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ORIGINAL ARTICLE

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Mycophenolic acid reduces renin-angiotensin-system activity in cultured mouse medullary thick ascending limb cells

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Abstract Mycophenolate mofetil (MMF) is a promising immunosuppressive agent. The intra-renal renin-angiotensin system (RAS) plays an important role in the regulation of intra-renal hemodynamics. Mycophenolic acid (MPA) is the bioactive metabolite of MMF. The interaction between MPA and intra-renal RAS is still unclear. We hypothesized that MPA might affect intra-renal RAS activity. We chose models of cultured mouse medullary thick ascending limb (mTAL) cells for the experiments, as the mTAL is one of the major sites of intra-renal RAS. We investigated the angiotensin-converting enzyme (ACE) activity by means of enzymatic assay, and the angiotensin-receptor activity by means of a binding study with radiolabeled angiotensin II, and measured the intracellular calcium concentration in cultured cells treated with and without MPA. ACE activity changed neither in cells incubated with MPA nor in those treated without MPA. The binding study also indicated decreased angiotensin-II binding in MPA-treated (MPA 10^{-7} M) cells, up to 43.7%. The decreased intracellular calcium concentration in MPA-treated cells further confirmed the MPA-inhibitory effect on the angiotensin receptor. We conclude that MPA reduces intra-renal RAS activity mainly through the decrease of AT1 receptor activity without affecting ACE activity. The results suggest that the inhibitory effect of MPA in the intra-renal RAS might play a role in the extra-immunosuppressive effect of MMF.

Keywords Mycophenolate mofetil · Angiotensin · Thick ascending limb cells

Introduction

Mycophenolate mofetil (MMF) is a potent immunosuppressive agent, which improves acute [1] and chronic graft-rejection rates [2] in kidney transplant recipients. MMF exerts its immunosuppressive action via the inhibition of purine biosynthesis [3]. Azathioprine (AZA) also interferes with the purine synthesis [4]. Mycophenolic acid (MPA) is the bioactive metabolite of MMF [3]. However, MMF has been shown to be more effective in improving kidney graft survival than AZA [1, 2]. The observation indicated that MMF might include, together with its immunosuppressive effect, auxiliary properties of renal protection. The intra-renal renin-angiotensin system (RAS) plays an important role in the progression of renal failure [5, 6]. The interaction between MPA and intra-renal RAS is still unclear. We hypothesized that MPA can modify the intra-renal RAS. Previous studies have indicated that the medullary thick ascending limb (mTAL) is one of the major sites of the intra-renal RAS with angiotensin-II receptor [7] and angiotensin-converting enzyme (ACE) activity [8]. In the present work, we investigate the effects of MPA on intra-renal RAS activity in sub-cultured mouse mTAL cells

with retained main features of the cells from which they were derived [9, 10].

Materials and methods

Cell culture

The experiments were carried out on sub-cultured cells derived from isolated mTALs micro-dissected from the kidney of normal, 1-month-old mice fed on a standard diet, as previously described [9]. mTAL segments isolated from the outer medulla by micro-dissection were isolated to establish cultured cells. The kidneys were removed and cut into thin sections. The kidney slices were incubated for 1 h at 37 °C in culture medium (DMEM: HAM's F12, 1:1 vol/vol) supplemented with 0.1% (w/vol) collagenase (type 1, Sigma). Thereafter, TAL segments were micro-dissected under stereomicroscopic observation at room temperature. Isolated TAL segments (0.2 to 0.5-mm long, 2–5/well) were transferred to 24-well trays coated with rat-tail collagen, and 1 ml of fresh medium was added to each well. The tubules were maintained in a modified culture medium (DMEM: HAM's F12, 1:1 vol/vol; 60 nM sodium selenate; 5 μg/ml transferrin; 2 mM glutamine; 5 μg/ml insulin; 50 nM dexamethasone; 1 nM tri-iodothyronine; 10 ng/ml epidermal growth factor; 2% fetal calf serum; 20 mM HEPES, pH 7.4) at 37 °C in 5% CO_2-95% air atmosphere. The primary cultures reached confluency after 4 weeks. Cell growth accelerated after the first passage, and the medium was changed every 4 days. After three passages, cells were routinely sub-cultured, and the medium was changed every 3 days. A line of mouse TAL cells could thus be subcultured for a long time (to date, over 35 passages). Sub-cultured mTAL cells were routinely grown in a modified culture medium (DMEM: HAM's F12, 1:1 vol/vol; 60 nM sodium selenate; 5 μg/ml transferrin; 2 mM glutamine; 5 μg/ml insulin; 50 nM dexamethasone; 1 nM tri-iodothyronine; 10 ng/ml epidermal growth factor; 2% fetal calf serum; 20 mM HEPES, pH 7.4) at 37 °C in 5% CO_2-95% air atmosphere. All experiments were performed on the 6th and 15th passages of confluent cells grown on Petri dishes. The experiments were performed in the presence and in the absence of MPA. MPA was dissolved in pure ethanol. Control experiments were performed in the same concentration of pure ethanol in the same set of cells.

