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Molecular Mechanism of the Urate-lowering Effects of Calcium Channel Blockers

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SUMMARY

Hyperuricemia has recently been recognized as one of the risk factors for cardiovascular diseases. Some calcium channel blockers (CCBs), commonly used in the treatment of hypertension, have been reported to decrease serum urate level. Here, we tried to elucidate the molecular mechanism of the urate-lowering effects of CCBs. We performed [^{14}C]urate uptake in cells stably expressing human urate transporter 1, a major contributor of renal urate reabsorption and a major target of uricosuric drugs such as benzbromarone and losartan (HEK-URAT1), together with mock (HEK-mock) cells to analyze the uricosuric action of CCBs. We also measured the activity of human xanthine oxidase (XO) to determine whether CCBs have inhibitory effects on urate production. The CCBs tested were nifedipine, nilvadipine, nitrendipine, benidipine, nisoldipine, nicardipine, efonidipine, amlodipine, azelnidipine, verapamil and diltiazem. We found for the first time that at least seven CCBs in the dihydropyridine subgroup interacted with URAT1-mediated urate uptake in HEK-URAT1 cells. Among these CCBs, nifedipine, nilvadipine and nitrendipine strongly inhibited URAT1-mediated urate uptake. Their IC_{50}s were 15.8, 0.018 and 0.40 μM, respectively. In contrast, urate production mediated by XO was weakly inhibited by nifedipine and nisoldipine. In summary, URAT1 interacted with various CCBs differently, whereas XO, a major enzyme for urate production in the liver, did not interact with most of CCBs. Although CCBs were not excreted from the urine basically, their urate-lowering effects may be associated with the inhibition of renal urate reabsorption mediated by renal urate transporters such as URAT1 with their metabolites, and the results for structure-activity information in this study will provide a clue for developing new uricosuric drugs targeting URAT1.

Key words : calcium channel blockers, uricosuric drugs, urate, transporters, URAT1, xanthine oxidase

INTRODUCTION

Calcium channel blockers (CCBs), originally named calcium antagonists, inhibit calcium entry by interacting with voltage-gated calcium channels[1]. CCBs are commonly used for treatment of cardiovascular disorders such as hypertension and they are classified into

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several classies including the dihydropyridine subgroup (e.g., nifedipine) and non-dihydropyridine subgroup (e.g., verapamil and diltiazem).

In addition to their established therapeutic uses, clinical studies have shown that some CCBs have uricosuric actions[2~5]. Since CCBs have vasodilatory effects on afferent arterioles, they induce glomerular filtration and, as a result, increase the clearance of urate, leading to a reduction of its serum level[6]. The anti-hypertensive drug losartan, an angiotensin II receptor blocker known to have an uricosuric effect, has been reported to inhibit renal urate reabsorption by interacting with tubular urate transporters[7]. However, the molecular mechanism of the urate-lowering effect found in CCBs is still uncertain.

In humans, serum urate level is determined by the balance between enzymatic production mainly in the liver by xanthine oxidase (XO) and urinary excretion mediated by renal tubular urate transporters[8]. Human renal urate transporter URAT1 is thought to be an essential molecule that mediates the initial step of urate reabsorption at the apical side of the proximal tubule[9]. To determine the molecular mechanism of the urate-lowering effects of CCBs, we examined their interaction with XO and renal apical urate transporter URAT1 *in vitro*.

METHODS

Materials

[^{14}C]urate (50 mCi/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA). Nifedipine, nitrendipine, benidipine, nisoldipine, nicardipine, amlodipine, azelnidipine, verapamil, diltiazem and benzbromarone were obtained from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Nilvadipine and azelnidipine were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Efonidipine was purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum, trypsin, geneticin and Lipofectamin were obtained from Invitrogen (Carlsbad, CA).

