Influence of haemodialysis and left ventricular failure on peripheral $A_2A$ adenosine receptor expression

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Abstract

Background. Haemodialysis (HD) sometimes accelerates left ventricular failure (LVF). As adenosine (ADO) is strongly implicated in cardiovascular functions, particularly via $A_2A$ receptor activation and as changes of peripheral $A_2A$ receptors mirror changes occurring in the cardiovascular system, we examined the influence of HD and LVF on both ADO plasma concentration and the expression of $A_2A$ receptors (i.e. $B_{max}$, $K_D$ and mRNA amount) of peripheral blood mononuclear cells.

Methods. This cross-sectional study included 61 chronic renal failure (CRF) patients: 41 without LVF (24 haemodialysed and 17 undialysed) and 20 with LVF (9 haemodialysed and 11 undialysed). Ten LVF patients without CRF and 10 healthy subjects were also examined.

Results. (i) $B_{max}$ values of CRF patients without LVF were significantly decreased in undialysed patients compared with haemodialysed patients, and compared with controls ($69 \pm 25$ vs $98 \pm 33$ vs $180 \pm 60 \text{ fmol/mg of protein}$, $P < 0.05$). $B_{max}$ values of CRF patients with LVF were lower in undialysed patients than in haemodialysed patients ($60 \pm 27$ vs $101 \pm 27 \text{ fmol/mg of protein}$, $P < 0.05$). $B_{max}$ values of LVF patients without CRF were lower than in controls ($51 \pm 19$ vs $180 \pm 60 \text{ fmol/mg of protein}$, $P < 0.05$). (ii) $A_2A$ mRNA expression was increased in haemodialysed patients compared with controls ($20.2 \pm 0.75$ vs $17.6 \pm 1.3$, $P < 0.05$). (iii) ADO plasma levels were high in haemodialysed patients and further increased during the HD sessions.

Conclusion. The number of $A_2A$ receptors was decreased by CRF with or without LVF. However, this decrease was less important in haemodialysed patients. The changes in peripheral $A_2A$ receptor expression suggest a significant inflammatory response to HD and heart or kidney failure. Whether these changes do reflect alterations in cardiomyocytes needs further investigation.

Keywords: adenosine; cardiac failure; chronic renal failure; haemodialysis

Introduction

Because of the scarcity of organs and because all patients cannot undergo graft replacement of a kidney, haemodialysis (HD) remains a common therapy for end-stage renal left ventricular failure (LVF). Both immune defects [1] and cardiovascular complications [2] observed in chronic renal failure (CRF) patients are not improved and sometimes worsened by HD sessions (HDS) [2,3]. Cardiovascular complications such as coronary artery diseases [4], LVF [5], accelerated atherosclerosis [6,7] or intradialytic hypotension [8] are often observed in the HD population. We and others have already reported high levels of adenosine (ADO) in HD patients, which may participate in these complications [8–10]. ADO is a powerful cardiovascular modulator [11,12] that acts on blood vessel tone and the sinoatrial node via activation of ADO receptors which are subdivided into four subtypes ($A_1$, $A_2A$, $A_2B$ and $A_3$) depending on their primary sequence and pharmacology [13,14]. While stimulation of $A_1$ and $A_3$ receptors in the heart has been associated with cardioprotection and ischaemic pre-conditioning [15], activation of the $A_2A$ receptor subtype of smooth
muscle cells, results in vasodilatation [16]. However, nothing is known about the behaviour of A$_{2A}$ receptors in CRF patients and also about the influence of HD treatment on these receptors, whereas their expression is crucial for the cardiovascular system regulation [17,18]. Previous reports, using a model of congestive LVF have shown that the behaviour of mononuclear cells' A$_{2A}$ receptors reflects that of these receptors in the myocardium [17]. We therefore examined the influence of CRF and the consequences of HDS on the expression of A$_{2A}$ receptors of peripheral blood mononuclear cells (PBMC). Because A$_{2A}$ receptors are implicated in the contractile performances of the heart [19,20] and because LVF is a frequent complication of CRF, we also addressed the possible relationship of LVF with A$_{2A}$ receptor expression.

**Patients and methods**

**Patients**

Patients were included in a cross-sectional study. Based on statistical considerations, since variations of at least 30% in Bmax values, mRNA and ADO levels were considered, we used groups composed of at least eight patients.

