Molecular mechanism of urate-lowering effects of anserine nitrate

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Introduction

Hyperuricemia is a risk factor of gout, metabolic syndrome, chronic kidney disease (CKD), and cardiovascular diseases. The serum urate level in humans is regulated by the balance between urinary excretion mediated by renal tubular transporters in the kidney, especially by URAT1, and enzymatic production mainly in the liver by xanthine oxidase (XO). There are two kinds of medications for hyperuricemia: XO inhibitors (allopurinol and febuxostat) and uricosurics (benzbromarone and probenecid), but new medications for hyperuricemia are still needed.

Anserine (β-alanyl-3-methyl-L-histidine) is a dipeptide containing an imidazole ring. Imidazole peptides are abundant in the skeletal muscle and brain of animals and large migratory fish. Many supplements containing anserine are sold in Japan. Anserine and other imidazole dipeptides have been reported to exhibit anti-inflammatory, anti-fatigue, and anti-oxidant effects. Recently, there were two conference reports that anserine had an effect of lowering serum urate levels in mice and humans, and there were also reports that some dipeptides had an effect of lowering serum urate levels. However, the molecular mechanism of the anserine urate-lowering effect is still uncertain, and so we aimed to elucidate this by examining whether anserine inhibits urate excretion or production.

Materials and Methods

Materials

[14C] urate (50 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA). Beta-Alanine, 1-methyl-histidine, 3-methyl-histidine, β-alanyl-L-histidine (carnosine), anserine nitrate, sodium nitrate, and G418 antibiotics were purchased from Sigma Aldrich (Saint-Louis, MO, USA). Fetal bovine serum, trypsin, and lipofectamine were obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture

HEK-URAT1 cells were established according to a method previously described. Briefly, the full-length cDNA of URAT1 was subcloned into pcDNA3.1 (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 a control (HEK-mock cells). The cells were grown in a humidified incubator at 37°C and in 5% CO₂ using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 500 µg/mL G418 antibiotics. The cells were subcultured in a medium containing 0.05% trypsin-EDTA solution (containing 137 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 4 mM NaHCO₃, 0.5 mM EDTA, and 5 mM Hepes; pH 7.2) and used at 15-25 passages.

Uptake experiments

Uptake experiments were performed as previously described. HEK-URAT1 and HEK-mock cells were seeded in 24-well plates at a density of 3 x 10⁵ cells/well. After the cells had been cultured for 24 hours, they were washed three times with serum-free and chloride-free Hanks’ balanced salt solution (HBSS) containing 125 mM Na gluconate, 4.8 mM K gluconate, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 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°C for 10 min. The cells were then incubated in a solution with 5 µM [¹⁴C] urate at 37°C for 2 min. The uptake was stopped by adding ice-cold HBSS, and the cells were washed two times with the same solution. The cells in each well were lysed with 0.5 mL of 0.1 N sodium hydroxide and 3 mL of INSTA-GEL PLUS (Perkin Elmer, Waltham, MA, USA), and radioactivity was determined using a β-scintillation counter (LSC-3100, Aloka, Tokyo, Japan) and normalized to the cellular protein content measured using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA).

Inhibition study

To evaluate the inhibitory effects of the substrates described above on urate uptake via URAT1, HEK-URAT1 and HEK-mock cells were incubated in a solution with 5 µM [¹⁴C] urate in the presence or absence of substrate solutions. After incubation, the cells were washed and lysed as described above, and radioactivity was determined as described. The inhibition was calculated by comparing the uptake in the presence of substrate to that in the absence of substrate, normalized to the cellular protein content. The substrates used in this study included β-alanine, 1-methyl-histidine, 3-methyl-histidine, Carnosine (β-alanyl-histidine), and Anserine (β-alanyl-3-methyl-histidine).

Figure 1. Chemical structures of substrates used in this study.
absence of 10 mM of the substrates at 37°C for 2 min.

