Cyclosporin inhibits nitric oxide production in medullary ascending limb cultured cells

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Abstract

Background. Nitric oxide (NO) has been shown to play a role in cyclosporin (CsA) nephrotoxicity, but its mechanism of action is still unclear. As inducible NO synthase (iNOS) mRNA has been found to be expressed in rat medullary thick ascending limb (mTAL) cells, we investigated the effects of CsA on NO production in a model of mouse cultured mTAL cells.

Materials and methods. The experiments were carried out on sub-cultured cells derived from isolated mTAL microdissected from the kidney of C57BL/6 mice. The identification of the iNOS mRNA in mTAL microdissected segment and cultured cell was confirmed by RT–PCR and Rsal digestion. Nitrite (NO⁻²) released by mTAL cells was determined using the modified Griess reagent method and taken as an index of nitric oxide production. The cultured cells were treated with various concentrations of CsA and different signal transduction regulators to assess the effect and possible pathway(s) of action of CsA on NO production in mTAL cells.

Results. The basal production of NO by mTAL cells increased by 1.8-fold following incubation with bacterial lipopolysaccharide (LPS). Both aminoguanidine and l-NAME inhibited NO production. CsA (10–300 ng/ml) also inhibited NO production in a dose-dependent manner and prevented its increase induced by LPS. Phorbol 12-myristate 13-acetate (PMA), a PKC stimulator, enhanced slightly the production of NO under basal conditions and prevented the inhibitory action of CsA on NO production. These results suggest that the NO secreted by mouse cultured mTAL cells is dependent on the PKC pathway.

Conclusion. These results show that CsA may down-regulate the production of NO by cultured mTAL cells expressing iNOS mRNA and that the PKC pathway is involved in this process.

Key words: cultured cells; cyclosporin; kidney; nephrotoxicity; nitric oxide; thick ascending limbs

Introduction

Nitric oxide (NO) plays an important role in the regulation of vascular tone [1]. It also represents an important intercellular messenger in a variety of physiological and pathological conditions [2]. NO, first described as an endothelium-derived relaxing factor (EDRF) [3,4], is produced by a variety of non-epithelial and epithelial cells and has multiple actions under many biological conditions [5]. Its production is mediated by the activity of NO synthase (NOS). At least three different isoforms of NOS have been described. The isoform I (bNOS) has been identified in brain neuronal cells, peripheral non-adrenergic and non-cholinergic neurons, and in some specialized epithelial cells [6]. Macrophages stimulated by bacterial endotoxin and/or cytokines have been shown to express the isoform II (iNOS) [7]. Endothelial cells express isoform III (eNOS) of NO synthase that seems to be unique for this cell type [8]. All NOS synthesize NO by oxidizing a terminal guanidino nitrogen of l-arginine, requiring oxygen and NADPH as substrates, but the expression of these isoforms is subjected to different kinds of regulations. Recent studies indicate that NO has potent effects on kidney functions [9,10] and tubular epithelial cells may constitutively generate NO [11]. bNOS has been specifically identified in macula densa cells and inner medullary collecting ducts [12]. iNOS mRNA, distinct from the vascular smooth muscle iNOS, has been shown to be expressed mainly in rat kidney medullary thick ascending limb (mTAL) cells and medullary collecting ducts (MCD) without any NO inducer [13,14]. These results demonstrate constitutive expression of iNOS mRNA in rat medulla and suggest that NO may play a role in the haemodynamic regulation in this part of the kidney [15]. Cyclosporin (CsA), a potent immunosuppressive
agent used widely in the prevention of transplant organ rejection, is nephrotoxic and responsible for progressive renal failure in some transplant patients [16]. Although the exact mechanism of CsA nephrotoxicity is still unknown, CsA has been shown to enhance renal arterial vasoconstriction and decrease renal blood flow [17], in part by altering the balance between vasodilating and vasoconstricting mediators, such as endothelin and NO/EDRF [18]. CsA may decrease NO production in various cell types from different tissues, including vascular smooth muscle cells [19], endothelial cells [20] and macrophages [21]. These results suggest that CsA may inhibit NO production. As mTAL cells have been shown to be the main site of steady iNOS gene expression in the kidney [13,14], the question arises whether CsA modulates the production of NO within these cells. To address this issue, we investigated the effects of CsA on the production of NO in a model of sub-cultured mouse mTAL cells which have kept the specific functions of the parent cells from which they were derived [22].