**Haemodialysed patients without LVF.** Twenty-four patients (16 men and 8 women; mean age 65±15 years, range 22–84) were included in the study. The causes of CRF were as follows: diabetic nephropathy (n=3), chronic glomerulonephritis (n=3), interstitial nephritis (n=4), polycystic kidney disease (n=4), nephroangiosclerosis (n=4), and undetermined (n=6). The characteristics of the sessions were the same for all the patients. They underwent HD 3 times a week for 5±1 h with high permeability cellulose diacetate and triacetate membranes (DICEA and TRICEA 110, Baxter Healthcare Corporation, McGaw Park, IL, USA). The haemodialysers were not reused. The composition of the dialysate was 140 mM sodium, 2 mM potassium, 1.75 mM calcium and 32 mM bicarbonate. Vascular access was a native radial arteriovenous fistula. An endotoxin-free, nonpyrogenic ultrapure bicarbonate dialysate was used. Dialysis water was tested every week with the Limulus amoebocyte lysates (LAL) assay. Endotoxin counts were consistently 0.01 endotoxin unit/ml. Blood and dialysate flow rates were 250 and 500 ml/min. The mean haemoglobin concentration was 11.4±1.2 g/100 ml, and mean haematocrit was 33±5%. Nine patients were receiving recombinant human erythropoietin (9000±3500 IU/week). Twelve were under β-adrenergic receptor blockers for coronary disease (atenolol 50 mg/day, n=6; propranolol 40 mg/day, n=4; carvedilol 7.5 mg/day, n=2). Before the session, the mean serum creatinine level was 754±163 μM and blood urea nitrogen (BUN) was 57±19 mM. The mean Kt/V was 1.5±0.3 (range 1–2.2). Intradialytic weight gain between two sessions was 2±0.3 Kg.

**CRF undialysed patients without LVF.** Seventeen patients (10 men and 7 women; mean age 60±9 years, range 28–77) without LVF were included in the study. The causes of CRF were as follows: diabetic nephropathy (n=4), chronic glomerulonephritis (n=3), interstitial nephritis (n=1), and undetermined (n=4). The mean haemoglobin concentration was 10.4±1.3 g/100 ml and mean haematocrit was 31±4%. The mean serum creatinine level was 562±125 μM and BUN was 46±18 mM. Four patients were under β-adrenergic receptor blockers for coronary disease (carvedilol 7.5 mg/day, n=2; atenolol 50 mg/day, n=2).

Except β-adrenergic receptor blockers for coronary disease, anti-hypertensive drugs were stopped 72 h before samples collection.

**Chronic LVF patients.** LVF was defined as a fraction of ejection below 45% at least at two consecutive echocardiographies in a patient with symptoms of LVF (NYHA class II to IV). Thirty patients 17 men and 13 women were included. In most patients, pro brain natriuretic peptide (proBNP) was evaluated (Table 1). Among LVF undialysed patients (seven men and four women; mean age 73±6 years, range 46–85). The causes of CRF were: diabetic nephropathy (n=4), chronic glomerulonephritis (n=1), interstitial nephritis (n=2), nephroangiosclerosis (n=2), polycystic kidney disease (n=1) and undetermined (n=1). The mean haemoglobin concentration was 10.1±1.7 g/100 ml and mean haematocrit was 32±3%. The mean serum creatinine level was 430±160 μM and BUN was 39±18 mM.

Nine were HD patients (five men and four women; mean age 69±4 years, range 55–79). The causes of the CRF were diabetic nephropathy (n=3), chronic glomerulonephritis (n=1), diabetic neuropathy (n=1), and interstitial nephritis (n=2).

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<table>
<thead>
<tr>
<th>Table 1. Clinical data about left ventricular failure (LVF) patients</th>
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<tbody>
<tr>
<td><strong>CRF + LVF</strong></td>
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<tr>
<td><strong>Cardiopathies:</strong></td>
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<td>CHD</td>
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<td>VHD</td>
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<td>HHD</td>
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<td>LVEF (mean ± SD)</td>
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<td><strong>NYHA:</strong></td>
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<tr>
<td>III</td>
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<tr>
<td>IV</td>
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<tr>
<td><strong>ProBNP (ng/ml)</strong> (mean ± SD)</td>
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<td><strong>Treatments:</strong></td>
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<tr>
<td>Beta(−)</td>
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<td>Beta(+)</td>
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<tr>
<td>Alpha(−)</td>
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<td>ACEI</td>
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<td>ARB</td>
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<tr>
<td>CCB</td>
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<tr>
<td>Diuretics</td>
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<td><strong>CRF:</strong> chronic renal failure; CHD, coronary heart disease; VHD, valvular heart disease; HHD, hypertensive heart disease; LVEF, left ventricular ejection fraction (determined by echography or ventriculography); NYHA, new york heart association classification for cardiac failure; proBNP, pro brain natriuretic peptide; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker; Beta(−), beta-adrenergic receptor blocker; Beta(+), beta adrenergic receptor agonist; Alpha(−), alpha-adrenergic receptor blocker.</td>
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</table>
Influence of HD and LVF on adenosine receptor expression