Cell culture

HEK-URAT1 cells were established according to a method previously described by us[10]. Briefly, the full-length cDNA of URAT1 was subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA), a mammalian expression vector. HEK-URAT1 cells were obtained by transfecting HEK293 cells with pcDNA3.1-URAT1 using Lipofectamin according to the manufacturer's instructions. HEK293 cells transfected with pcDNA3.1 lacking an insert were used as control (HEK-mock cells). The cells were grown in a humidified incubator at 37℃ and in 5% CO2 using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen) and 400 μg/ml geneticin (SIGMA). The cells were subcultured in a medium containing 0.05% trypsin-EDTA solution (containing in mM : 137 NaCl, 5.4 KCl, 5.5 glucose, 4 NaHCO3, 0.5 EDTA and 5 Hepes ; pH 7.2) and used for 15～25 passages.

Uptake experiments

Uptake experiments were performed as previously described[11]. HEK-URAT1 and HEK-mock cells were seeded in 24-well tissue culture plates at a density of 1×10^5 cells/well. After cells were cultured for 2 days, they were washed three times with serum- and chloride-free Hanks' balanced salt solution (HBSS) containing 125 mM Na gluconate, 4.8 mM K gluconate, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM Ca gluconate, 5.6 mM glucose and 25 mM HEPES, pH 7.4 and then preincubated in the same solution in a water bath at 37℃ for 10 min. The cells were then incubated in the HBSS containing 5 μM [^{14}C]urate at 37℃ for the indicated time. The uptake was stopped by adding ice-cold HBSS, and the cells were washed three times with the same solution. The cells in each well were lysed with 0.5 ml of 0.1 N sodium hydroxide and 2.5 ml of INSTA-GEL PLUS (Perkin Elmer), and radioactivity was determined using a β-scintillation counter (LSC-3100, Aloka, Tokyo, Japan) and normalized to the cellular protein content measured by using the BCA assay (Pierce, Rockford, IL, USA).

Inhibition study

To evaluate the inhibitory effects of various CCBs on urate uptake via URAT1, HEK-URAT1 cells were incubated in a solution containing 5 μM [^{14}C]urate in the absence or presence of various concentrations of drugs at 37℃ for 2 min. The drugs were dissolved in dimethylsulfoxide. The final concentration of dimethyl-

