A semi-quantitative method for the estimation of adenosine $A_1$ receptor mRNA levels in rat kidney

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ABSTRACT

Objective: To develop Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) method for the estimation of adenosine $A_1$ receptor mRNA levels in rat kidney.

Material and Methods: Total cellular RNA was isolated from whole rat kidney by small-scale total RNA preparation protocol and it was reverse transcribed into cDNA. The cDNA was subjected to PCR amplification using gen specific primers. The amplified cDNA was evaluated by gel electrophoresis and the intensity of the bands were visualized and quantitated with a FujiBAS 1000 PhosphorImager. Then the adenosine $A_1$ receptor mRNA levels were extrapolated from the standard curve.

Results: Adenosine $A_1$ receptor mRNA levels in rat kidney were measured as: $1.30 \pm 0.17 \times 10^7$ copies of adenosine $A_1$ receptor transcript/mg total RNA.

Conclusion: The RT-PCR method developed for the estimation of adenosine $A_1$ receptor mRNA levels in rat kidney is sensitive and reliable.

KEY WORDS: RT-PCR, cellular RNA, cDNA

Introduction

Adenosine is an endogenous nucleoside that modulates many physiological processes via its receptors including $A_1$, $A_2a$, $A_2b$, and $A_3$.

However, a functional role has especially been ascribed to $A_1$ receptors in the kidney. Therefore, adenosine $A_1$ receptors are extensively characterized in this tissue. But a sensitive method for the estimation of the mRNA levels of these receptors is still quite important, since the common methods including in situ hybridization, northern blot and slot blot analysis are no longer practical. In the present study, an attempt has been made to develop a sensitive RT-PCR method for the detection of adenosine $A_1$ receptor mRNA levels in rat kidney.

Material and Methods

Animals

Male Wistar rats (200-250 g) were housed in polypropylene cages in controlled temperature (27±2°C) and light. They were fed standard rat pellets. Food and water were provided ad libitum. The care and use of animals was carried out according to the Code of Practice set out by the (UK) Animals (Scientific Procedures) Act 1986.

Experimental protocols

Small-scale total RNA isolation for the extraction and purification of total cellular RNA from whole rat kidney: 1) Male Wistar rats (200-250 g) were anesthetized with sodium thiobutabarbitone (180 mg/kg, i.p.) and were killed by a blow to the head followed by exsanguination. Kidneys were removed and immediately freeze-clamped in liquid nitrogen and then stored at -70°C until required. 2) The kidney was homogenized in 10 ml of ice-cold denaturing solution (0.5 g/ml guanidium thiocyanate) with a Polytron homogeniser (2x15 second bursts). 3) 1.0 ml sodium acetate (2 M, pH 4.0) was added with mixing. 4) Five ml citrate-buffered phenol (pH 4.0) and 5 ml Chloroform-isoamyl alcohol (48:2 v/v) mix were added, mixed and chilled on ice for 15 min. 5) The mixture was then transferred to an ependorf tube (1.5 ml) and centrifuged (13 krpm, 10 min), then the top aqueous phase was pipetted into a fresh tube. After the addition of an equal volume (10 ml) of isopropanol, the mixture was chilled at -20°C for 30 min. 6) The total cellular RNA was pelleted by centrifugation (13 krpm, 10 min). Then the supernatant was decanted and the pellet dried by inversion on a paper towel and it was resuspended in 500 µl denaturing solution with a brief heating at 65°C. 7) Total cellular RNA was again precipitated with 500 µl isopropanol and
centrifugated (13 krpm, 10 min), then it was washed with 500 µl of ice-cold ethanol (70%) and re-centrifugated (13 krpm, 10 min). The pellet was dried at 37°C for 15 min and dissolved in 50 µl of DEPC (diethyl pyrocarbonate)-treated water with brief heating to 65°C to aid solubilisation. The resulting total cellular RNA was stored at -70°C.

