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Adenosine Receptor mRNA Levels During Postnatal Renal Maturation in the Rat

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Abstract

Adenosine may affect the pattern of intrarenal blood flow during renal development. It provides an angiogenic stimulus for the growth of new blood vessels and may be involved in compensatory renal growth. It is therefore of interest to investigate the expression of adenosine receptor genes during postnatal renal development. In the present study this was carried out by measuring adenosine receptor mRNA levels in rats aged between 2 and 60 days.

The order of abundance of adenosine receptor mRNA levels in 60-day-old rats was $A_{2A} > A_{2B} \geq A_1 > A_3$. A_1 receptor mRNA levels showed only small changes with increasing age although, by contrast, A_3 receptor mRNA increased markedly with age with levels at 60 days twenty-fold greater than at 2 days. A_{2A} receptor mRNA levels declined during renal maturation with transcript numbers four- to fivefold that at 12–18 days compared with numbers at 60 days. By contrast to the A_{2A} receptor, there were no significant changes in the renal levels of A_{2B} receptor mRNA during kidney maturation.

During postnatal renal maturation, the levels of mRNA for A_{2A} and A_3 adenosine receptor subtypes undergo marked changes which may be related to functional maturation, morphological development, or both.

Adenosine is thought to act as a paracrine regulator of renal function in the kidney. It affects various aspects of renal function in mature and neonatal animals including renal blood flow and its distribution within the kidneys, glomerular filtration rate, renin release, sodium excretion and urine flow (McCoy et al 1993). Moreover, studies by Balakrishnan et al (1993, 1996) of the renal effects of exogenous adenosine, and the selective adenosine A_1 receptor antagonist FK-453, have confirmed that this purine has a significant regulatory influence on normal human renal function. In addition, adenosine appears to be an important haemodynamic mediator of some forms of acute renal dysfunction in adult (Collis et al 1994) and neonatal animals (Gouyon & Guignard 1988)) and man (Erley et al 1994). For example, adenosine antagonists have been shown to ameliorate myohaemoglobinuric acute renal failure in the rat (Kellett et al 1989), a form of renal failure in which there are increases in renal adenosine A_1 receptor numbers and the abundance of A_1 receptor mRNA (Gould et al 1997).

The actions of adenosine are mediated by at least four different membrane-bound receptors, termed A_1, A_{2A}, A_{2B} and A_3 (Collis & Hourani 1993). Autoradiography (Weber et al 1988), in-situ hybridization (Weaver & Reppert 1992) and reverse transcriptase–polymerase chain reaction assays (Yamaguchi et al 1995) indicate that in rat kidney, A_1 receptors and transcripts are mainly located on glomeruli, the thick ascending limb and medullary collecting ducts. A_{2A} and A_{2B} transcripts have been detected in rat kidney (Weaver & Reppert 1992) and recently Kreisberg et al (1997) found mRNA for these receptors in rat outer medulla descending vasa recta. A_3 receptor mRNA has been found in rat kidney, but only at low levels, and its distribution is unknown (Zhou et al 1992). However, a functional role has only been ascribed to A_1 and A_2 receptors in the kidney (Navar et al 1996).

The above studies of renal adenosine receptor mRNAs were conducted with tissue from adult rats but there have been no investigations of gene expression in immature rat kidneys. The rat kidney

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exhibits nephrogenesis; new nephrons form in the cortex during postnatal development, and maturation of renal function is complete between 40–50 days post-parturition (Solomon 1977). Furthermore, at birth an uneven distribution of blood flow exists within the mammalian kidney with a greater perfusion of the inner cortex and medulla than the outer cortex, and as the kidney develops, there is a shift in blood flow to the outer cortex (Hook & Bailie 1979). Thus it seemed appropriate to investigate the expression of adenosine receptor genes during postnatal renal development since adenosine affects the pattern of intrarenal blood flow (McCoy et al 1993), provides an angiogenic stimulus for the growth of new blood vessels (Adair et al 1990) and may be involved in compensatory renal growth (Bergeron & Hoang 1983). To investigate which subtypes of adenosine receptor may be involved in postnatal renal development, we have measured the levels of individual receptor messenger RNAs in rats between 2 and 60 days of age.