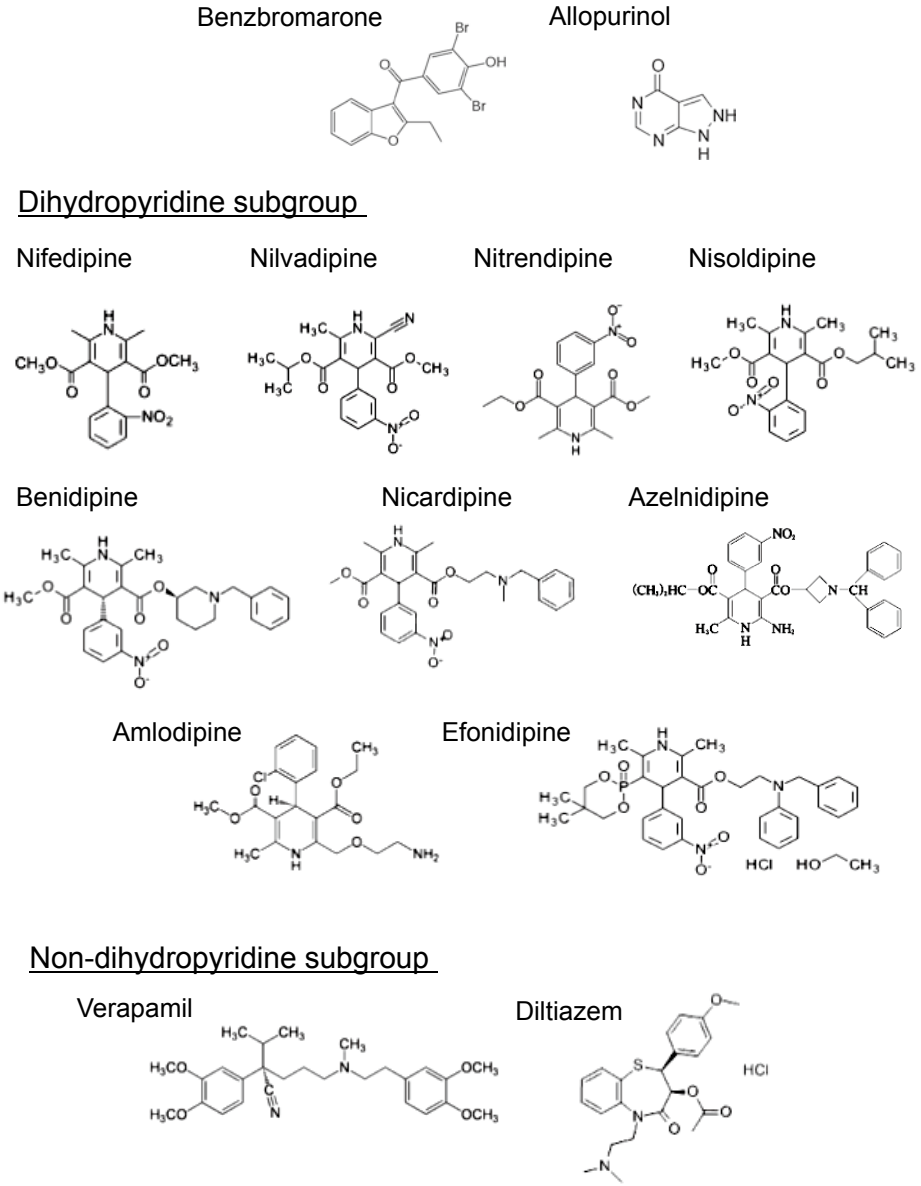

Figure 1 Chemical structures of drugs used in this study.

sulfoxide in the incubation medium was adjusted to be less than 0.5% .

Xanthine oxidase (XO) assay

A Xanthine Oxidase Fluorometric Assay Kit (Cayman Chemical Co.) was used to quantify xanthine oxidase activity according to the instructions of the manufacturer[12,13]. Urate production by XO was assessed in the presence or absence of allopurinol (50 μM) and CCBs (50 μM).

Statistical Analysis

Data are expressed as means ± S.E. Statistical differences were determined using one-way ANOVA with Dunnett's post-hoc test. Differences were considered significant at $P<0.05$.

RESULTS

Inhibitory effects of CCBs on utrate uptake mediated by URAT1

We elucidated the effects of CCBs on urate uptake mediated by HEK-URAT1 cells. As shown in Figure 1, we tested eleven drugs including nifedipine, nilvadipine, nitrendipine, benidipine, nisoldipine, nicardipine, efonidipine, amlodipine and azelnidipine (dihydropyridine subgroup) and verapamil and diltiazem (non-