ACE activity assay

ACE activity was measured according to the method described by Santos et al. [11]. Briefly, medium supernatant (50 μl) from confluent cultured mTAL cells was incubated for 2 h at 37 °C with 250 μl of assay solution containing 5 mM hippuryl-L-His-Leu (HHL) in 0.4 M sodium borate buffer, pH 8.3, and 0.9 M NaCl. The enzymatic reaction was stopped by addition of 1.40 ml of 0.28 M NaOH; 100 μl of 1% *o*-phthaldialdehyde (10 mg/ml) in methanol was then added and the mixture left at 37 °C for 10 min. The fluorescent reaction was stopped by the addition of 200 μl of 3 N HCl, and the sample was centrifuged (3,000 rpm for 5 min) at 4 °C. The L-His-Leu product was measured fluorimetrically (365-nm excitation and 495-nm emission). As control, we prepared a blank reaction by reversing the sequential order of adding enzyme and NaOH to the assay solution. The standard curve was obtained from varying concentrations of L-His-Leu (0.1–100 pmol/ml) in the blank reaction incubation mixture. Passive control experiments were performed in the presence of 10^{-4} M captopril (ACE inhibitor) and L-His-Leu (100 pmol/ml). Captopril (10^{-2} M) suppressed the ACE activity for almost 80% (control: 6.56 ± 0.19; +captopril: 1.39 ± 0.05 nmol/10^6 cells, $n = 10$).

Angiotensin-II binding studies

Angiotensin-II receptors binding assay was performed on cultured mTAL cells, as previously described [12]; 5×10^4 mTAL cells were seeded into 24-well trays (Corning, N.Y., USA) and grown for 5 days. Cells were then incubated with 10 nmol of ^{3}H-angiotensin II (specific activity: 50 Ci/mmol; NEN Life Science, Boston, Mass., USA) in DMEM medium supplemented with 1% bovine serum albumin (BSA) at 22 °C for 2 h. Afterwards, cells were rinsed three times with ice-cold phosphate buffered saline (PBS) to remove unbound ^{3}H-angiotensin II. Cells were then lysed in 100 ml 1 M NaOH plus 0.1% Triton X-100, and the radioactivity was measured. Non-specific ^{3}H-angiotensin-II binding was measured on cells incubated with a 1,000-fold excess of unlabeled angiotensin II. The specific ^{3}H-angiotensin-II binding, expressed as fmol/mg protein was calculated as the difference between total and non-specific binding. We performed Scatchard-plot analyses to calculate the dissociation constant (K_D) and the maximum binding capacity (B_{max}). Protein content was measured according to the method of Lowry [13] with BSA used as standard.