Xanthine oxidase (XO) assay

A xanthine oxidase Fluorometric Assay Kit from Cayman chemical Co. (Ann Arbor, MI, USA) was used to quantify xanthine oxidase activity according to the instructions of the manufacturer13, 14. Urate production by XO was assessed in the presence or absence of allopurinol (50 µM) and the substrates described above (10 mM).

Statistical Analysis

Uptake experiments were conducted three times, and each was performed in triplicate. Values are presented as the mean ± standard deviation (SD). Significance was determined by Student's t-test. Differences were considered significant at p<0.05.

Results

Inhibitory effects of anserine on urate uptake mediated by URAT1

We investigated the effects of anserine on urate uptake mediated by HEK-URAT1 cells. As shown in Figure 1, we tested 5 substrates: β-alanine, 1-methyl-histidine, 3-methyl-histidine, carnosine (β-alanyl-L-histidine), and anserine nitrate. Among these substrates, anserine nitrate markedly inhibited URAT1-mediated urate uptake, and 3-methyl-histidine and carnosine moderately inhibited it (Figure 2). Anserine nitrate and sodium nitrate markedly inhibited urate uptake mediated by URAT1, but there was no significant difference in urate uptake via URAT1 and mock between anserine nitrate and sodium nitrate (Figure 3).

Inhibitory effects of anserine on xanthine oxidase

We also examined the effects of β-alanine, 1-methyl-
histidine, 3-methyl-histidine, carnosine (β-alanyl-L-histidine), anserine nitrate, and sodium nitrate on urate production by XO. As shown in Figure 4, urate production mediated by XO was markedly inhibited by anserine and moderately inhibited by 3-methyl-histidine and carnosine. Figure 5 shows the dose-dependent inhibitory effects of anserine nitrate. Only anserine nitrate showed a marked inhibitory effect and its IC₅₀ value was 6.45 ± 1.57 mM.

**Discussion**

Anserine (β-alanyl-3-methyl-L-histidine) is one of the dipeptides containing an imidazole ring. Carnosine and homocarnosine are well-known imidazole peptides. It has been reported that they show anti-inflammatory, anti-fatigue, and anti-oxidant effects. Some reports stated that anserine had an effect of lowering the serum urate level in mice and humans. However, the mechanism remains unknown, so we aimed to elucidate it. We concluded that anserine lowered the serum urate level through its XO inhibitory effect.

However, there were several issues that need to be considered in our study. The first one was that anserine itself was not commercially available and we had to use anserine nitrate in our experiments. Anserine is a free and water-soluble substance, so anserine nitrate can be used instead of anserine if nitrate does not affect URAT1 or XO activity. Actually, in the XO activity assay, anserine nitrate could be a substitute because nitrate did not show an inhibitory effect. However, URAT1 is a urate/anion exchanger and it was reported that intracellularly injected nitrate increased urate uptake via URAT1. We examined the effect of nitrate, and found that extracellularly increased nitrate markedly inhibited urate uptake via URAT1 and there was no difference in uptake inhibition via URAT1 or endogenous transporter between anserine nitrate and sodium nitrate. Considering these results, we cannot exclude the possibility that anserine has an inhibitory effect on URAT1. Further investigation of the inhibitory effects of anserine on URAT1, in which nitrate is removed by an ion-exchange column, is still necessary.

The second one is the anti-oxidant effect of anserine. It has been reported that imidazole dipeptides including anserine and carnosine have an anti-oxidant effect on superoxide and hydrogen peroxide. In the presence
of XO, hypoxanthine changes to xanthine and then to urate in the liver. Superoxide generated in this process is converted to hydrogen peroxide by superoxide dismutase, and finally to water and oxygen by catalase and peroxidase. In the XO fluorometric assay, the final product of this enzymatic reaction, resorufin, was measured. Resorufin is a highly fluorescent substrate which can be measured at an excitation wavelength of 520-550 nm and emission wavelength of 585-595 nm. The absorbance of resorufin in the XO fluorometric assay may have been low as a result of anserine reacting with superoxide or hydrogen peroxide, and not with XO directly. However, only anserine showed a marked inhibitory effect and nor carnosine, even at a concentration higher than 10 mM (data not shown). There was a report refuting the anti-oxidant effect of anserine\(^6\). Although the anti-oxidant activity of imidazole dipeptides is still controversial, these facts support our finding that anserine has inhibitory effects on XO activity.