Materials and Methods

Materials

α-[^{32}P] 2′-Deoxycytidine 5′-triphosphate (α-[^{32}P] dCTP) was obtained from ICN Pharmaceuticals Ltd (Thame, Oxfordshire, UK). All oligonucleotide primers were obtained from Genosys Biotechnologies Ltd (Cambridge, UK). M-MLV reverse transcriptase was obtained from Gibco-BRL (Life Technologies, Renfrewshire, Scotland). The Ambion mMESSAGE mMACHINE In Vitro Transcription Kit was obtained from ams Biotechnology (Oxfordshire, UK).

RNA preparation

Male Wistar rats, aged 2–60 days post-parturition, were used in this investigation. The diet of these animals consisted of maternal milk (0–11 days); maternal milk and standard rat pellets (11–21 days) and from 21 days onwards, when animals were weaned, standard rat pellets only (Banting & Kingman, Hull, UK). Rats were killed by a blow to the head followed by exsanguination, after which both kidneys were removed and immediately freeze-clamped in liquid nitrogen and then stored at −70°C until required. A group of 60-day-old rats were anaesthetized with thiobutabarbital (180 mg kg^{-1}), a cannula inserted into the abdominal aorta at a point below the left renal artery and the abdominal aorta occluded at a position just caudal to the origin of the right renal artery. The animals were killed with an overdose of anaesthetic and 10 mL of sterile saline (0·9% NaCl) was immediately infused via the abdominal aortic cannula over a period of 1 min. The saline-flushed left kidney and non-flushed right kidney, which acted as a control, were removed and freeze-clamped in liquid nitrogen.

Total cellular RNA was isolated from whole kidneys by the acid-guanidium thiocyanate-phenol-chloroform extraction method of Chromoczynski & Sacchi (1987) and adjusted to a concentration of approximately 1 μg μL^{-1} in diethyl-pyrocarbonate-treated water.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Two micrograms of total RNA were reverse transcribed in a total volume of 20 μL which included: 50 mM Tris buffer (pH 8·3), 75 mM KCl, 3 mM $MgCl_2$, 10 μM dithiothreitol, 50 μM deoxynucleoside triphosphates, 200 units M-MLV reverse transcriptase and 0·2 μg oligo $(dT)_{12-18}$ primer. The reaction was carried out at 37°C for 10 min. Five microlitres of the reverse transcribed material were then subjected to PCR in a total volume of 50 μL containing 10 pmol of each primer (specific oligonucleotide primer sequences are given in Table 1), 50 μM deoxynucleoside triphos- phates, 10 mM Tris buffer (pH 8·4), 50 mM KCl, 2 mM $MgCl_2$ (1 mM for A_3-specific PCR), 0·1 g dL^{-1} Triton X-100, 1 μCi α-[^{32}P] dCTP (spec. act. 3000 Ci mmol^{-1}) and 2·5 units of Taq DNA polymerase prepared according to the method of Pluthers (1993). Tubes were subjected to 26–30 PCR cycles, each cycle consisting of 94°C for 45 s, an annealing temperature (see Table 1) for 1 min and 72°C for 1 min. PCR for β-actin and A_{2B} mRNA employed a

Table 1. Specific-primer sequences and annealing temperature (T_{ann}) used in each polymerase chain reaction.

Transcript	N-terminus primer (5′-3′)	C-terminus primer (5′-3′)	T_{ann} (°C)
A_1	CTCGCCATTGCTGTGGATCGA	GTGTGTGAGGAAGATGGCGAT	64
A_{2A}	ATCGCCATCCGAATTCCACTC	ACGTGGGTTCGGATGATCTTC	66
A_{2B}	GCCATCACCATCAGCCTGGGC	CAGGAGGATGGCCACATTCAT	74–66*
A_3	CAAGCTGAACCGCACTCTGAG	CGCGTCTCTCTGAAGCCAGTC	66
β-Actin	TTGTAACCAACTGGGACGATATGG	GATCTTGATCTTCATGGTGCTAGG	69–61*

*Touchdown PCR (Don et al 1991).

touchdown PCR protocol (see Table 1) (Don et al 1991).