Intracellular calcium assay

The level of intracellular calcium $[Ca^{2+}]_i$ was determined with the calcium-sensitive fluorescent probe fura-2/AM [14] according to the method described by Haller et al. [15] with slight modifications. Briefly, confluent cells grown on 60-mm-diameter Petri dishes were scraped off with a rubber scraper, rinsed twice, and resuspended in calcium-free buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 6 mM glucose, 10 mM HEPES,). Suspended cells were then incubated in the buffer, supplemented with 5 μmol of fura-2/AM for 20 min at room temperature. Afterwards, the extracellular dye was removed with three rinses with a large excess of calcium-free buffer. Cells were then resuspended (10^6 cells/ml) in the calcium-free buffer supplemented with 1.5 mM Ca^{2+}. Fluorescence intensity (F) was measured as a function of time in thermostat-controlled quartz cuvettes at 37 °C under continuous stirring in a Hitachi F-4000 spectrofluorometer at 340/380 nm excitation wavelengths and 505-nm emission wavelength. After a 5-min equilibration period, recordings were taken before and after the addition of 10^{-7} M angiotensin II. At the end of the experiment, the level of $[Ca^{2+}]_i$ was determined according to the following protocol: maximal fluorescence (F_{max}) was determined by addition of an excess of 10 mM $CaCl_2$, and minimal fluorescence (F_{min}) was measured by lysing of the cells with Triton X-100 at pH > 8.4 and adjustment of free calcium to < 2 nmol/l by addition of 10 mM EGTA to the cell suspension. The $[Ca^{2+}]_i$ corresponding to the fluorescence emitted by intracellular fura-2 (F) was calculated from the following formula: $[Ca^{2+}]_i = 224 \text{ nmol/l} \times (F - F_{min})/(F_{max} - F)$, where 224 nmol/l represents the dissociation constant of the fura-2-Ca^{2+} complex [14].

Statistical analysis

Results are expressed as means ± SEM from (n) experiments performed in duplicate or triplicate. Significant differences from paired and unpaired experiments were analyzed by Student's t-test.

Results

Effects of MPA on ACE activity

Confluent mTAL cells grown on a collagen-coated dish were of uniform cobblestone shape, as described

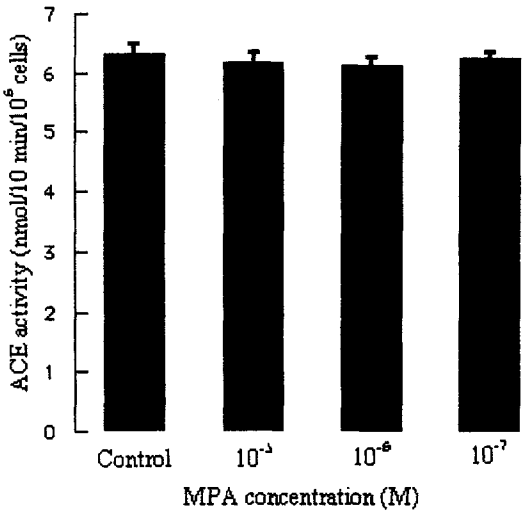

Fig. 1 Effect of MPA on ACE activity in mTAL cells. ACE activity was measured in mTAL cells incubated without (control) and with various concentrations of MPA (10^{-5} to 10^{-7} M) for 24 h. Values are means ± SDs from eight separate experiments

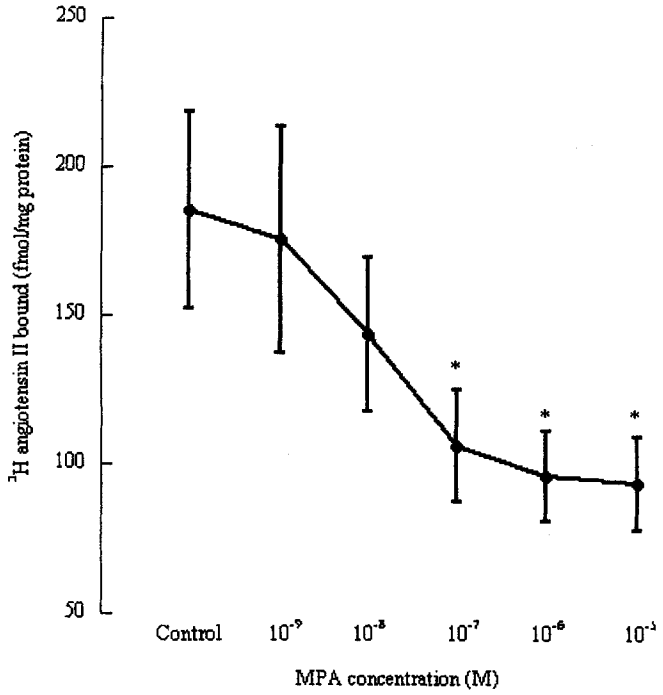

Fig. 2 Effect of MPA on ^{3}H-angiotensin-II binding in mTAL cells. ^{3}H-angiotensin-II binding was measured in mTAL cells incubated *without (control)* and with various concentrations of MPA (10^{-5} to 10^{-7} M) for 24 h. Values are means ± SDs from six separate experiments. $^*P < 0.05$ vs control values

previously [9]. Pre-incubation of mTAL with various concentrations of MPA for 24 h did not alter ACE activity (control: 6.33 ± 0.17; MPA 10^{-5} M: 6.21 ± 0.15; MPA 10^{-6} M: 6.16 ± 0.12; MPA 10^{-7} M: 6.28 ± 0.12 nmol/10^6 cells, $n = 8$) (Fig. 1).