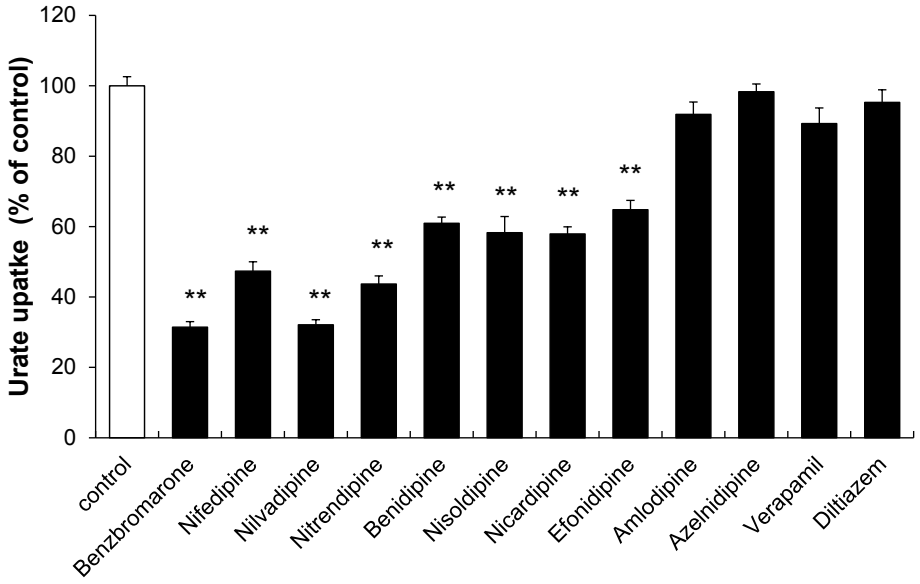

Figure 2 Effects of various CCBs on urate uptake mediated by HEK-URAT1 cells. HEK-URAT1 cells were incubated in a solution containing 5 μM [^{14}C]urate in the absence or presence of 10 μM CCBs for 2 min at 37℃. Each value represents the mean ± S.E. of eight to twelve monolayers from two separate experiments. $^{**}P<0.01$ vs. control.

dihydropyridine subgroup). Among these drugs, nifedipine, nilvadipine, and nitrendipine strongly inhibited URAT1-mediated urate uptake (47%, 32%, and 43%, respectively ; Figure 2) and benidipine, nisoldipine, nicardipine and efonidipine moderately inhibited URAT1-mediated urate uptake (60%, 59%, 58%, and 64%, respectively ; Figure 2). These drugs did not show any inhibitory effects on urate uptake in HEK-mock cells (data not shown). Figure 3 shows the dose-dependent inhibitory effects of nifedipine (A), nilvadipine (B) and nitrendipine (C) on urate uptake in HEK-URAT1 cells. Their IC50 values for urate uptake by HEK-URAT1 were 15.8, 0.018, and 0.40 μM, respectively.

Inhibitory effects of CCBs on xanthine oxidase

We also examined the effects of CCBs on urate production mediated by xanthine oxidase (XO). As shown in Figure 4, urate production mediated by XO was significantly ($p<0.05$), but slightly inhibited by nifedipine and nisoldipine (50 μM each), while most of the CCBs tested in this study did not show any inhibitory effect on urate production by XO.

DISCUSSION

In this study, we examined the interaction of CCBs with XO and with renal apical urate transporter URAT1 *in vitro* to clarify the molecular mechanism of the urate-lowering effects of CCBs. URAT1, a major molecule for renal urate reabsorption, interacted with CCBs to various degree, whereas XO, a major enzyme for urate production in the liver, did not interact with CCBs.

Serum urate level in humans are determined by the balance between production by XO mainly in the liver and urinary excretion by renal proximal tubules. Several tubular urate transporters are thought to be involved in urinary urate excretion. One important transporter is URAT1 (encoded by *SLC22A12*), a urate-anion exchanger localized at the apical side of the renal proximal tubule that mediates the initial step of urate reabsorption. The physiological importance in renal urate reabsorption of URAT1 has been established on the basis of the fact that several loss-of-function mutations in *SLC22A12* cause renal hypouricemia[14,15] and on the basis of results of a genome-wide association study (GWAS) for hematological and biochemical traits from approximately 14,700 Japanese

