

# Adiponectin receptor expression in the human adrenal cortex and aldosterone-producing adenomas

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**Abstract.** Adiponectin is an adipocyte-derived circulating peptide that plays an important role in adipose tissue metabolism, insulin sensitivity and cardiovascular disease. The adrenal gland, by secreting glucocorticoid and mineralocorticoid hormones, intervenes in cardiovascular and glucose metabolism regulation and is surrounded by adipose tissue. Hence, we investigated the hypothesis that adiponectin receptor types 1 and 2 (adipo-R1 and adipo-R2) are expressed in the human adrenal gland and in adrenocortical zona glomerulosa cell-derived aldosterone-producing adenoma (APA) tissue. We used real-time reverse transcription-polymerase chain reaction to demonstrate the mRNA of adipo-R1 and adipo-R2 in 10 histologically normal human adrenal cortexes that were obtained from patients with renal cancer undergoing nephrectomy with ipsilateral adrenalectomy and in 10 APAs. Melting curve analysis and sequencing were used to confirm the specificity of the amplicons obtained. Results consistently showed the expression of specific mRNAs of adiponectin receptors in all histologically normal human adrenal cortexes and APAs. This novel finding suggests that adiponectin could play a regulatory role in adrenocortical function and growth in humans.

## Introduction

Adiponectin, an adipose tissue derived peptide (1, reviewed in ref. 2), exerts anti-inflammatory and anti-atherogenic effects by inhibiting crucial steps of atherogenesis (3-6), stimulating nitric oxide production (7) and blunting the expression of adhesins on the endothelium (3, reviewed in ref. 8). The

evidence linking blood adiponectin levels with arterial hypertension remains conflicting (9-14), even though low plasma adiponectin concentrations have been implicated in endothelial dysfunction, a hallmark of arterial hypertension and other conditions associated with high cardiovascular risk (15,16). Low plasma adiponectin levels have been also described in obesity, where they increase during weight reduction (17-19) and in type 2 diabetes (20). Moreover, because supplying physiological doses of recombinant adiponectin were found to correct hyperinsulinemia and insulin resistance (21), low plasma adiponectin concentrations have been suggested to play a causal role in insulin resistance (18,22,23). Therefore, blunted blood adiponectin levels would appear to be a hallmark of high cardiovascular risk conditions and hyperinsulinemia.

Two recently identified adiponectin receptor subtypes, referred to as adipo-R1 and adipo-R2, mediate the biological effects of adiponectin. The cloning and sequencing of the genes coding these receptor subtypes have been recently accomplished, thus providing novel opportunities for the investigation of their tissue distribution at the mRNA level (2,24). As the adrenal gland is not only exposed to circulating adiponectin but is deeply embedded in adipose tissue, endocrine-paracrine interactions might exist between the adrenal cortex and adipocytes. Hence, we decided to test the hypothesis that adipo-R1 and adipo-R2 genes are expressed in the histologically normal human adrenal cortex and in tumors that are derived from the adrenal zona glomerulosa.

## Materials and methods

**Adrenal specimens.** Histologically normal adrenocortical tissue was obtained at surgery from 10 patients with renal cancer undergoing unilateral nephrectomy and ipsilateral adrenalectomy. Furthermore, 10 patients with an aldosterone-producing adenoma (APA) were enrolled. In these patients the diagnosis was based on strict predefined criteria that were based on lateralization of aldosterone secretion at adrenal vein sampling, on surgery, pathology and, more importantly, follow-up data. For the latter, we required demonstration of normokaliemia and, according to the AHA guidelines (25), cure or improvement of hypertension at least 120 days after adrenalectomy. Cure was defined as a systolic blood pressure

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Table I. Anthropometric and biochemical features of the patients with APA.

Age (years)	52±12
Gender (male/female)	4/6
Systolic blood pressure (mmHg)	172±17
Diastolic blood pressure (mmHg)	107±11
Serum K <sup>+</sup> concentration (mmol/l)	3.1±0.5 [3.6-4.5]
Plasma renin activity (ng Ang-I/ml/h)	0.19 (0.09-0.36) [0.65-2.65]
Aldosterone plasma concentration (pg/ml)	397 (221-660) [<110]
Cortisol plasma concentration (ng/ml)	147 (46-259) [<46]
ACTH plasma concentration (pg/ml)	13.2 (4.5-46.3) [15-22]

Means ± SEM, n=10; ranges in parentheses. Normal values are shown in square brackets.

of <140 mmHg and diastolic blood pressure of <90 mmHg without medications; and improvement as a systolic and diastolic blood pressure of <140/90 mmHg, respectively, on the same or reduced number of medications and/or reduced defined daily doses, as described by the World Health Organization (26). The main anthropometric and clinical features of patients with APA are shown in Table I. Tissue specimens were obtained at surgery under sterile conditions, immediately frozen in liquid nitrogen, and stored at -80°C until extraction, as previously described (27). Peri-adrenal adipose tissue obtained from three APA patients was similarly processed as control.