The third one is the concentration of anserine in the human liver, where XO acts. As described previously, its IC\(_{50}\) value for an inhibitory effect on XO was 6.45 ± 1.57 mM. Whether anserine is present at such a high concentration in the human liver is controversial. Anserine is present in the millimolar range in cardiac tissue and skeletal muscle\(^7\), and its plasma concentration is at most 2.72 µM at 100 min after ingesting 150 g of chicken breast\(^8\). The tissue distribution of anserine in the human liver remains uncertain because of difficulty in sampling. It is almost impossible to conduct a tissue biopsy after a meal, especially of the human liver. To elucidate the concentration of anserine in the liver, animal experiments may be an alternative.

In this study, we found that 3-methyl histidine and carnosine had an inhibitory effect on URAT1 and XO, but not β-alanine or 1-methyl histidine. Based on these results, the imidazole ring itself or difference in the methyl position may have an important role in the effects on URAT1 and XO. Imidazole dipeptides are known to have several bioactivities\(^6,7\). To clarify this issue, further experiments to investigate the inhibitory effect on URAT1 and XO using histidine, anserine itself, and balenine (β-alanyl-1-methyl-histidine) as inhibitor substances are needed.

**Conclusion**

We clarified the urate-lowering effect of anserine nitrate in HEK293-URAT1 cells for the first time. Although the effect of anserine nitrate on URAT1 remains unclear, anserine nitrate showed a marked inhibitory effect on XO activity. This indicates that the effect of anserine to lower the serum urate level in humans is partly due to the inhibition of urate production by XO activity.

**Financial Disclosure**

The authors declare no conflict of interest associated with this study.

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SUMMARY
Recently, anserine (beta-alanyl-3-methyl-L-histidine), one of the dipeptides, was reported to lower the serum uric acid level in humans. This level in humans is regulated by the balance between urinary excretion mediated by renal tubular transporters in the kidney and enzymatic production mainly in the liver xanthine oxidase (XO). The mechanism of anserine lowering the serum uric acid level is still unknown, so we investigated it by examining whether anserine inhibits urate excretion or production. To analyze the uricosuric action, we performed a \([14C]\)urate uptake experiment in human embryonic kidney cells stably expressing human urate transporter 1 (HEK293-URAT1) or mock (HEK293-mock) cells with 10 mM of the following substrates: beta-alanine, 1-methyl-L-histidine, 3-methyl-L-histidine, carnosine (beta-alanyl-L-histidine), and anserine nitrate. Because only anserine nitrate was commercially available and there was a report that sodium nitrate injected intracellularly increased urate uptake mediated by URAT1, we also examined the effect of sodium nitrate on urate uptake via URAT1. Next, we carried out a XO activity assay to analyze the inhibitory effect on urate production with 10 mM of substrates, as previously mentioned. In the uptake experiment, anserine nitrate and sodium nitrate markedly inhibited urate uptake mediated by URAT1. 3-Methyl-L-histidine and carnosine showed weak inhibitory effects. Beta-alanine and 1-methyl-L-histidine did not show inhibitory effects. In the XO activity assay, anserine nitrate markedly inhibited XO activity and its IC50 was 6.45 ± 1.57 mM. 3-Methyl-L-histidine and carnosine showed weak inhibitory effects. Beta-alanine, 1-methyl-L-histidine, and sodium nitrate did not show inhibitory effects. Although the effect of anserine nitrate on URAT1 was unsettled, it showed a marked inhibitory effect on XO activity. This suggests that the effect of anserine to lower the serum urate level in humans is partly due to the inhibition of urate production by XO activity.

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