PCRs were optimized with respect to several parameters which included the amount of reverse transcribed material used as a template for each PCR, concentrations of magnesium and *Taq* DNA polymerase and reaction pH. PCR cycle times and annealing temperatures were adjusted as appropriate. After PCR, a 10-μL aliquot of each sample was electrophoresed on a $1\,g\,dL^{-1}$ agarose gel using Tris–acetate–EDTA buffer and the PCR product was visualized and quantitated on the FujiBAS 1000 PhosphorImager. Under optimal PCR conditions, there exists a linear relationship between the amount of PCR product and the initial amount of starting material.

Quantitation of mRNA levels using RT-PCR

Quantitation of adenosine A_1, A_{2A}, A_{2B}, A_3 receptor and β-actin mRNA levels was performed using RT-PCR according to the method of Robinson & Simon (1991). Standard curves were generated using varying amounts of each adenosine receptor and β-actin cRNA. These were produced by in-vitro transcription of the respective cDNA using a T7 RNA polymerase-based transcription kit (Gould et al 1997). 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.

Analysis of data

mRNA levels are expressed as transcript number or as a percentage of the levels recorded in 60-day-old rats. Data are given as mean ± s.e.m. and statistical comparison of means was made using a Student's paired *t*-test or one-way analysis of variance with mean data obtained from rats aged 2–42 days compared with the means from 60-day-old rats using Dunnett's *t*-test. A value of $P < 0.05$ was considered statistically significant.

The intensities of adenosine receptor mRNA bands were normalized relative to that of the β-actin bands. β-Actin was chosen as the internal control since it is the product of a house-keeping gene, that is, gene expression is constitutive and remains constant regardless of activation or proliferation state of the cell (Rappolee et al 1988). By using an endogenously expressed RNA as an internal control template, co-amplified along with the target RNA(s) of interest, 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

Expression of adenosine receptor subtypes in 60-day-old rats

The absolute levels of adenosine receptor and β-actin transcripts in kidneys of 60-day-old rats are shown in Table 2. The levels of adenosine A_{2A} receptor mRNA were twentyfold the levels of A_1 and A_{2B} receptor mRNA and fiftyfold those of A_3 receptor mRNA. mRNA transcript numbers for adenosine receptor subtypes in 60-day-old rats were not significantly different in saline-flushed kidneys compared with unflushed controls (Table 3). Compared with adenosine receptor subtype mRNAs, transcripts of β-actin were present in greater quantites with levels ten-fold higher than those for A_{2A} receptor mRNA (Table 2). This is consistent with the fact that β-actin represents one of the abundant proteins expressed in most cell types.

Adenosine receptor mRNA levels in the developing kidney

β-Actin mRNA in kidneys did not show statistically significant variations during postnatal maturation whereas A_1 receptor mRNA transcipt number displayed a modest decline with increasing age (Table 4). Compared with values at 60 days, A_1 receptor mRNA levels were 76–65% higher ($P < 0.05$) at 2 and 12 days, and declined to 38%

Table 2. Number of copies of transcripts for adenosine receptors and β-actin in kidneys of 60-day-old rats.

Transcript	Copies of transcript per μg total mRNA
A_1	$1.26 \pm 1.30 \times 10^7$
A_{2A}	$2.26 \pm 2.90 \times 10^8$
A_{2B}	$1.42 \pm 0.29 \times 10^7$
A_3	$2.27 \pm 0.69 \times 10^6$
β-Actin	$2.70 \pm 0.80 \times 10^9$

Results are given as mean ± s.e.m. mRNA was extracted from the kidneys of five rats.

Table 3. Adenosine receptor transcript levels in saline-flushed and control kidneys of 60-day-old rats.

Transcript	Copies of transcript per μg total mRNA	
	Control	Saline-flushed
A_1	$1.25 \pm 0.24 \times 10^7$	$1.42 \pm 0.90 \times 10^7$
A_{2A}	$2.26 \pm 0.22 \times 10^8$	$2.28 \pm 0.98 \times 10^8$
A_{2B}	$1.42 \pm 0.15 \times 10^7$	$1.35 \pm 0.65 \times 10^7$
A_3	$2.28 \pm 1.22 \times 10^6$	$1.92 \pm 0.18 \times 10^6$

Results are given as mean ± s.e.m. mRNA was extracted from the kidneys of four rats.