Reverse transcriptase polymerase chain reaction (RT-PCR)

a) Reverse transcription (RT)
The concentration of total cellular RNA, isolated from whole rat kidney, was adjusted by UV spectrophotometry to 1 µg µl⁻¹ in diethylpyrocarbonate-treated water. Then 2.0 µl of total RNA (1µg/µl) and 1.0 µl of oligo (dT)₁₂₋₁₈ primer (0.2 µg/µl) were added into a DEPC-treated eppendorf tube and heated at 65°C for 3 min and then the tube was cooled slowly to room temperature. After that 2 µl of DTT (dithiothreitol; 0.1 M), 4 µl of 5x first strand buffer (250 mM Tris.Cl [pH 8.3], 375 mM KCl and 15 mM MgCl₂), 1 µl of dNTPs (deoxynucleoside triphosphates; 10 µM), 9 µl of RNA grade H₂O and 1 µl of Murine leukemia virus reverse transcriptase (MMLV RT; 200 U/µl) were added and mixed. Then 50 µl of ice-cold ethanol (70%) was added. After that the cRNA was pelleted by using a centrifuge (13 krpm, 10 min), then it was washed with 500 µl of ammonium acetate were added to the reaction. Then it was cleaned by phenol/CHCl₃ extraction; 15 µl Phenol mix (Phenol:CHCl₃:isoamylalcohol; 50:48:2 v/v) was added and centrifuged (13 krpm, 5 min). The top layer was taken into a fresh eppendorf tube and 151 µl CHCl₃:isoamylalcohol (48:2) was added. After mixing, the aliquot was centrifuged (13 krpm, 5 min). The top layer was taken into a fresh eppendorf tube, 6 µl of RNase free H₂O, 2 µl of 10xBuffer, 2 µl of each nucleotide (ATP, GTP, CTP, UTP), 2 µl of template (βA or adenosine receptor cDNA; 1.0 µg/µl) and 2 µl of enzyme mix (TF RNA polymerase and placental ribonuclease inhibitor) were added and incubated at 37°C for 15 min. 115 µl of RNA free H₂O and 15 µl of ammonium acetate were added to the reaction. Then it was cleaned by phenol/CHCl₃ extraction; 15 µl Phenol mix (Phenol:CHCl₃:isoamylalcohol; 50:48:2 v/v) was added and centrifuged (13 krpm, 5 min). The top layer was taken into a fresh eppendorf tube and 151 µl CHCl₃:isoamylalcohol (48:2) was added. After mixing, the aliquot was centrifuged (13 krpm, 5 min). The top layer was taken into a fresh eppendorf tube, then 20 µl of sodium acetate (3M, pH 5.2) and 151 µl isopropanol were added and kept at -20°C for 30-60 min. After that the cRNA was pelleted by using a centrifuge (13 krpm, 10 min) and the supernatant was removed, then 500 µl of icecold ethanol (70%) was added. After centrifuging for 10 min at 13 krpm, ethanol was removed and the cRNA pellet was dried at 37°C for 10-15 min. Then it was dissolved in 10 µl water.

b) Polymerase chain reaction (PCR)

