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Effects of Naofen on Enzyme Activities of Serine Proteases and Matrix Metallo-proteinases

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Abstract: The aim of this study was to investigate whether naofen affected on the activities of metallo and serine proteases. Naofen, found in both intra- and extra-cellular spaces, increased in the livers especially under pathological conditions such as CCl_4-induced cirrhosis of rats. Moreover, naofen seemed to be digested into fragments which might be closely correlated to the pathological alterations of proliferations and fibrosis. Recent studies showed that metallo and serine proteases degrade the fibrous tissues. Therefore, we investigated possible influences of naofen fragment (s) on the activities of metallo-protease, gelatinase/collagenase and serine protease, trypsin, *in vitro* by using quenching fluorescence method. It was found that 1.2×10^{-8} and 4×10^{-8} M naofen C-fragment had inhibitory effect on trypsin but not gelatinase/collagenase activity. Naofen N-fragment of 1.2×10^{-7} and 4×10^{-7} M did not change gelatinase/collagenase activity but did enhance trypsin activity in a dose-dependent manner. Kunitz type serine protease inhibitor, bikunin inhibited both gelatinase/collagenase and trypsin activities, at bikunin concentrations of 1.2×10^{-7} and 1.2×10^{-6} g mL^{-1}. Interestingly, naofen N-fragment depleted the reduction ability of bikunin on trypsin from 80 to 50%. These findings indicated that naofen C-fragments may be an endogenous serine protease inhibitor like bikunin, whereas naofen N-fragment may be an enhancer of serine protease and further counteracts the action of the inhibitor, bikunin. Therefore, naofen may be a precursor for active fragments which interacts with serine proteases.

Key words: Naofen, bikunin, gelatinase/collagenase, trypsin, kunitz type

INTRODUCTION

Naofen (Gene Bank ID: EF613262), a protein in WD-repeat2-protein family (Neer *et al*., 1994; Smith *et al*., 1999) has been found through cloning as anti-verotoxin-2 antibody immunoreactive proteins from rat spinal cord cDNA library. In-situ hybridization showed that naofen mRNA is expressed in the vascular endothelial cells, besides neurons and glial cells, intestinal nerve plexuses, kidney tubule epithelial cells, testis, hepatocytes and so on (Ishikawa *et al*., 2004; Feng *et al*., 2008). Evidently, naofen might have some roles on cell growth, proliferation, development or cell death: Naofen mRNA expressions were enhanced at the early phases of carbon tetrachloride (CCl_4)-induced cirrhosis development and during regenerative phases after partial hepatectomy, more remarkable in the cirrhosis. Moreover, partial hepatectomy caused a small increase of naofen expressions in the whole hepatocytes and significantly in the endothelial cells of portal veins and hepatic arterioles. Whereas, some fragments of naofen located in the extracellular fibrosis tissues in the cirrhosis livers and interestingly co-located with Transforming Growth Factor (TGF)-β1 in the rat cirrhotic hepatocytes (An *et al*., 2008). Furthermore, since the physiological roles of naofen are still unknown, double hybrid analyses in our preliminary studies have been performed to clarify the binding proteins for naofen, showing that naofen seemed to bind to one of Kunitz type serine protease inhibitors.

The present study was undertaken to evaluate the relationship between naofen and protease activities. Since naofen is distributed in the connective tissues such as collagen in the late phases of cirrhosis (An *et al*., 2008),

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collagen catalyzing assay system was utilized with trypsin for the serine proteases and gelatinases/collagenases for matrix metalloproteinases (MMPs). *In-situ* hybridization and immuno-histochemistry for the distribution in the cirrhosis and partial hepatectomy livers showed that naofen mRNA produced in the cell bodies was transcribed to naofen which then may be catalyzed into fragments, since naofen staining with antibodies showed both intra- and extra-cellular existences. Especially naofen fragment which was recognized with an antibody against C-terminal peptide has located only in the intracellular spaces, whereas that with an antibody against N-terminal in both intra- and extra-cellular spaces (An *et al*., 2008). Therefore, besides the effect on metallo and serine proteases, the objective of our study was whether naofen might affect the actions of bikunin, a Kunitz-type serine protease inhibitor, or not. Furthermore two fragmental proteins of naofen were artificially synthesized, purified and utilized in the present study to observe their influence on the actions of the protease inhibitor, bikunin.