**Measurement of adiponectin-receptor mRNAs.** Total RNA was isolated from frozen tissue using the guanidine isothiocyanate method (Omnizol Kit™, EuroClone, Milan, Italy). The method used to investigate adipo-R1 and adipo-R2 mRNAs entails a novel real-time reverse transcription (RT)-polymerase chain reaction (PCR) that was applied for the first time in this study. We used the LightCycler Instrument™ and LightCycler Design Probe™ software (both from Roche, Monza, Italy). For this approach, it is crucial that the RNA preparation is free of contaminating DNA which can lead to overestimation of the amount of RNA. Hence, the quality of the RNA was systematically checked using lab-on-a-chip technology in an Agilent Bio-analyzer 2100 with the RNA6000 nano assay (Agilent Technologies, Palo Alto, CA). To exclude the possibility of cross-amplification between the two genes, which share 67% homology (2), we blasted the mRNA sequences of the adipo-R1 versus adipo-R2 to identify the homologous regions; then, we designed primers that were selective for each adiponectin receptor gene. Primers were chosen to span exon-intron boundaries, in order to prevent occurrence of co-amplification of genomic DNA. Adipo-R1 and adipo-R2 (1 µg) encoding mRNA was reverse-transcribed with Iscript™ (Bio-Rad, Milan, Italy), and 2 µl of each RT reaction was amplified with specific primers by using a Fast Start SYBR-Green Plus Kit™ (Roche) in a glass capillary according to the manufacturer's instructions. The mRNA encoding for porphobilinogen deaminase (PBGD) was similarly processed for use as a housekeeping gene in a separate one-step RT-PCR, as detailed earlier (28). This served as a control for RT-PCR performance. Primer sequences are shown in Table II. Cycle conditions were the same for all genes tested: initial denaturation at 95°C for 10 min, followed by 40 PCR cycles consisting of 95°C for 5 sec, 58°C for 10 sec and 72°C for 15 sec. SYBR-Green I fluorescence emissions were monitored after each cycle. Expression of adipo-R1, adipo-R2 and PBGD mRNA was quantified by the second derivative maximum method of the LightCycler software (Roche), by determining the crossing points of individual samples by an algorithm which identifies the first turning point of the fluorescence curve. Adipo-R1 and adipo-R2 expression was calculated relative to PBGD, which was used as an internal control. Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 50°C and

Table II. RT-PCR primers and PCR products.

Primers	Sequence	Accession no.	Amplicon size (bp)
Adipo-R1			
Sense	5'-CCAAAGCTGAAGAAGAGCAAA-3'		
Antisense	5'-TCCCTCCCAGACCTTGTA-3'	NM_015999	134
Adipo-R2			
Sense	5'-ACATCTGGTTTCACTCTCATC-3'		
Antisense	5'-ATCAGTAGCCAGCAGGGA-3'	NM_024551	214
PBGD			
Sense	5'-TGCCAGAGAAGAGTGTGGT-3'		
Antisense	5'-ATGCTATCTGAGCCGTCT-3'	NM_000190	463