Materials and methods

Materials

Soluble cyclosporin in cremophor was obtained from Sandoz® (Basel, Switzerland). FK506 (Tacrolimus) was kindly supplied by Fujisawa Co. (Osaka, Japan). Rapamycin (AY-22989-21) was a generous gift from Wyeth-Ayerst Research (Princeton, NJ). Furosemide was purchased from Hoechst Laboratories (German Remedies Co., Taiwan) and all other reagents were from Sigma (Sigma Chemical Co., St Louis, MO). Culture media (DMEM, HAM’s F12) were obtained from Gibco BRL Laboratory (Life Technologies® Taiwan). The permeable filters were Millicell-CM filters (0.4 h μm pore size; Millipore Continental Water Systems, Bedford, MA).

Cultured cells

The experiments were carried out on sub-cultured cells derived from isolated medullary thick ascending limbs (mTAL) microdissected from the kidneys of 1-month-old C57BL/6 mice as described previously [22]. Sub-cultured mTAL cells were routinely grown in a modified culture medium [DMEM: HAMS F12, 1:1 vol/vol; 60 mM sodium selenate; 5 μg/ml transferin; 2 mM glutamine; 5 μg/ml insulin; 50 nM dexamethasone; 1 nM triiodothyronine; 10 ng/ml epidermal growth factor; 2% fetal calf serum (FCS); 20 mM HEPES, pH 7.4] at 37°C in 5% CO2—95% air atmosphere. All experiments were performed on the sixth and 15th passages of confluent cells grown on Petri dishes.

RNA extraction and reverse transcriptase polymerase chain reaction (RT–PCR)

RNA was extracted from isolated mTAL segments microdissected from an adult mouse kidney and confluent mTAL cells using the method of Chomczynski and Sacchi [23]. Total RNA concentration was treated with RNase-free DNAse I (Boehringer Mannheim, Germany) at 37°C for 30 min and the RNA concentration was evaluated by spectrophotometry. RNA (100 μg) was reverse transcribed with avian myeloblastosis virus reverse transcriptase (RT AMV, Boehringer Mannheim) at 42°C for 60 min. cDNA (150 ng) and non-reverse transcribed RNA were amplified for 30–42 cycles in 100 μl total volume containing 50 mM KCl, 20 mM Tris–HCl pH 8.4, 10 mM dNTP, 1.5 mM MgCl2, 1 unit Taq polymerase and 10 pmoles of iNOS primers. The thermal cycling protocol was as follows: 94°C for 1 min, 60°C for 1 min and 72°C for 3 min. The two primers from the iNOS gene [7] were: antisense strand 5'-agt cgg cta gtag cag agg-3' and sense strand 5' -gtc ttc cac cgc gaa tgg-3'. Amplification products were separated on a 4% agarose gel with ethidium bromide and photographed. The identity of the amplified products (508 bp) was controlled by digestion with RsaI (Boehringer Mannheim, Germany).

NO measurement

The stable end product of NO oxidation is nitrite (NO2−). NO2− was measured in the medium by the Griess reagent method to determine the amount of NO produced by cultured mTAL cells [24]. Briefly, 1 ml culture medium, kept at −20°C before use, was mixed with 1 ml Griess reagent (0.1% naphthylethlenediamine dihydrochloride, 1% sulfanilamide in 5% concentrated H3PO4, vol:vol). After 15 min incubation at room temperature, the colorimetric reaction was read by spectrophotometry at 546 nm (Beckman DU-600). For each set of experiments, a standard curve was generated by the addition of various concentrations of sodium nitrite (0.01–0.30 μg/ml) to the culture medium. To avoid the influence of endotoxin on NO production, endotoxin was determined within the medium with the Limulus amebocyte lysate (LAL) automated-turbimetric procedure (LAL-S000, Associates of Cape Cod Inc., MA). The levels were always below 0.001 EU/ml.

The effects of various agents and compounds on the production of NO by cultured mTAL cells were tested. Confluent cells grown on Petri dishes were incubated either with 5 mM aminoguanidine (AG), 5 mM Nω-nitro-L-arginine-methylester hydrochloride (LNAME), 100 ng/ml bacterial lipopolysaccharide (LPS), or 500 μg/ml interferon-γ (IFN-γ), for 6 h at 37°C. Cells were also incubated with CsA in cremophor (Sandoz®, Basel, Switzerland) diluted in water at the final concentrations of 10–300 ng/ml, 10−7 M FK506 (Fujisawa®, Osaka, Japan) or 10−7 M rapamycin (AY-22989–21, Wyeth-Ayerst Research, Princeton, NJ) for 1–48 h at 37°C. As a control, experiments using CsA were carried out with the vehicle (cremophor) alone. Cells were also incubated with 10 μg/ml phorbol 12-myristate 13-acetate (PMA), or 100 μM H7, used as a PKC inhibitor [25] for 6 h at 37°C. In all cases, the NO production was measured on sets of cells from the same passage incubated with or without the agents to be tested. All measurements were performed in duplicate. Results are expressed as μM NO2− per 106 cells and corrected by the background level of nitrite measured in fresh culture medium.