system with a diode array detector was used (Hewlett Packard, Palo Alto, CA, USA). Lyophilized samples (500 μl) were mixed with 1 ml of phosphate buffer (NaH₂PO₄/Na₂HPO₄, pH 4), injected into a 1-ml loop and eluted with a methanol gradient on a Lichrospher C18 column (0% methanol for 3 min, then 10-25% over 15 min). The intra- and inter-assay coefficients of variation for nucleosides ranged from 1–3%. The limit of detection at 254 nm was 1 pmol/ml. Retention times and spectra were compared with those of exogenous ADO and metabolites. Quantifications were conducted by comparing areas obtained for samples with areas of known quantities of nucleosides.

Isolation of peripheral blood mononuclear cells (PBMC). Blood from the brachial vein (32 ml/sample) was drawn into Vacutainer tubes (8 ml per tube, Ficoll-based CPT system®; Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged (20°C, 1500 g, 30 min) within 2 h of blood collection. A 5 ml sample of interphase cells was collected (4.9 ± 1 x 10⁶ cells/ml) and washed three times with PBS, resuspended in 2 ml ice-cold distilled water and frozen (−80°C).

PBMC membrane preparation. Frozen cells were subjected to three freeze-thaw cycles (−80°C, +20°C) and centrifuged (4°C, 5000 g, 20 min). The supernatant was pipetted off and the pellet resuspended in 4.5 ml of binding buffer (50 mM Tris–HCl, 10 mM MgCl₂, pH 7.4). The suspension was homogenized with an Ultra Turrax® (30 s) in order to obtain a homogeneous cell membrane preparation, just prior to the binding assay. Protein concentration was determined (Beckman Synchron LX® apparatus).

βmax and KD determination. The methodology described by Varani et al. [23] was used with some modifications: [3H]-ZM 241385 is a potent and selective A₂A receptor ligand [24,25]. Saturation binding experiments were performed in duplicate by incubating (90 min, 4°C) homogenates of mononuclear cell membranes (200 μl for a total volume of 250 μl) with increasing concentrations of ligand (0.5 to 6 nM). Bound and free radioactivities were separated by vacuum-filtration of the sample through Whatman GF/C glass-fibre filters. Cold binding buffer (1 ml) was added to the sample before filtering. The filter was washed three times, and bound radioactivity was determined with a Beckman LS-1800 liquid scintillation spectrometer. A weighted nonlinear least-square curve fitting program, Graph Pad Prism® (Graph Pad Software Inc., San Diego, CA, USA) was used for the computer analysis of saturation experiments. Non-specific binding of [3H]-ZM 241385 was defined as binding in the presence of 10 μM of unlabelled ligand and was about 40% of total binding, in the range of a previous publication [17].

RNA extraction. Total RNA from PBMC was extracted with the MagAttract RNA tissue Mini M48 kit Qiagen, according to the manufacturer's recommendations (Bio-Robot M48). The cDNA was prepared from 250 ng of total RNA.

Reverse transcriptase-polymerase chain reaction (RT–PCR). Real-time PCR was performed with a Light-Cycler (Roche®) using the Light-Cycler FastStart DNA Master plus SYBR green I kit. The 18S primers were 5’GGT-GAC-GCG-GAA-TCA-GG-3’ (forward) and
5'-GCT-GCT-GGC-ACC-AGA-C-3' (reverse), generating a 218-bp DNA fragment. The A2A primers were 5'-TCC-CAT-GCT-AGG-TTG-GAA-CA-3' (forward) and 5'-GGA-GAGA-TCC-GGA-GTT-AGA-CAC-3' (reverse), generating a 191-bp DNA fragment. PCR conditions were as follows: after an initial 10 min pre-incubation step activation of the FastStart Taq polymerase, at 95°C, 45 amplification cycles were performed (95°C for 15 s, 60°C for 5 s and 72°C for 10 s).