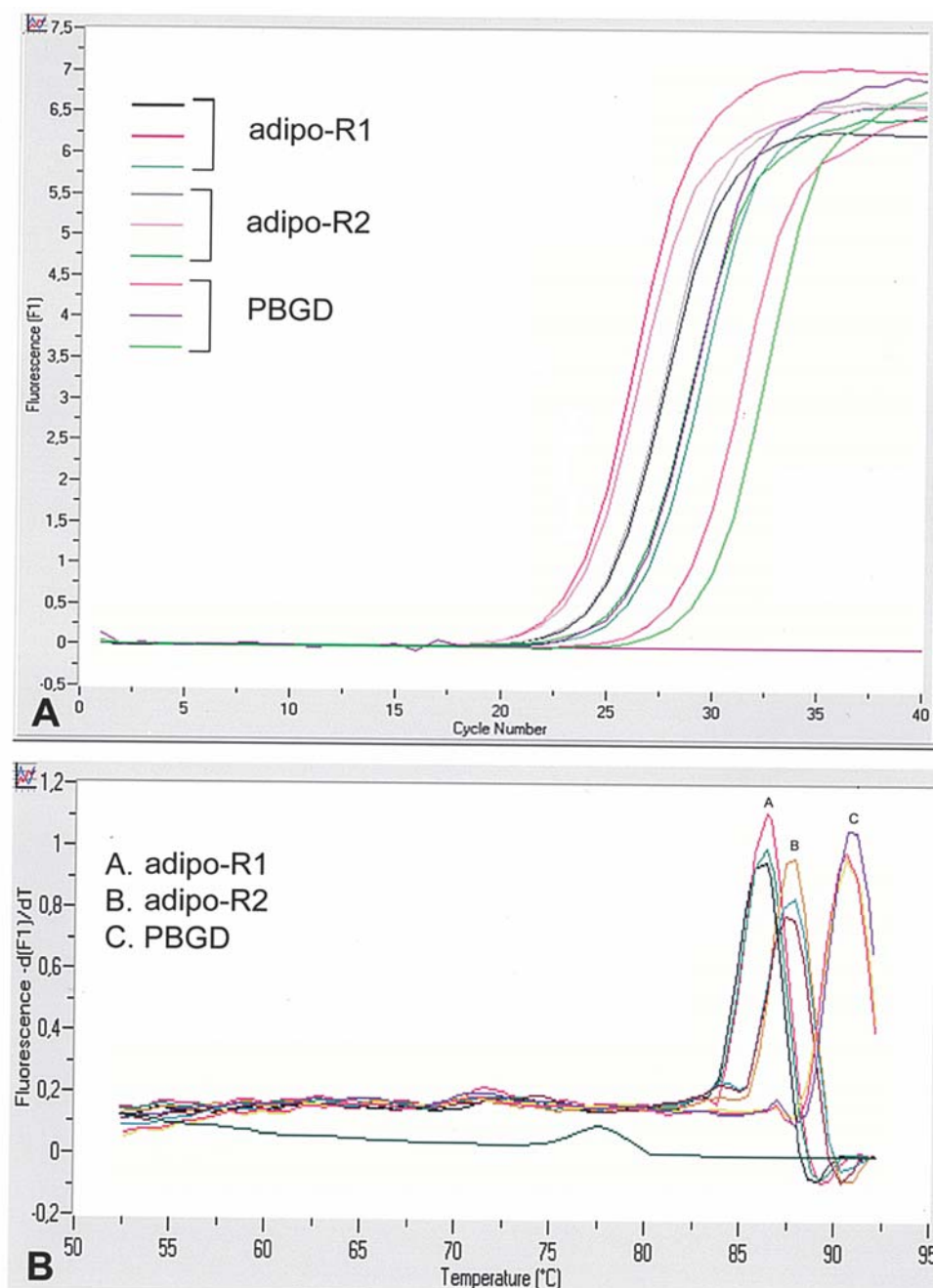


Figure 1. Exemplary results of real-time PCR amplification of adipo-R1 and adipo-R2 from three different specimens of histologically normal adrenal cortex (A). Corresponding melting curve results, which suggest the specificity of the amplicons and the lack of contaminants (B).

heating slowly to 95°C with measurement of fluorescence) at the end of each PCR cycle, using the specific routine built-up in the LightCycler instrument. The specificity of the PCR was further verified by sequencing analysis. The products of PCR were cleaned using a GenElute™ PCR Clean-up kit (Sigma-Aldrich Corp., St. Louis, MO), dehydrated, re-amplified using the original primers, and sequenced by the BigDye Terminator cycle sequencing reaction and an ABI PRISM 3100 Genetic Analyzer with support from CRIBI (Centro Ricerca Interdipartimentale Biotecnologie Innovative) at the University of Padua, Italy. Quantification of gene expression was carried out relative to PBGD by determining the threshold cycle (Ct) of the target genes, as described previously (29).

**Statistical analysis.** Comparison of the expression of adipo-R1 and adipo-R2 genes between the normal adrenal cortex and APA tissue was performed using the non-parametric Mann-Whitney test. Statistical analysis was performed using SPSS for Windows software (Vers. 13.0, SPSS Italy Inc., Bologna, Italy).