Cell viability

Cell viability was estimated by counting the number of cells that excluded trypan blue. Lactic dehydrogenase (LDH) activity, a marker of non-specific cell injury, was assayed on cell lysates and supernatants from untreated or CsA-treated cells as described [26] using a Technitron RA-500 autoanalyzer (Technitron, Tarrytown, NY). Protein content was
measured using the method of Lowry [27] using bovine serum albumin (BSA) as standard.

**Statistical analysis**

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

**Results**

**iNOS expression in microdissected mouse mTAL and derived cells**

iNOS isoform in microdissected mouse mTAL segments and sub-cultured mTAL cells was detected by RT–PCR using specific primers. As shown in Figure 1, a single 508-bp band of expected size was amplified in both microdissected segments and cultured cells. As negative control, no amplified products were obtained using non-reversed transcribed RNA from mTAL segments and cultured cells. The identity of the amplified products (508 bp) was controlled by digestion with RsaI. The RsaI digestion products were 469 bp and 39 bp (Figure 1), indicating that mouse mTAL segments and sub-cultured mTAL cells expressed the iNOS mRNA, as reported for the rat kidney [13,14].

**NO production by mouse mTAL cultured cells**

The kinetics of NO production and the effects of various NOS inhibitors or inducers were analyzed in mouse mTAL cells grown on Petri dishes. At confluence, the culture medium was removed and fresh medium was added to the dishes. The amount of NO− secreted in the culture medium, reflecting the NO produced by the cells, was measured after various incubation periods (Figure 2). In the absence of any effector, the production of NO by mTAL cells was detectable within 1 h, and became maximal after 6 h. The production of NO remained in plateau for at least 48 h (Figure 2). Accordingly, a 6-h incubation period was chosen for all subsequent experiments. To test whether the serum and dexamethasone present in the culture medium may affect the production of NO by mTAL cells, NO produced by the cells was also measured in the presence or absence of the serum (2% FCS) or dexamethasone (50 nM). We found no significant difference in NO production when cells were grown in the presence or absence of serum or dexamethasone (complete medium: 0.357 ± 0.017; dexamethasone-free medium: 0.380 ± 0.019; serum-free medium: 0.335 ± 0.022 μM NO−/10^6 cells). We then tested the effects of two NOS inhibitors, aminoguanidine and l-NAME,
The effects of CsA on the production of NO by cultured mTAL cells

To test the action of CsA on the production of NO, the release of NO in mTAL cultured cells (Figure 3). These two agents significantly decreased the production of NO compared with untreated cells. Aminoguanidine, a rather specific iNOS inhibitor [28], had a similar inhibitory effect on NO production as the non-specific inhibitor L-NAME [28]. Aminoguanidine (AG, 5 mM) and L-NAME (5 mM) significantly decreased the production of NO compared with untreated cells. Values represent the mean ± SEM from 10–12 separate experiments.

**P < 0.001 vs control (C) values.

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The effects of CsA on the production of NO by cultured mTAL cells

To test the action of CsA on the production of NO, the release of NO in mTAL cultured cells incubated with various concentrations of CsA for 6 h at 37°C. CsA decreased production of NO in a dose-dependent manner (Figure 4A). Maximal inhibitory effect was achieved with 100 ng/ml CsA (Figure 4A). We also tested the effects of FK506 and rapamycin, two other immunosuppressive agents similar to CsA [29], on the production of NO. Compared with untreated cells, 100 ng/ml CsA (corresponding to 0.8 × 10⁻⁷ M) CsA significantly reduced the NO release by mTAL cells by 43% (Figure 4B). FK506 (10⁻⁷ M) also reduced, although to a lesser extent (13%), the production of NO, whereas rapamycin (10⁻⁷ M) had no effect (Figure 4B). These results indicate that CsA had a more potent inhibitory effect than FK506 and rapamycin on the production of NO by cultured mTAL cells. However, RT-PCR experiments using specific sets of iNOS and β-actin primers showed that the amount of iNOS transcripts compared with the level of β-actin primers (used as internal standard), although slightly lower, were not dramatically altered after the cells were incubated with either CsA or FK506 (data not shown).