MATERIALS AND METHODS

All the experiments were done in 2008

Reagents: EnzChek® Gelatinase/Collagenase Assay Kit was purchased from Molecular Probes Company and trypsin from Invitrogen (CA, USA). Bikunin was kindly given by Prof. Emeritus Koji Kimata (Department of Molecular Science of Medicine, Aichi Medical University, Japan) who purchased it from Mochida Pharmaceutical Co. Ltd., Japan and purified with a DEAE-Sepharose column.

Purification of naofen N-terminal or C-terminal proteins: Amplified cDNA of N-terminal (coding 1-245 amino acids, aa) or C-terminal (coding 973-1170 aa) proteins of naofen, named as naofen-N (NF-N) and naofen-C (NF-C) fragments, respectively, were separately inserted into the pET32 Ek/LIC plasmid, encoding the TRX-His-naofen N-terminal or C-terminal fusion proteins. The sequence of inserted nucleotides for naofen was confirmed with the aid of a genetic analyzer (ABI PRISM 3100, Applied Biosystems Inc., Foster City, CA, USA). *E. coli* BL21 (DE3) competent cells were transformed with expression plasmids and grown at 37°C and protein synthesis was induced by adding 1mM isopropyl-β-D- thiogalactoside (IPTG). The bacterial cells were collected by centrifugation and suspended in ice-cold TEN buffer and sonicated. The inclusion body was collected by centrifugation and was incubated in TEN buffer followed by centrifugation. Then the inclusion bodies were washed twice to remove Triton X-100 and dissolved in digestion buffer and centrifuged to remove insoluble particles. The supernatant was applied to Ni-NTA His•bind® resin to purify naofen terminal-fragment fusion proteins. The eluted naofen terminal fusion proteins were detected with the Western blotting technique using mouse monoclonal anti-His antibody. The fraction containing naofen terminal fusion proteins was diluted with 7 volumes of digestion buffer and insoluble particles were removed by centrifugation. Thereafter, naofen terminal fusion proteins were digested with enterokinase for 16 h at 22°C to separate naofen from His-tag.

Then the solution was applied to a reverse-phase chromatograph (RP-300, Aquapore Octyl, C8, 7 μm, 300 Å, 25 cm×4.6 mm i.d., Brownlee Conventional Column, PerkinElmer Inc., Shelton, CT, USA), utilizing a high-performance liquid chromatograph (HPLC LC-VP series, Shimazu Seisakusho Co. Ltd., Tokyo, Japan). Eluent solution was pumped at a speed of 1 mL min^{-1} and proteins were detected at a wavelength of 280 nm. Acetonitrile-gradient eluent solution containing 0.1% trifluoroacetic acid (TFA) solution and 99.9% acetonitrile plus 0.1% TFA solution made by two pumps was infused into the column, to separate and to isolate the fractions of naofen N- or C- fragment protein of naofen terminal proteins. Detection was performed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue R-250 (Sigma) staining. Then the fractions containing naofen-N or -C fragments were dried with a freeze dryer (EDU-830, Eyela, Tokyo Rika Kikai Co. Ltd., Tokyo, Japan). Naofen-N and -C fragments were dissolved in dimethyl sulfoxide, then diluted with diluted buffer.

Enzyme activity assays: All the experiments were carried out at Aichi Medical University School of Medicine, Japan in 2008. Assay of gelatinase/collagenase and trypsin activity employed substrate DQ™ gelatin, fluorescein conjugate-gelatin labeled with fluorescien that the fluorescence is quenched. The increase in fluorescence which is proportional to proteolytic activity can be measured by Fluorescence microplate reader (Fluoroscan Ascent, Helsinki, Finland) in a unit of the Relative Florescence Unit (RFU) at excitation wave length of 485 nm and emission of 538 nm. The RFU results of the experiments will be subtracted from control substrate blank. The enzyme activity was continuously measured for 2 h. To analyze effects of substances, the enzyme, gelatinase/collagenase at a concentration of 0.5 U mL^{-1} or trypsin at 0.009 mg mL^{-1}, was preincubated with serine protease inhibitor, bikunin (wBKN, 1.2×10^{-7}-1.2×10^{-6} g mL^{-1}) in the absence or presence of naofen N-fragment (1.2×10^{-7} and 4×10^{-7} M) or naofen C-fragment

(1.2×10^{-8}-1.2×10^{-7} M) for 2 h at 37°C before adding substrate in comparison with enzyme control, neither inhibitor nor naofen C-fragment / naofen N-fragment.