## Results

The novel real-time RT-PCR methodology developed in this study illustrates the feasibility of detecting adipo-R1 and adipo-R2 gene specific mRNAs in human tissues (Fig. 1A). The melting curve analysis revealed a clean well-defined peak (Fig. 1B) at the temperature of 86.3 and 87.7°C for the

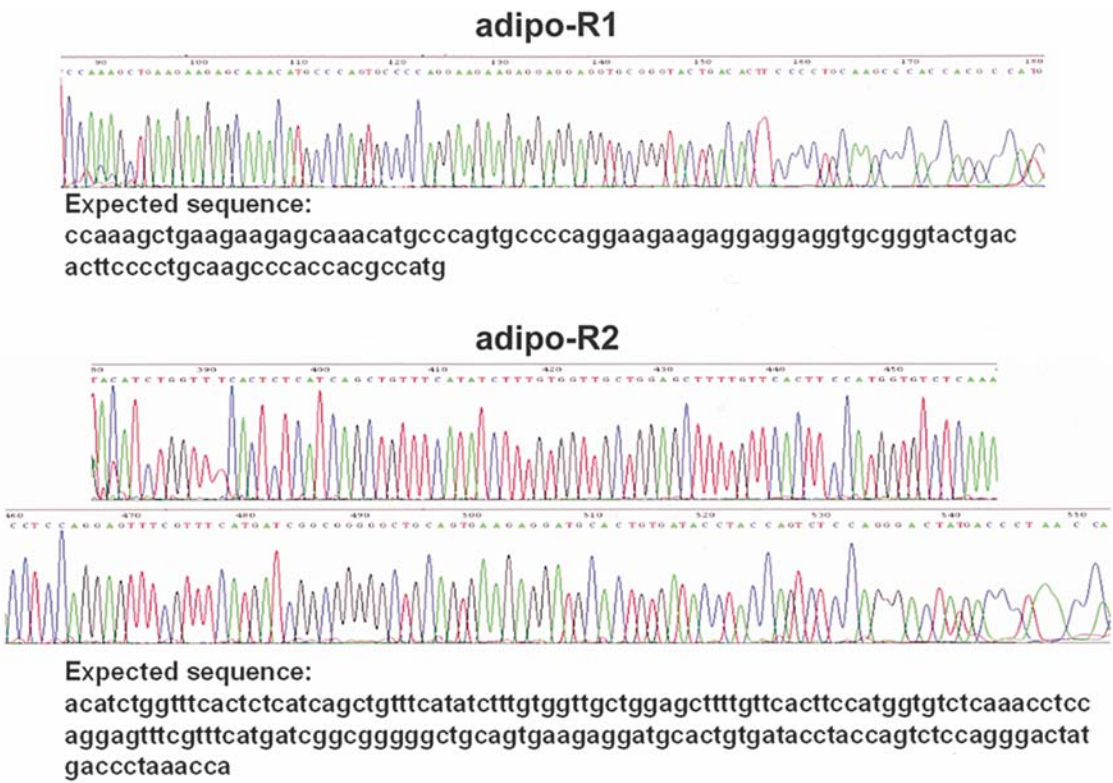


Figure 2. Results of sequencing of real-time PCR amplification products of adiponectin receptor subtypes.

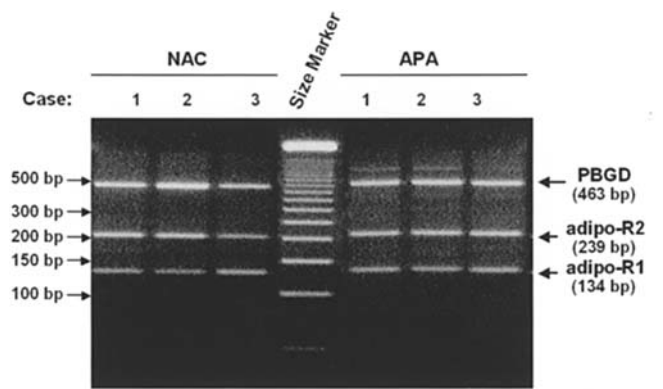


Figure 3. Ethidium bromide-stained 3% gel electrophoresis showing exemplary results of RT-PCR in three histologically normal human adrenal cortex (NAC) specimens and in three APA tissues. Lanes were loaded with amplification products of all three genes (adipo-R1, adipo-R2 and PBGD).

adipo-R1 and adipo-R2 genes, respectively, thus ruling out the amplification of non-specific products. Furthermore, the sequencing of the amplicons conclusively confirmed the exact correspondence between the obtained and the expected sequences (Fig. 2).