To ensure that the decrease in NO production induced by CsA was not related to cell damage, cell viability and LDH activity, used as an index of cell injury [26], were analyzed on CsA-treated (100 ng/ml CsA for 6 h at 37°C) and untreated mTAL cultured cells. The percentage of viable cells was not significantly different between treated and untreated cells. The protein content per cell, the LDH activity within cells and medium were also not significantly different in untreated and CsA-treated cells (Table 1).
Table 1. Influence of CsA on protein content and LDH activity

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<tr>
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<th>Control</th>
<th>+ CsA</th>
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<tr>
<td>Cell protein content (μg/10⁶ cells)</td>
<td>95 ± 32</td>
<td>97 ± 27</td>
</tr>
<tr>
<td>LDH activity (μmol/min/g protein)</td>
<td>1329 ± 318</td>
<td>1414 ± 216</td>
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<tr>
<td>% of total LDH secreted in medium</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.2</td>
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Cell protein and LDH activity in cells and culture medium from sets of confluent mTAL cells grown on Petri dishes incubated without (control) or with 100 ng/ml CsA (+ CsA) for 6 h at 37°C. Values represent the mean ± SEM from five separate experiments.

Table 2. Effects of CsA on the production of NO under unstimulated and stimulated conditions

<table>
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<th>NO production (μM NO₂⁻/10⁶ cells)</th>
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<tr>
<td></td>
<td>− CsA</td>
</tr>
<tr>
<td>Control</td>
<td>0.395 ± 0.008</td>
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<tr>
<td>LPS + IFN-γ</td>
<td>0.361 ± 0.001</td>
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</table>

The production of NO was measured on confluent mTAL cells incubated without (Control) or with LPS (100 ng/ml) plus IFN-γ (500 μg/ml) in the absence (−) or presence (+) of CsA (100 ng/ml) for 6 h at 37°C. Values represent the mean ± SEM from 12 experiments. ***P < 0.001 vs CsA values.

As LPS or LPS plus IFN-γ enhanced the production of NO by mTAL cells, we then measured the release of NO₃⁻ by mTAL cells co-incubated with CsA (100 ng/ml) and LPS plus IFN-γ for 6 h at 37°C. CsA also prevented the stimulating effect of LPS plus IFN-γ on the production of NO by mTAL cells (Table 2).

The question arises whether CsA exerts its action on NO production by interfering with signal transduction pathways. Therefore, the effects of PMA were tested on untreated and CsA-treated cells. As shown in Figure 5A, PMA slightly enhanced the production of NO in the basal state (control: 0.351 ± 0.008; + PMA: 0.396 ± 0.011 μM/10⁶ cells, n = 10), whereas it significantly increased the production of NO in CsA-treated cells (CsA: 0.189 ± 0.010; CsA plus PMA: 0.361 ± 0.010 μM/10⁶ cells, n = 12, P < 0.001). As a result, no significant differences in NO production were observed between untreated and CsA-treated cells incubated with PMA (Figure 5A). To further access the role of PKC pathway in the production of NO by mTAL cells, H7, a PKC inhibitory agent [25], was tested in both untreated and CsA-treated cells. H7 (100 μM) significantly reduced the production of NO in the basal state (control: 0.356 ± 0.013; + H7: 0.224 ± 0.019 μM/10⁶ cells, n = 12, P < 0.001, Figure 5B), and did not affect the reduced production of NO of CsA-treated cells (Figure 5B). These results strongly suggest that CsA altered the PKC-dependent NO production in this model of cultured mTAL cells.