Analysis of data: Data are presented as Means+SEM and were analyzed by two way analysis of variance ANOVA, with Bonferroni methods (Graphpad prism 5 Free Download, 2008) for testing the significance between means. Significant level was <0.05.

RESULTS

Effect of naofen and bikunin on trypsin activities: Bikunin of 1.2×10^{-7} and 1.2×10^{-6} g mL^{-1} markedly depleted the velocity of trypsin activity ($p<0.001$ and $p<0.05$, respectively) (Fig. 1). Moreover, 1.2×10^{-8} and 4×10^{-8} M naofen C-fragment significantly decreased the velocity of enzyme activity as well (both $p<0.01$). However, the combination of naofen C-fragment plus bikunin had no further inhibitory effect on enzyme activity.

The highest velocities of trypsin were significantly enhanced in dose-dependent manner by naofen N-fragment of 4×10^{-7} M compared to the absence of naofen (Fig. 2): RFU velocities obtained without or with bikunin of 1.2×10^{-6} g mL^{-1} in the presence of naofen N-fragment were significantly greater than those in its absence ($p<0.001$) and furthermore the velocities with bikunin of 1.2×10^{-7} or 4×10^{-7} g mL^{-1} significantly greater than those in the absence of naofen N-fragment ($p<0.05$).

Either concentration of bikunin in the presence of 1.2×10^{-7} M naofen N-fragment significantly diminished the velocities of trypsin activities ($p<0.05$ or 0.01, Fig. 2).

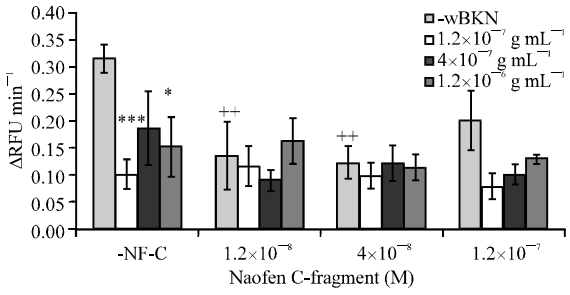

Fig. 1: Effects of naofen C-fragment and bikunin on trypsin activity. Columns displayed the highest velocities of trypsin activity in the absence or presence of naofen C-fragment (NF-C, 1.2×10^{-8}, 4×10^{-8}, 1.2×10^{-7} M) or bikunin (wBKN, 1.2×10^{-7}, 4×10^{-7}, 1.2×10^{-6} g mL^{-1}) or the combination of naofen C-fragment plus bikunin. Data represent the Means±SEM (n = 6); *, *** $p<0.05$, <0.001, respectively, vs. without bikunin; ++$p<0.01$ vs. without naofen (-NF-C)

And 1.2×10^{-7} g mL^{-1} bikunin in the presence of 4×10^{-7} M naofen N-fragment significantly depleted the velocities ($p<0.01$) but higher concentrations of bikunin diminished them only to a small extent.

The effect of naofen and bikunin on the gelatinase/collagenase activity: The highest velocities of gelatinase/collagenase were significantly reduced by bikunin (wBKN, 1.2×10^{-7} g mL^{-1}) compared to those of the enzyme without bikunin ($p<0.05$) (Fig. 3). However,

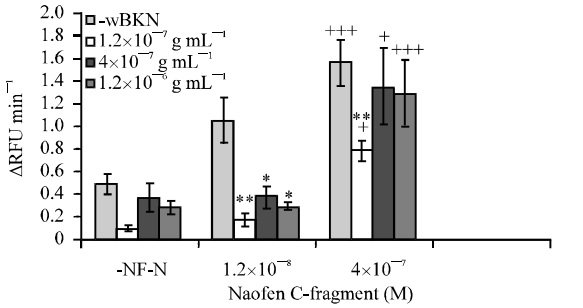

Fig. 2: Effects of naofen N-fragment and bikunin on trypsin activity. Columns illustrated the velocities of the enzyme activity in the absence or presence of naofen N-fragment (NF-N, 1.2×10^{-7}, 4×10^{-7} M) or bikunin (wBKN, 1.2×10^{-7}, 4×10^{-7}, 1.2×10^{-6} g mL^{-1}) or the combination of naofen N-fragment and bikunin. Data represent the Means±SEM (n = 4); *, ** $p<0.05$, <0.01, respectively, vs. without bikunin, +, +++ $p<0.05$, <0.001, respectively, vs. without naofen (-NF-N)

