 8 independent experiments performed in duplicate. Data were analyzed using two-tailed paired t-test. Statistically significant differences with the activity of the pGL3/ADRA2B-5505/+3[del/G] construct are indicated by asterisks (* $P < 0.01$; ** $P < 0.001$).

Figure 1 B/W

A



B

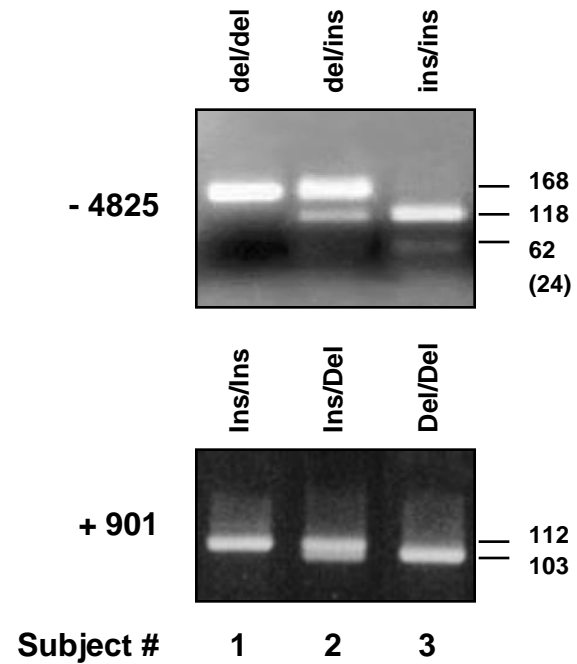
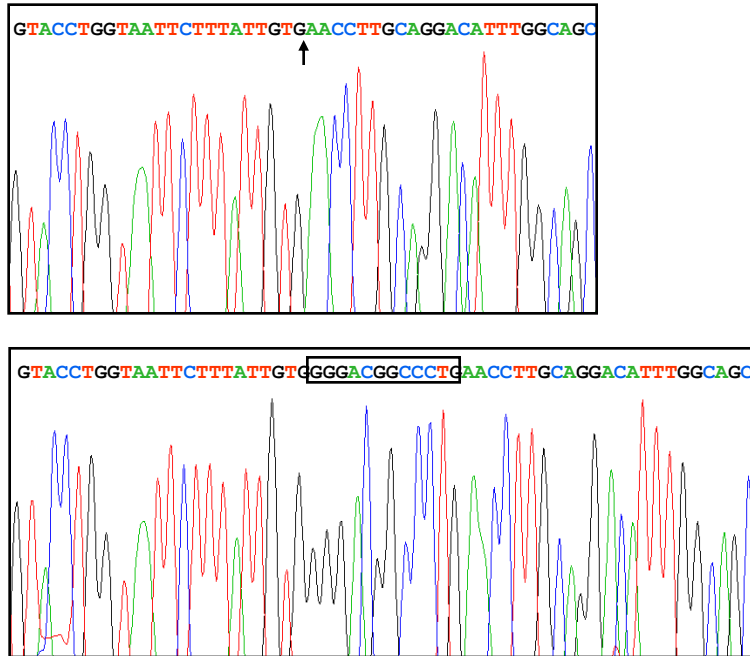


Figure 1 Color

A



B