Into a small eppendorf tube 5 µl of RT-template (cDNA), 5 µl of 10xPCR buffer (Tris.Cl [100 mM; pH 8.4], 0.5 M KCl, 1% Triton X-100), 4 µl MgCl₂ (25 mM), 1 µl of dNTPs (deoxynucleoside triphosphates; 2 mM), 1 µl of N-terminus primer (5'-3'; 500 pmol), 1 µl of C-terminus primer (5'-3'; 500 pmol) (primer sequences were given in Table 1), 1 µl of a-[^32]P]-dCTP (2'-deoxycytidine-5'-triphosphate; 50 µCi spec. act. 3000 Ci:mmol⁻¹) and 31 µl of MilliQ H₂O were added and mixed. Then 50 µl of mineral oil was added. After heating to 95°C for a few minutes, 1 µl of Taq (Thermophilus aquaticus) DNA polymerase (125 units) prepared according to the method of Pluthers (1993), 4 was added (The PCR protocol for adenosine A₁ receptors and β-actin are given in Table 2). After thermocycling, 10 µl of PCR product was subjected to agarose gel (1% agarose gel and 0.1 mM ethidium bromide) electrophoresis (Figures 1 and 2). To visualize the PCR product, the gel was dried overnight by sandwiching between 20-25 paper towels and applying a 1 kg weight. The dried gel covered with diethylpyrocarbonate-treated eppendorf tube, 6 µl of RNase free H₂O, 2 µl of 10xBuffer, 2 µl of each nucleotide (ATP, GTP, CTP, UTP), 2 µl of template (βA or adenosine receptor cDNA; 1.0 µg/µl) and 2 µl of enzyme mix (TF RNA polymerase and placental ribonuclease inhibitor) were added and incubated at 37°C for 4 h. 2 µl RNA free DNA was added to the reaction and was incubated at 37°C for 15 min. 151 µl of RNA free H₂O and 15 µl of ammonium acetate were added to the reaction. Then it was cleaned by phenol/CHCl₃ extraction; 15 µl Phenol mix (Phenol:CHCl₃:isoamylalcohol; 50:48:2 v/v) was added and centrifuged (13 krpm, 5 min). The top layer was taken into a fresh eppendorf tube and 151 µl CHCl₃:isoamylalcohol (48:2) was added. After mixing, the aliquot was centrifuged (13 krpm, 5 min). The top layer was taken into a fresh eppendorf tube, then 20 µl of sodium acetate (3M, pH 5.2) and 151 µl isopropanol were added and kept at -20°C for 30-60 min. After that the cRNA was pelleted by using a centrifuge (13 krpm, 10 min) and the supernatant was removed, then 500 µl of icecold ethanol (70%) was added. After centrifuging for 10 min at 13 krpm, ethanol was removed and the cRNA pellet was dried at 37°C for 10-15 min. Then it was dissolved in 10 µl water.

### Table 1

<table>
<thead>
<tr>
<th>Transcript</th>
<th>N-terminus primer (5'-3')</th>
<th>C-terminus primer (5'-3')</th>
<th>Size of PCR product (b.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>CTCGCCATTGCTGTGGATCGA</td>
<td>GTGTTGAGGAAGATGGCGAT</td>
<td>540 b.p.</td>
</tr>
<tr>
<td>β-actin</td>
<td>TGTAAACCAGTGCGGAGATG</td>
<td>GATCTGGATCTCATGTTGACTG</td>
<td>740 b.p.</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Transcript</th>
<th>PCR Protocol</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>94°C, 40 sec.; 64°C, 1 min.; 72°C, 1 min.</td>
<td>30</td>
</tr>
<tr>
<td>β-actin</td>
<td>94°C, 40 sec.; 69 to 61°C, 1 min.; 72°C, 1 min.</td>
<td>35 (15)*</td>
</tr>
</tbody>
</table>

*a Touchdown PCR was performed as described by Don et al (1991): To begin, the annealing temperature was decreased 1°C every 2 cycles. Once the baseline annealing temperature was reached, the number of cycles given in brackets were performed.*
Preparation of standard curve

Standard curves were generated using varying amounts (1/3, 1/9, 1/27, 1/81, 1/243) of in vitro transcribed adenosine A₁ receptor and β-actin cRNAs. These serially diluted cRNAs were subjected to RT-PCR and the amount of the PCR product determined by densitometry. The band densities of the test samples were adjusted for concentration differences (dividing by the concentration in mg/ml) and then mRNA levels were extrapolated from the standard curve. To improve assay reproducibility, where numerous assays were performed at a time, a master-mix of all reagents was prepared and aliquoted to ensure that all assays received equal amounts and concentrations of substrate and reagents.