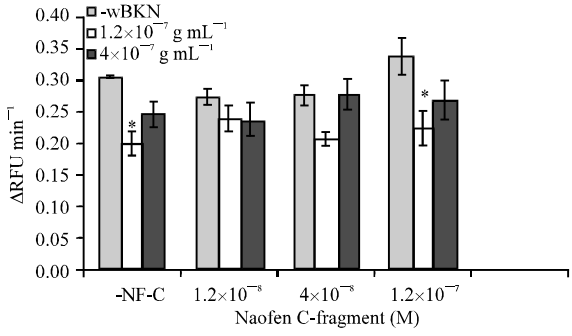

Fig. 3: Effects of naofen C-fragment and bikunin on gelatinase/collagenase activity. The highest velocities of the enzyme (0.5 U mL^{-1}) activity were shown cin columns, in the absence or presence of naofen C-fragment (NF-C, 1.2×10^{-8}, 4×10^{-8}, 1.2×10^{-7} M) or bikunin (wBKN, 1.2×10^{-7}, 4×10^{-7} g mL^{-1}) or the combination of naofen C-fragment and bikunin. Data represent the Mean±SEM (n = 4); * $p<0.05$ vs. without bikunin (-wBKN)

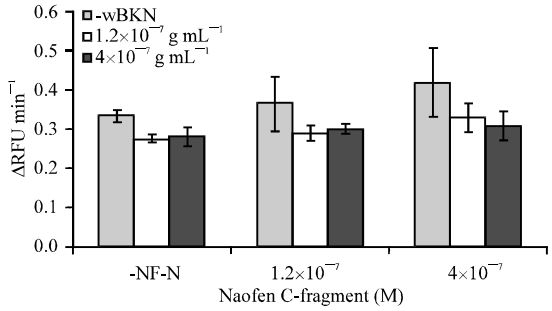

Fig. 4: Effects of naofen N-fragment and bikunin on gelatinase/collagenase activity. Columns showed the velocities of the enzyme activity in the absence or presence of naofen N-fragment (NF-N, 1.2×10^{-7}, 4×10^{-7} M) or bikunin (wBKN, 1.2×10^{-7}, 4×10^{-7} g mL^{-1}) or the combination of naofen N-fragment and bikunin. Data represent the Mean±SEM (n = 4)

naofen C-fragment (NF-C) showed, not significantly, a small reduction of the velocity of the enzyme activity at low concentration, 1.2×10^{-8} and 4×10^{-8} M) while showed a little enhancement at highest concentration (1.2×10^{-7} M). The combination of bikunin (1.2×10^{-7} g mL^{-1}) plus naofen C-fragment (1.2×10^{-7} M) caused a significant reduction of the enzyme activity compared to the absence of bikunin ($p<0.05$) which it was possibly caused by the little enhancement plus a constant degree of a small reduction of naofen C. Moreover, at higher doses bikunin may not bind properly to the enzyme pocket which is likely a critical factor that leads to a loss of inhibitory effect.

Naofen N-fragment (NF-N, 1.2×10^{-7}, 4×10^{-7} M) also did not alter the velocity of the enzyme activity in either absence or presence of bikunin (Fig. 4).

DISCUSSION

Firstly, due to the first recent discovery by Prof. Ishikawa and his colleges (United States Patent 7847132), naofen has not been known widely. Therefore, present investigation of the influence of naofen on the proteases were first emerged. The study investigated the relationship between naofen C- and N-terminal fragments and protease activities which may play roles on cell proliferation, tumor invasion and metastasis. Using quenching fluorescence method, it was found that naofen C-fragment had inhibitory effect on trypsin but naofen N-fragment enhanced the trypsin activity in a dose-dependent manner. Kunitz type serine protease inhibitor, bikunin, inhibited both gelatinase/collagenase and trypsin activities and naofen N-fragment attenuated the inhibition of bikunin on trypsin. The conclusion was that naofen C-fragments may be an endogenous serine protease inhibitor, whereas naofen N-fragment may be an enhancer of serine protease and counteract the action of inhibitor, bikunin. It was possibly speculated that naofen could be a precursor of active fragments which interacts with serine proteases.