Table 4. mRNA levels for β-actin and adenosine receptors in the kidneys of rats at various times following parturition.

	Time (days)				
	2	12	18	42	60
β-Actin	103±26	121±28	89±15	95±1	100±9
A_1	176±9*	165±36*	112±21	38±3*	100±10
A_{2A}	334±117	494±27**	431±128*	208±42	100±58
A_{2B}	141±15	131±7	136±7	88±16	100±15
A_3	5±2**	52±6**	42±7**	41±2**	100±8

mRNA levels are expressed relative to those in 60-day-old rats. mRNA was extracted from the kidneys of five rats. Results are given as mean±s.e.m. *$P < 0.05$; **$P < 0.01$ relative to values at 60 days.

($P < 0.05$) at 42 days. The levels recorded for A_3 receptor mRNA showed an opposite pattern of expression to that of the A_1 receptor, since the abundance of A_3 receptor mRNA increased with age (Table 4). A_3 receptor mRNA levels in rats of between 2 and 42 days of age were significantly lower ($P < 0.01$) than those measured at 60 days, with a tenfold increase in levels from 2 to 12 days and a further twofold increase from 12 to 60 days.

A_{2A} receptor mRNA levels decreased with age since in rats of 12 and 18 days of age levels were four- to fivefold ($P < 0.05$) those in 60-day-old rats (Table 4). Changes in the renal levels of A_{2B} receptor mRNA during maturation were less marked than those noted for other adeno- sine receptor subtypes with no statistically significant changes noted in mRNA levels in rats of 2–42 days of age compared with levels recorded in 60-day-old rats (Table 4).

Discussion

The present study shows that there are some pronounced differences in the expression of various subtypes of adenosine receptor in kidneys from mature 60-day-old rats. The rank order of transcript abundance was $A_{2A} > A_{2B} \geq A_1 > A_3$, with the difference in transcript number between A_{2A} and A_3 receptors being almost two orders of magnitude. The results for A_1 and A_2 receptor mRNAs contrast with those of Weaver & Reppert (1992) who found, by Northern blot analysis, that the relative abundance of these receptor subtype mRNAs was $A_1 > A_{2A} \gg A_{2B}$. There is no clear explanation for these contrasting results although, in contrast to RT-PCR, Northern blot analysis is only a semi-quantative method of measuring mRNA levels. We did use Northern blot analysis in initial studies but the resulting signals were too weak to allow any accurate assessment of renal adenosine receptor mRNA levels (unpublished observations). The adenosine receptor mRNA levels measured in the current study are unlikely to be distorted by adenosine receptor mRNA from nucleated blood cells, as we have found that there were no statistically significant differences in transcript numbers between saline-flushed and non-flushed kidneys. Compared with other adenosine receptor subtypes, transcript numbers for the A_3 receptor were low and suggests that this adenosine receptor subtype is poorly expressed in the adult rat kidney. There are few studies of adenosine receptor gene expression at the level of anatomical structures. However, Kreisberg et al (1997) identified A_1, A_{2A} and A_{2B} transcripts in outer medulla descending vasa recta from rat kidneys, although A_3 receptor mRNA was not detected in these vessels.

This study has shown that renal maturation is associated with changes in adenosine receptor mRNA levels with the most striking changes occurring with A_{2A} and A_3 receptor transcripts. Such changes could be the result of either an alteration in the pattern of expression of adenosine receptors during renal maturation or different rates of proliferation of cells which predominately express a particular adenosine receptor subtype. Diet may also influence renal adenosine receptors. During the period of study, the diet of rats changed from maternal milk to pellets, and therefore sodium intake may also have changed, a factor that has been shown to affect adenosine receptor expression (Smith et al 1998). However, at least for the A_1 receptor, alterations in the sodium content of the diet need to be large to evoke changes in receptor density and mRNA levels. We have recently shown that 10-fold increases or decreases in the sodium content of the diet produce approximately twofold changes in A_1 receptor density and mRNA levels (Smith et al 1998). Such large changes in sodium intake, as enforced in the above study, are unlikely to occur during the transition from maternal milk to food pellets.