**Statistical analysis**

The Wilcoxon sign rank test was used for intragroup comparisons (ADO plasma concentrations, Bmax values and mRNA levels before and after the HDS in the same group). A two-way analysis of variance (ANOVA) was used for inter-group comparisons. A P-value 0.05 was considered significant. The Spearman coefficient of correlation was used for correlation studies.

**Results**

**Bmax and KD of A2A receptors**

The KD was defined as the concentration of unbound ligand at which binding sites are 50% occupied (equilibrium constant, a measure of affinity). Bmax represents the total number of binding sites (i.e. in our assay, the number of membrane A2A receptors expressed as fmol/mg protein). That our conditions specifically address the expression of A2A receptors was verified by incubating PBMC with caffeine, a non-specific ADO receptor antagonist. In these conditions, ligand binding increased by 60% at the cell surface (data not shown), in agreement with previous findings [26].

Bmax values of CRF patients without LVF were significantly decreased in undialysed patients compared with haemodialysed patients, and compared with controls (69 ± 25 vs 98 ± 33 vs 180 ± 60 fmol/mg of protein, $P < 0.05$, Table 2 and Figure 1). Bmax values of CRF patients with LVF were higher in haemodialysed patients than in undialysed patients (101 ± 27 vs 60 ± 27 fmol/mg of protein, $P < 0.05$). LVF patients without CRF had lower Bmax values than controls (51 ± 19 vs 180 ± 60 fmol/mg of protein, $P < 0.05$).

**ADO plasma concentrations**

While mRNA amounts (expressed as the A2A receptor/18S ratio) measured in undialysed patients were not significantly different from those of controls, high levels of A2A mRNA were present in HD patients before the HDS, with no significant variations during the sessions (Table 2). In HD patients, before HDS, the ADO plasma concentrations were higher in patients with or without LVF than in undialysed patients. After HDS, ADO plasma concentrations increased significantly (Table 2).

**Discussion**

The main findings of this study were (i) CRF and/or LVF were accompanied by a decrease in the membrane expression and adenosine (ADO) plasma levels of CRF patients with LVF were higher in haemodialysed patients than in undialysed patients (101 ± 27 vs 60 ± 27 fmol/mg of protein, $P < 0.05$). LVF patients without CRF had lower Bmax values than controls (51 ± 19 vs 180 ± 60 fmol/mg of protein, $P < 0.05$).

K_D values of patients were in the same range as those of controls. CRF, LVF and HDS did not change these values (Table 2).

In summary, patients with CRF and/or LVF had lower expression of A2A receptors than controls, whereas HD patients displayed higher expression than undialysed patients.

**A2A receptor mRNA expression**

While mRNA amounts (expressed as the A2A receptor/18S ratio) measured in undialysed patients were not significantly different from those of controls, high levels of A2A mRNA were present in HD patients before the HDS, with no significant variations during the sessions (Table 2). Bmax values and mRNA expression were not correlated (Spearman’s $R = 0.1$, $P > 0.05$).

**Table 2.** Influence of chronic renal failure (CRF) and left ventricular failure (LVF) on peripheral blood mononuclear cell A2A receptor expression and adenosine (ADO) plasma levels

<table>
<thead>
<tr>
<th>CRF patients without LVF</th>
<th>CRF patients with LVF</th>
<th>LVF patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undialysed</td>
<td>Haemodialysed</td>
<td>Undialysed</td>
<td>Haemodialysed</td>
</tr>
<tr>
<td>$n = 17$</td>
<td>$n = 24$</td>
<td>$n = 11$</td>
<td>$n = 9$</td>
</tr>
<tr>
<td>Bmax (fmol/mg of protein)</td>
<td>69 ± 25*</td>
<td>98 ± 33***</td>
<td>101 ± 39***</td>
</tr>
<tr>
<td>KD (nM)</td>
<td>2.4 ± 1.6</td>
<td>3.2 ± 1.5</td>
<td>1.8 ± 1.5</td>
</tr>
<tr>
<td>mRNA (A2A/18S)</td>
<td>18.0 ± 1.1</td>
<td>20.2 ± 1.4*</td>
<td>19.7 ± 0.7*</td>
</tr>
<tr>
<td>ADO (μM)</td>
<td>0.80 ± 0.25</td>
<td>2.40 ± 0.28*</td>
<td>3.15 ± 0.60***</td>
</tr>
</tbody>
</table>