This methodology allowed the detection of specific mRNAs of adipo-R1 and adipo-R2 genes, not only in adipose tissue but also in adrenocortical tissue specimens. Histologically normal adrenocortical tissue (Figs. 1 and 3), APA specimens (Fig. 3) and control adipose tissue (data not shown) were found to consistently express both adiponectin receptor subtypes. Results of quantification relative to the housekeeping gene, PBGD, showed that both the adipo-R1 and the adipo-R2

mRNAs were expressed at a significantly higher level in APAs than in the normal adrenal cortex (Fig. 4).

**Discussion**

The intimate relationship between the amount of adipose tissue on one hand and the sensitivity to insulin, and blood pressure levels on the other, suggests the hypothesis that adipose tissue modulates the release of hormones that are involved in glucose metabolism. The present results demonstrate for the first time that the genes of the two known adiponectin receptor subtypes are expressed in the normal human adrenal cortex. Hence, the possibility of a cross-talk between adipose tissue and the adrenal cortex is suggested. However, as we used RNA extracted from tissue homogenates, our gene expression experiments do not allow any precise localization of adiponectin receptors in the human adrenal cortex. Moreover, they do not conclusively prove that these receptor subtypes are functionally expressed at the protein level. Accordingly, studies with *in situ* hybridization, immunocytochemistry, autoradiography and binding displacement are necessary to address these limitations.

Other peptides produced by adipocytes, e.g. leptin (30-34) and beacon (35-38), or regulating fat metabolism and energy homeostasis, e.g. orexins (39-42), neuropeptide-Y (43), neuropeptides-B and -W (44,45) and cholecystokinin (46-48), may control adrenal functions. Hence, it is tempting to hypothesize that adiponectin can regulate the release of cortisol and aldosterone from the adrenal cortex, with ensuing effects on gluconeogenesis, fat deposition, insulin sensitivity, electrolyte and water homeostasis, and ultimately on blood pressure levels.

By studying high cardiovascular risk patients, we recently found, in accordance with previous studies (13,49), that body



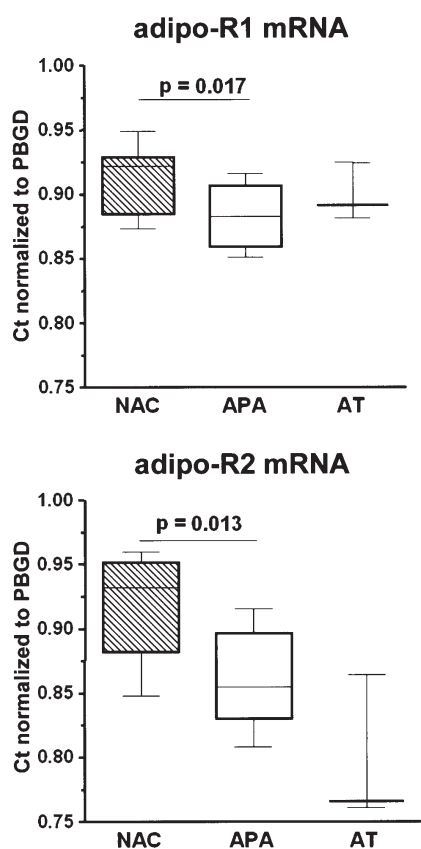


Figure 4. The graphs show the median, interquartile range and extreme values of the expression of adipo-R1 and adipo-R2 relative to the housekeeping gene PBGD measured with real-time PCR in the histologically normal human adrenal cortex (NAC), APA and adipose tissue (AT). The significantly lower Ct ratio of APA tissue for both adipo-R1 and adipo-R2 indicates a higher mRNA expression level as compared to NAC.

mass index, along with other variables, significantly predicts plasma adiponectin levels (unpublished results). Thus, the present demonstration of adiponectin receptors in the adrenal cortex may explain a functional link between body mass index (e.g. the amount of adipose tissue), blood pressure and the adrenal cortex. It has to be acknowledged, however, that the evidence from cross-sectional studies implicating plasma adiponectin in arterial hypertension is conflicting (11-14), and there are no data linking adiponectin to aldosterone secretion. Therefore, the hypothesis that adiponectin can modulate aldosterone secretion by acting on adiponectin receptor subtypes in the adrenal cortex remains to be addressed. The present results show that both adipo-R1 and the adipo-R2 genes are expressed at a higher level in APAs than in the normal adrenal cortex, a finding that merits further study.

In summary, our results document for the first time the gene expression of the adiponectin receptor subtypes in the human adrenal cortex and, at a higher level, in APA tissue. While the functional significance of this finding remains to be investigated, the possibility that adipose tissue can regulate the function of the human adrenal cortex is suggested.

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