Compared with levels at 60 days, A_{2A} mRNA transcript numbers at 12–18 days of age were

increased four- to fivefold. Since levels of A_{2A} mRNA are at least 20 times greater at 60 days than any other adenosine receptor subtype mRNA, this suggests that the peak in abundance at 12–18 days, if translated to receptor protein, is of functional or possibly morphological significance. The rat kidney undergoes a developmental surge during weaning (16–20 days old) which is characterized by a rapid increase in both Na/K-ATPase activity and ability to concentrate urine (Fryckstedt et al 1993). Such a developmental surge is associated with cortical nephrogenesis and a shift in blood flow from the medulla and inner cortex to the outer cortex (Aperia & Larsson 1979; Hook & Bailie 1979). A possible role for a rise in A_{2A} receptors at 12–18 days is stimulation of angiogenesis since adenosine, which in adult rats produces renal vasodilation via A_2 receptors (Agmon et al 1993), has been shown to increase the proliferation of coronary venular endothelial cells in culture, an effect blocked by the non-selective adenosine antagonist 8-phenyltheophylline (Meininger & Granger 1990). Furthermore, endogenous adenosine, when produced by hypoxia, induces vascular endothelial growth factor mRNA in U-937 cells via stimulation of A_2 receptors (Hashimoto et al 1994), and adenosine may also stimulate compensatory renal hypertrophy (Bergeron & Hoang 1983).

The A_{2B} receptor mediates vasodilatation in some blood vessels, for example the guinea-pig aorta (Collis & Hourani 1993) and rat renal artery (Martin & Potts 1994). However, no other aspect of renal function has been specifically attributed to the A_{2B} receptor. Moreover, there were no statistically significant changes in A_{2B} mRNA levels throughout renal maturation and thus, by comparison with A_{2A} receptor mRNA, there is no rise in levels during the developmental surge at 16–20 days. In contrast to the A_{2B} receptor, a number of renal effects resulting from stimulation of the A_1 receptor have been proposed including mediation of tubuloglomerular feedback and inhibition of renin release (Navar et al 1996). Levels of A_1 receptor mRNA showed relatively minor changes during renal maturation particularly when compared with those recorded for A_{2A} and A_3 receptors. This suggests that, if translation of mRNA to receptor protein occurs, the A_1 receptor-mediated actions of adenosine are active during renal maturation. Human A_1 adenosine receptor expression is regulated by post-transcriptional mRNA processing (Ren & Stiles 1994) while the exact mode of regulation of A_1 receptor expression in the rat is unknown. However, studies of rat A_1 receptors during the development of acute renal failure and in the heart during foetal and postnatal maturation have shown parallel changes in A_1 receptor numbers and mRNA levels (Matherne et al 1996; Gould et al 1997). These findings suggest that in rodents there is transcriptional control of A_1 receptor density and therefore mRNA levels for the receptor may give an indication of receptor protein concentration.

RT-PCR studies have previously identified mRNA for the A_3 adenosine receptor in rat kidneys, but at lower abundance than in the testes where a reproductive role for the receptor has been proposed (Zhou et al 1992). No physiological role has been identified for the A_3 receptor in the kidney, but transcript numbers during renal development show interesting changes, particularly in comparison with other adenosine receptor subtypes. The most striking change is a tenfold increase in mRNA levels from 2 to 12 days with a further smaller rise at 60 days. The initial rise in transcript number from a low baseline may have relevance to the development surge at 16 to 20 days. Alternatively, the general increase in transcripts during the 60-day period might reflect the accumulation of mast cells in the kidney since this a particular cell type that has been associated with the A_3 receptor (Linden 1994).

In conclusion, this study demonstrates that the adult rat kidney expresses all currently cloned subtypes of the adenosine receptor with A_{2A} receptor mRNA present in the greatest amounts. During postnatal renal maturation, the levels of mRNA for adenosine receptor subtypes undergo a number of changes which are most marked for A_{2A} and A_3 receptors. These changes may relate to the period of rapid renal development which occurs at 16–20 days of age, although studies designed to localize the various receptor proteins at a regional and cellular level will be required to substantiate this proposal.

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