Data are means and standard deviations. Saturation experiments were performed using [3H]-ZM241385, a selective ligand of A2A receptors [29]. Estimates of the equilibrium dissociation constant ($K_D$ in nM) and the density of binding sites (Bmax in fmol/mg of proteins) were deduced from non-linear regression curves using a computer program (PRISM™ Graph Pad Software). The A2A receptor’s mRNA was assessed using semi-quantitative real-time PCR and is reported as the A2A receptor/18S ratio of RT–PCR products. *$P < 0.05$ compared with controls; **$P < 0.05$ compared with undialysed patients. ***$P < 0.01$ compared with before HDS.
expression of peripheral A2A receptors, without correlation with mRNA synthesis (ii) HD patients had higher receptor expression than undialysed patients; (iii) ADO plasma levels were high in HD patients and increased further during HDS.

We found a high expression level of A2A receptors in HD patients compared with undialysed patients. In undialysed patients with or without LVF, the CRF state, however, prevents normal expression of these receptors since it remains lower than those of controls.

Therefore, observation of a relative increased number of A2A receptors in HD patients compared with undialysed patients may be due to high ADO release and to an up-regulation phenomenon. However, the cytokine release that occurs during HD treatment [27] may also participate in this up-regulation. Indeed, it was shown that TNF-α [28] or T-helper cell type 1 cytokines and polysaccharides [29] increased A2A receptor expression. It was also shown that diabetic status increased A2A mRNA amount in rats’ hearts [30]. Thus a lot of inflammatory factors may participate in the modulation of A2A receptor expression in HD patients. A high expression of peripheral A2A receptors was observed in a congestive model of LVF patients, this expression however returns to normal within few months after heart transplantation [17]. Here, we found that LVF is accompanied by low A2A receptor expression. This may be due to the treatment of LVF, since drugs acting on adrenergic receptors are known to modulate A2A receptor expression [30]. We also found that among CRF patients with LVF, HD patients had higher A2A receptor expression than undialysed patients. As A2A receptors are strongly involved in the regulation of the cardiovascular system [11,16,18–20], changes in their expression may have crucial consequences for CRF patients. Indeed, ADO increases coronary blood flow via the activation of A2A and A2B receptors [28] and activation of A2A receptors improves contractile performances of the myocardium [19,20]. Since changes of peripheral A2A receptors mirror changes occurring in the myocardium, it is likely that the variations in their expression that we reported have consequences on left ventricular function. Because HD patients have a higher expression of A2A receptors and higher ADO plasma levels than undialysed patients, we postulate that HD may have beneficial effects on the myocardium.

Since KD values of A2A receptors were in the same range in patients and in controls, CRF is not associated with the accumulation of a competitive inhibitor of A2A receptors.

Finally, we confirmed here that HD patients have high ADO plasma levels that increase still further during the HDS. There are three possible origins for these high ADO levels. First, we evidenced that chronic HDS decreases mononuclear cell ADO deaminase activity which participates in the accumulation of ADO [8–10]. Second, ischaemia—even minimal—that occurs in splanchnic territories during HDS [32] is sufficient to induce the release of ADO by splanchnic organs. Third, HDS is accompanied by a red-cell lysis which is associated with significant adenosine triphosphate (ATP) release which is then converted to ADO, participating in abnormally high ADO plasma levels.

**Conclusion**

We found that HD tends to reduce the decrease in the number of peripheral A2A receptors which results from CRF via an up-regulation phenomenon. The changes in peripheral A2A receptor expression suggest a significant inflammatory response to HD and kidney dysfunction.
or heart failure. Whether these changes do reflect alterations in cardiomyocytes remains to be seen.

Study limitations

While high ADO levels may have beneficial effects on the myocardium via the activation of $A_{2A}$ receptors, it has been established that the activation of $A_{2B}$ receptors precipitates atherosclerosis via apoptosis of arterial smooth muscle cells [33]. Yet $A_{2B}$ receptors with low affinity for ADO are activated by its high concentration in HD patients. As discussed above, it is generally admitted that HD does not improve and sometimes accelerates atherosclerosis in CRF patients. In this context, it would be interesting to evaluate the expression of $A_{2B}$ receptors in HD patients. Cardiovascular complications of HD treatment may partly depend on $A_{2A}/A_{2B}$ expression ratio.

Conflict of interest statement. None declared.

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