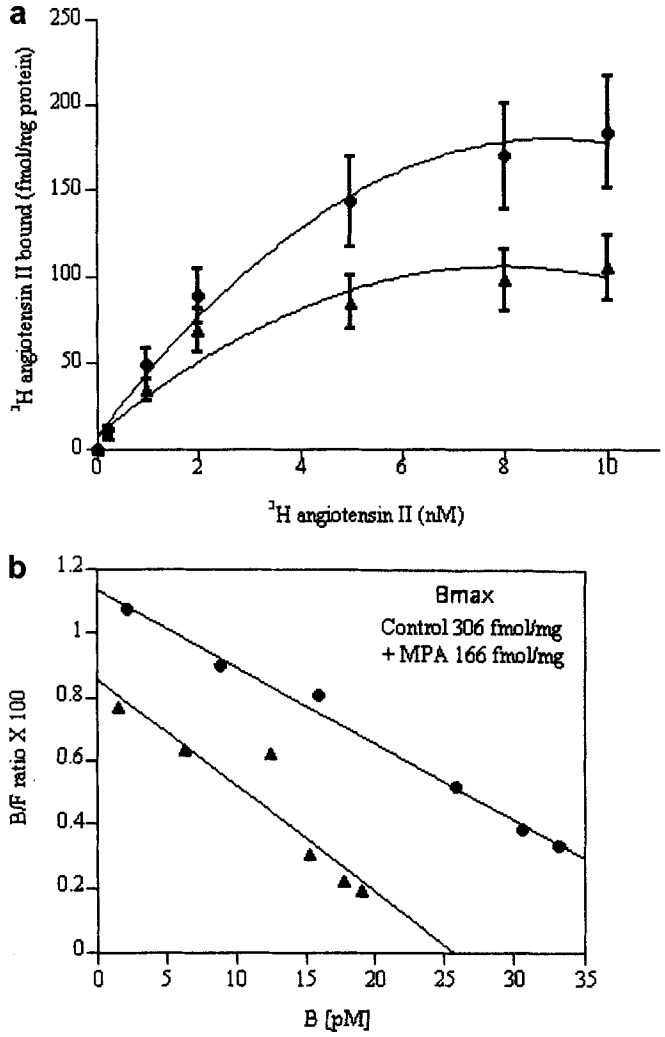

Fig. 3 Effect of MPA on ^{3}H-angiotensin-II binding in mTAL cells. **a** Untreated (*circles*) and MPA-treated (10^{-7} M, 24 h; *triangles*) mTAL cells were incubated with various concentrations (0.2 to 10 nM) of ^{3}H-angiotensin II for 2 h at 22 °C. Points are means ± SDs from six separate experiments. **b** Scatchard plots of ^{3}H-angiotensin-II binding to untreated (*circles*) and MPA-treated (*triangles*) mTAL cells. Each point is the mean of six determinations performed in duplicate

Effects of MPA on angiotensin-II receptor binding

^{3}H-angiotensin-II binding studies were performed to quantify angiotensin-II receptors in cultured mTAL cells. In contrast to the unchanged ACE activity, angiotensin binding decreased dose-dependently in MPA-treated (24 h) mTAL cells. (control: 185.3 ± 33.1; MPA 10^{-9} M: 175.8 ± 38.7; MPA 10^{-8} M: 143.3 ± 26.7; MPA 10^{-7} M: 106.4 ± 19.7; MPA 10^{-6} M: 96.2 ± 15.2; MPA 10^{-5} M: 93.8 ± 16.0 fmol/mg protein, $n = 6$) (Fig. 2). These results strongly suggested that MPA inhibited angiotensin-II receptor binding. To further evaluate the mechanism of the MPA action, we treated mTAL cells