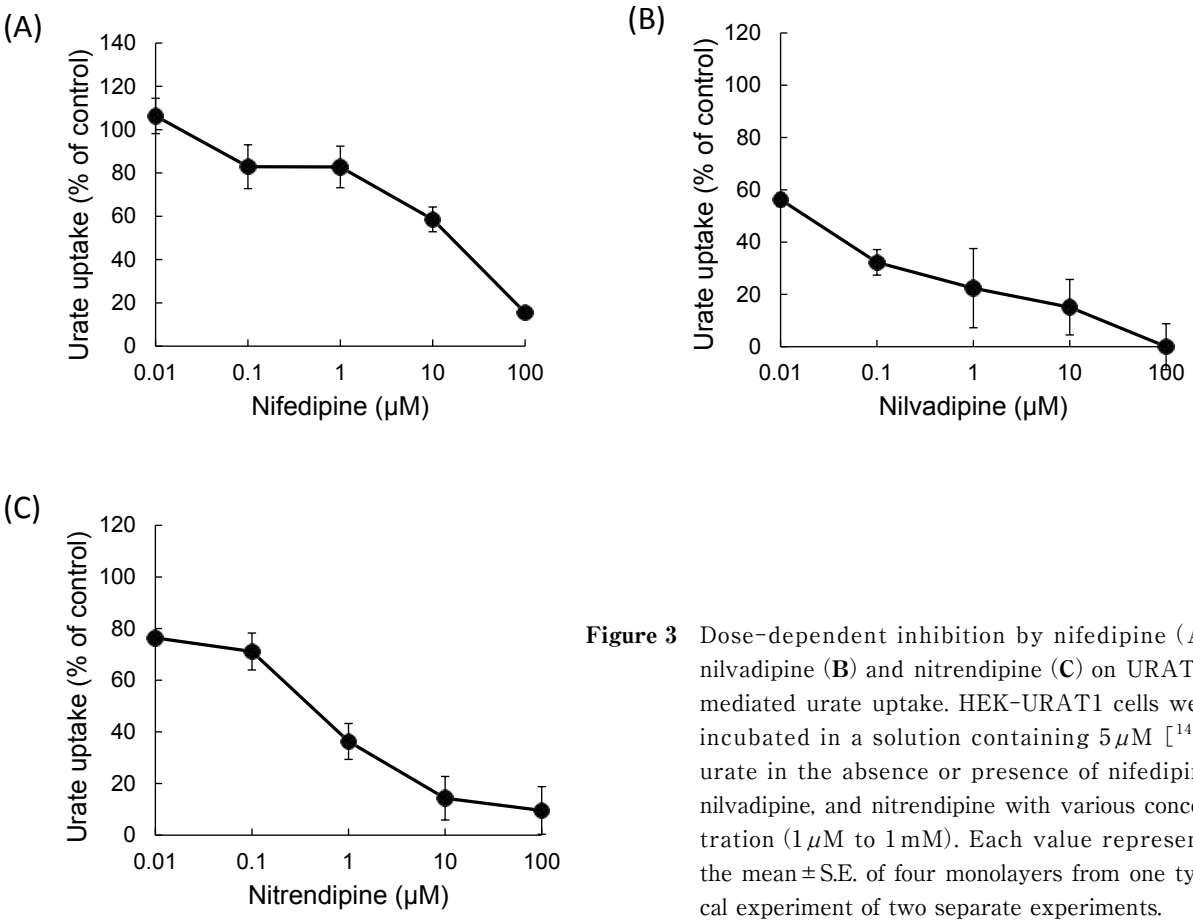

Figure 3 Dose-dependent inhibition by nifedipine (**A**), nilvadipine (**B**) and nitrendipine (**C**) on URAT1-mediated urate uptake. HEK-URAT1 cells were incubated in a solution containing 5 μM [^{14}C] urate in the absence or presence of nifedipine, nilvadipine, and nitrendipine with various concentration (1 μM to 1 mM). Each value represents the mean ± S.E. of four monolayers from one typical experiment of two separate experiments.