Materials

All oligonucleotide primers were obtained from Genosys Biotechnologies Ltd. Deoxyribonucleotides and oligo (dT)₁₂₋₁₈ primer were purchased from Pharmacia. M-MLV reverse transcriptase was obtained from Gibco-BRL (Life Technologies). α-[³²P] dCTP was obtained from ICN Pharmaceuticals. The Ambion mMESSAGE mMACHINE in vitro transcription kit was obtained from amls Biotechnology. Millipore water (18 MΩ, Millipore) was used in the preparation of all reagents. For procedures involving RNA, all reagents and plasticware were treated with diethylpyrocarbonate (DEPC; 0.5%).

Analysis of data

mRNA levels were expressed as transcript number. Data are given as mean ± SEM and statistical comparison was made using t-test (One-Sample test). A value of P<0.05 was considered statistically significant. The intensities of the adenosine receptor mRNA bands were normalized relative to that of β-actin bands by dividing the former by the β-actin-specific PCR product densities. β-actin acted as a control for sample to sample variation in reverse transcription and PCR conditions, and the extent of degradation and recovery of RNA.

Results

The absolute mRNA levels of adenosine A₁ receptors in rat kidney were 1.30 ± 0.17 x 10⁹ copies of adenosine A₁ receptor transcript/µg total RNA. The absolute mRNA levels of β-actin in the rat kidney were 2.26 ± 0.19 X 10⁹ copies of β-actin transcript/µg total RNA. mRNA was extracted from the kidneys of six rats. β-actin or adenosine A₁ receptor mRNA levels in kidneys did not show statistically significant variations.

Discussion

In the present study, a reverse transcriptase-polymerase chain reaction (RT-PCR) assay was developed and optimized to enable the absolute quantitation of the mRNA levels of adenosine A₁ receptors. Several techniques are currently available to measure changes in gene expression including Northern blot, RNase protection assay, in situ hybridisation, and RT-PCR. The Northern blot or the more sensitive RNase protection assay is sufficient to detect quantitative differences between samples. However, if the sample quantity is low or the target message is rare these techniques are no longer practical. In the RT-PCR method, which is developed in the present study, less than 10 copies of target RNA can be estimated. This method is, actually, a semi-quantitative method.

In the present study, small-scale total RNA isolation protocol for the extraction and purification of total cellular RNA from the rat kidney was also developed. This is a slight modification of the method described by Chromoczynski and Sacchi.
who used a large-scale total RNA isolation procedure. This modification was attempted since the new protocol of RNA isolation could be completed in half the time of the large-scale method, conducive to the handling of a large number of samples, and it used only a fraction of the reagents whilst maintaining the quality of the total cellular RNA isolated.

In conclusion, in the present study reverse transcriptase polymerase chain reaction (RT-PCR) technique was developed and optimized to quantify the adenosine A1 receptor mRNA levels. The precision of the method values indicates that the developed RT-PCR method for the estimation of adenosine A1 receptor mRNA levels is a very sensitive, reliable and accurate one. Such a sensitive method is essential for the determination of extremely small amounts of mRNA levels.

Acknowledgements

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References


ERRATA

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A study of the antimicrobial activity of oil of Eucalyptus
Page 93: Column 2, Para 4, Line 3
50 ml should read as 50 µl

The error is regretted

Dr. Shreesh K. Ojha, Department of Pharmacology, AIIMS, New Delhi has pointed out that the article entitled “N-Methyl-D-Aspartate (NMDA) receptor antagonists as potential therapeutic agents in neurodegenerative diseases” published under ‘Molecules of the Millennium’ in February 2004 issue of the Indian Journal of Pharmacology has already been published in the October 2003 issue. The Indian Journal of Pharmacology regrets the error and thanks Dr. Ojha for pointing it out.

– Chief Editor, IJP