Discussion

The results from the present study show that mouse mTAL cells in culture are able to maintain substantial expression of the iNOS mRNA isoform and, thus, provide a suitable ex vivo model to study the production of NO under various experimental conditions. As described previously for rat mTAL [13,14], the results from RT-PCR indicate that mouse mTAL segments and their derived cultured cells constitutively express the iNOS isoform. In a previous study, Kone et al. [30] reported that LPS plus IFN-γ increased the expression of iNOS mRNA in a similar model of immortalized mouse mTAL cells (ST-1), but failed to detect iNOS mRNA in basal state. One can not exclude that the differences in levels of iNOS mRNA expression in these immortalized ST-1 cells and our sub-cultured mTAL cells are linked to differences in proliferation and/or differentiation states. It is also possible that the composition of the media used for cell culture may affect the production of NO. Here, we found that neither serum nor dexamethasone affected the release of NO by cultured mTAL cells. The fact that NO produced by the cultured mTAL cells is inhibited by aminoguanidine, a specific iNOS inhibitor, and slightly increased by LPS, a component of bacterial endotoxin [7], suggests that the production of NO is, at least in part, mediated by the iNOS isoform. However, the increase in NO induced by LPS was much lower in the cultured mTAL cells than reported for other cell types, such as macrophages [5,6]. Although, there is no direct explanation for these discrepancies, it can be hypothesized that mTAL cells from the mouse poorly react with LPS.

The role of NO on intrarenal hemodynamics and specific kidney-tubule functions under physiological or pathological conditions still remain poorly understood. One important function of NO is the maintenance and...
regulation of medullary blood flow [15,31]. Using a partial cDNA homologous to the murine macrophage iNOS (macNOS), Mohaupt et al. [13] demonstrated that macNOS is the principal iNOS isoform expressed in microdissected tubules, mainly in mTAL and to a lower extent in inner medullary collecting ducts from the rat kidney. Based on these results, these authors emphasized that the basal NO production, mediated by iNOS within these tubular epithelial cells, may play a physiological role in an autocrine or paracrine fashion. In addition, previous studies have shown that NO exerts an influence on the medullary circulation [15,31] and that NO inhibitors caused vasoconstriction of in vitro perfused descending vasa recta from the inner layer of the outer medulla of the rat kidney [32]. In the present study, we found that sub-cultured mTAL cells produce NO under basal conditions. Thus, NO produced by mTAL cells may act at the level of peritubular vessels to modulate blood flow and oxygenation, although endothelial cells may also play an important role.

In clinical practice, chronic administration of CsA may cause progressive interstitial fibrosis [18], but the exact mechanism of CsA cytotoxicity still remains undefined. Several studies have shown that CsA inhibits NO production in endothelial cells [20], smooth muscle cells [19] and macrophages [21]. The question arises whether CsA affects the production of NO by mTAL cells located in renal outer medulla where associated interstitial fibrosis initiates. Our results show that the inhibitory effect of CsA on the production of NO reached maximal effect at a rather low concentration of CsA (100 ng/ml). Of interest, FK506 and rapamycin, two novel immunosuppressive agents [29] similar to CsA, but less nephrotoxic, have no or lower inhibitory action than CsA on the production of NO by cultured mTAL cells. Our results also suggest that the effect of CsA on NO produced by cultured mTAL cells is linked to the PKC transduction pathway. These results, in keeping with the association of CsA and interstitial fibrosis, indirectly suggest that the inhibition of NO by CsA may interfere with the renal medullary vascular tone and/or may have direct effects on medullary interstitial cells [33].

There is an increasing number of studies indicating that NO plays a role in the regulation of ionic transport within tubule epithelial cells. For example, Guzman et al. [34] showed that the production of NO induced by LPS/IFN-γ treatment inhibited the Na⁺–K⁺ ATPase activity and reduced Na-dependent solute transport in a model of rat proximal tubule culture cells. NO also inhibits sodium reabsorption [35] and is able to decrease anti-diuretic-hormone-stimulated water permeability by increasing cGMP in isolated perfused cortical collecting ducts [36]. Another study also showed that NO may modulate the H⁺–ATPase activity within the tubule segment [12]. In addition, Lu and Wang [37] demonstrated that NO stimulates the activity of a low-conductance K⁺ channel located in the basolateral membrane of the cortical collecting duct cells. Despite the fact that NO is known to increase the production of cGMP through activation of the guanylate cyclase [38] and that luminal and cellular cGMP inhibits net chloride reabsorption in mTAL, there is still very little information concerning the role of NO on the transport of solute in this segment. Preliminary experiments on cultured mTAL cells have shown that furosemide (10⁻⁵ M) significantly enhances the production of NO by L-6-fold (data not shown). These results suggest that NO may play a role in the control of ion transport and interfere, directly or indirectly, via cell volume changes, on the Na⁺–K⁺–Cl⁻ cotransporter present at the luminal side of mTAL cells [22,39]. Although this observation needs further investigation, the results from the present study indicate that CsA may directly alter the NO released by mTAL cells, and that our model of mouse sub-cultured mTAL cells may be a suitable model to analyse the interplay between the iNOS-dependent production of NO and ion transport processes.

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