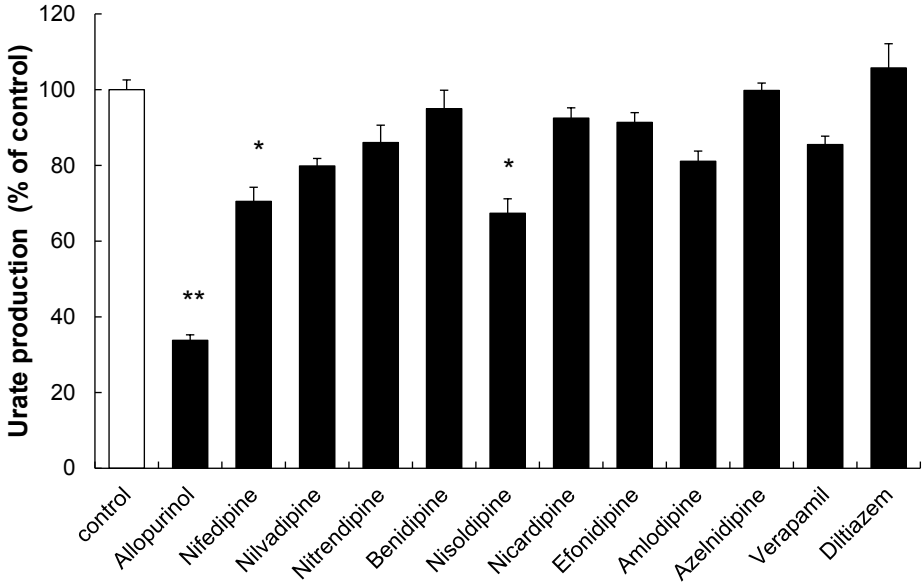

Figure 4 Effects of CCBs on urate production mediated by xanthine oxidase. Urate production was determined in the presence or absence of allopurinol (50 μM) and CCBs (50 μM). Each value represents the mean ± S.E. of six to nine samples from one typical experiment of two separate experiments. $^{*}P<0.05$ and $^{**}P<0.01$ vs. control.

individuals[16]. In the present study, we clarified the interactions of URAT1 with various CCBs using HEK-URAT1 cells and we proposed the possible mechanism of the urate-lowering effects of CCBs via inhibition of renal urate reabsorption by interacting with the major urate transporter URAT1.

Using HEK-URAT1 cells that exhibited a time- and dose-dependent uptake of urate (data not shown), we elucidated the interactions of URAT1 with CCBs including nifedipine, nilvadipine, nitrendipine, benidipine, nisoldipine, nicardipine, efonidipine, amlodipine, azelnidipine, verapamil, and diltiazem. Among these CCBs, nifedipine, nilvadipine, and nitrendipine strongly inhibited URAT1-mediated urate uptake and benidipine, nisoldipine, nicardipine, efonidipine moderately inhibited URAT1-mediated urate uptake, but the non-dihydropyridine subgroup did not show any interaction (Figure 2). Based on their strength, we chose nifedipine, nilvadipine, and nitrendipine and checked the dose-dependency for their URAT1 inhibition. Although IC50 of nilvadipine (0.018 μM) in HEK-URAT1 cells was comparable to Cmax in normal subjects (0.009 μM)[17], the uricosuric action of nilvadipine targeting URAT1 is less likely to occur because its unbound form is not excreted into urine[17]. From this point of view, the urate-lowering effects of CCBs are not the same as that of losartan, another anti-hypertensive drug that has been reported to have uricosuric action. However, since more than half of their metabolites are excreted in urine, those that have a chemical structure similar to that of the original CCBs may interact with renal urate transporters such as URAT1 and provide a urate-lowering effect observed in several clinical studies similar to the report of interaction of URAT1 with benzbromarone and its metabolite 6-OH-benzbromarone[18].

The different degrees of inhibition of URAT1 by CCBs shown in Figure 2 provide information on the structure-activity relation for the development of new uricosuric drugs. Based on the strong inhibition by three drugs of the dihydropyridine subgroup (nifedipine, nilvadipine and nitrendipine), a nitrophenyl group at the C4-position of the dihydropyridine ring seems to be important for interaction with unknown binding site (s) of URAT1 and inducing a strong interaction.

Urate production mediated by XO was not inhibited by most of the CCBs tested (except nifedipine and nisoldipine) in this study. This means that the urate-lowering effects of CCBs are not due to the inhibition of urate production. Hepatic enzymatic production by XO is important for the regulation of serum urate level in humans together with urinary excretion by tubular transporters, but only a limited number of drugs such as allopurinol and febuxostat have shown inhibition of XO. The urate-lowering effects of CCBs do not seem to be mediated by XO inhibition based on our results.

CONCLUSION

Using HEK-URAT1 cells, we clarified the interaction of CCBs with URAT1 for the first time. Although the unbound forms of most CCBs were not excreted into urine, their urate-lowering effects may be associated with the inhibition of renal urate reabsorption mediated by renal urate transporters such as URAT1 with their metabolites that may retain the similar chemical structure. In addition, the results of this study provide a useful clue for the development of new uricosuric drugs.

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Conflict of Interest.
No COI for all authors.

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