with guanosine 5′-triphosphate (GTP) in the presence and absence of 10^{-7} M MPA. GTP (10^{-4} M, 30 min) alone did not alter the angiotensin-II binding (control: 201.3 ± 42.1; +GTP: 198.5 ± 39.2 fmol/mg protein, $n=6$). GTP (10^{-4} M, for 30 min) prevented MPA (10^{-7} M) inhibitory effect on angiotensin-II binding (+MPA: 98.3 ± 27.3; +MPA +GTP: 173.2 ± 31.5 fmol/mg protein, $n=6$). Scatchard-plot analyses also showed that MPA (10^{-7} M) caused a significant decrease in the number of binding sites (B_{max}: control: 305.8 ± 33.5 fmol/mg protein; +MPA: 166.49 ± 23.6 fmol/mg protein, $n=10$, $P<0.001$) without affecting K_D (control: 7.06 ± 0.12; +MPA: 6.76 ± 0.19 nM, $n=10$, NS) (Fig. 3).

Effects of MPA on $[Ca^{2+}]_i$

The results from binding studies, indicating that MPA caused a decrease in angiotensin-II binding, led us to test the effects of MPA on the levels of $[Ca^{2+}]_i$ from cultured mTAL cells. $[Ca^{2+}]_i$ has been shown to be one of the most important intracellular signals induced by angiotensin II along the nephron [12, 16]. The basal level of $[Ca^{2+}]_i$ was slightly lower in MPA-treated cells than in untreated cells (untreated: 69.7 ± 9.8; +MPA: 41.1 ± 6.7 nM/10^6 cells, $n=6$, $P<0.05$). Angiotensin II induced the rise of $[Ca^{2+}]$i in both untreated and MPA-treated cells. However, the stimulatory action of angiotensin II was less pronounced in MPA-treated cells than in untreated cells (untreated: 110.8 ± 8.4; +MPA: 86.3 ± 10.6 nM/10^6 cells, $n=6$, $P<0.05$). The decreased $[Ca^{2+}]_i$ in either basal or angiotensin-II-stimulated status, in MPA-treated cells, further confirmed the MPA-inhibitory effect on angiotensin-II binding. MPA (10^{-7} M) showed no effect on cell viability in mTAL cells in our study.

Discussion

The intra-renal RAS was thought to be an important factor in the regulation of renal hemodynamics and cell hypertrophy [17, 18]. The activation of the intra-renal RAS had been shown to play an important role in the progression of renal failure [6, 19]. It is hypothesized that the local RAS acts at the peritubule level and affects the adjacent vascular tone [20]. The peritubular vessel, adjacent to the mTAL segment, is the key smooth muscle-cell-containing blood vessel in the regulation of renal medullary perfusion [21].

Previous studies have shown that the intra-renal angiotensin-II content per gram of tissue is five times greater in the medulla than in the cortex of rat kidney [22]. The mTAL is one of the major sites of intra-renal RAS with angiotensin-II receptor [7] and ACE activity [8]. This specific part of the nephron segment also plays an important role in the regulation of sodium reabsorption [23] and intra-renal hemodynamics [17]. Thus, cultured mTAL cells represent a suitable ex-vivo cell model for the analysis of the effects of MPA on local RAS activity. Our experiment suggested that MPA could reduce the local RAS by decreasing the angiotensin-II binding. The result suggested a possible role of MPA in modulating the intra-renal RAS, which might be responsible for the beneficial effect of MMF in the preservation of renal grafts.

However, the mechanism for the observed effect of MPA on angiotensin-II binding in renal epithelial cells is still unclear. MPA was known to exert its effect through non-competitive reversible inhibition of inosine monophosphate dehydrogenase (IMPDH). The inhibition of IMPDH reduces the cellular GTP. Our experiments indicated that excessive GTP partially prevented the inhibitory effect of MPA. The results indirectly suggested a possible role of the IMPDH pathway in reducing angiotensin-II binding.

There are some limitations of this ex-vivo study. The concentration we used in the experiment was not relevant to the therapeutic level. In addition, the local concentration in renal tissue was not necessarily the same as the systemic concentration. The application of these ex-vivo data to clinical application is far from mature. Further clinical investigation might be necessary to confirm the finding.

In conclusion, we have shown that MPA inhibits intra-renal RAS activity mainly through the reduction of angiotensin receptor binding without decreasing ACE activity, in a model of cultured mouse mTAL cells. The results also suggest that the suppression of local RAS might play a role in the MPA extra-immunosuppressive effect.

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