Trypsin, one of three serine proteases of the chymotrypsin-like clan including chymotrypsin and elastase as well (Pugia *et al*., 2007), was inhibited dose-independently by bikunin in either presence or absence of naofen N- or C-fragments. Possibly, started doses of the inhibitor was high. And such inhibitory actions of bikunin were much lower in the presence of N-fragment, approximately 50% of the trypsin activity obtained without bikunin, compared with 80% in the absence of N-fragment. Since from two-hybrid analyses naofen expression was correlated to the binding to a type of serine protease inhibitors (unpublished observation), such a change induced by the combination of N-fragment and bikunin may support that these two substances are able to bind and influence the trypsin activity. Bikunin, a Kunitz-type of serine protease inhibitors (Vetr and Gebhard, 1990; Gebhard *et al*., 1990) inhibits trypsin, chymotrypsin, human leukocyte elastase and plasmin but its effect on gelatinase/collagenase is still unclear.

The trypsin gene is widely expressed in normal human tissues including various epithelial tissues, brain, spleen, kidney and liver (Koshikawa *et al*., 1998). It has been established that extracellular matrix-degrading proteases play a critical role in tumor invasion and metastasis (Stetler-stevenson *et al*., 1993). Trypsin is a potent proteolytic enzyme which degrades a wide variety of extracellular matrix proteins such as laminin and fibronectin (Koshikawa *et al*., 1992) and also a potent activator of latent form of various MMPs and serine protease from zymogens (Birkedal-Hansen *et al*., 1975; Soreide *et al*., 2006). Therefore, it is conceivable that a naofen fragment such as naofen C-fragment may inhibit the trypsin activity, thereby preventing the deformation of extracellular matrix.

On the contrary, naofen N-fragment enhanced the catalytic activity of trypsin in the collagen degeneration, although N-fragment itself did not degrade the collagen (data not shown). The results indicated that naofen fragment may interact with trypsin, thus increasing the trypsin activity and augmenting the degradation of extracellular matrix. Thus the present study shows that naofen may elicit an inhibition and in the other part enhancement on the enzyme activity of serine protease, as bimodal proteins.

More importantly a fragment of naofen also exists in the extracellular spaces, bound to connective tissues, i.e., collagens and elastins. MMPs, including collagenases, gelatinases, elastases and stromelysins, a family of endopeptidases (Malemud, 2006), are shown to degrade the extracellular matrix molecules (ECM). Therefore, the enhanced or inhibited activities of serine proteases obtained in the presence of naofen fragments suggested a possible role of naofen fragments in the break-down of ECM, i.e., degrading gelatin, collagens, laminin, fibronectin and other matrix proteins (Woessner and Nagase, 2000). Thus, conceivably naofen fragments may participate in modifying the proteolytic actions of MMPs by influencing the serine protease activity. Furthermore, MMPs can target not only to ECM but also to many non-ECM proteins (Ii *et al*., 2006), including growth factors, growth factor receptors, cell-associated molecules and cytokines. As a result, over-expression of these proteases causes changes in the amount and organization of the ECM components and also cellular functions and naofen as well.

Naofen is produced in the cytoplasm, degraded into fragments which may or may not be active in the signal transmission. Our recent study demonstrated that in the course of CCl_4-induced cirrhosis development naofen expression appeared at the early phases compared to the expression times for TGF?1 and collagen and thus naofen fragments may also influence the fibrogenesis.

Naofen C-fragment diminished the trypsin activities and either naofen fragment showed no inhibitory action on gelatinase/collagenase, demonstrating the actions of serine protease inhibitors and its action was especially similar to bikunin. Serine proteases are shown inhibited by three types of serine protease inhibitors, i.e., Serpin and Kazal superfamilies, besides Kunitz (Roberts *et al*., 1995). Since trypsin activity can be inhibited with all types of inhibitors, whether naofen fragment may or may not interact with other types of serine proteases might enable to clarify the binding of naofen fragment to specific types of serine protease inhibitor. In the previous studies (Kobayashi *et al*., 2001), it was found that bikunin has tumor-suppressive potential in several malignant cell types, acting at the level of tumor invasion and metastasis. And furthermore, it is conceivable that such a naofen fragment as located in the extracellular spaces may play a role in influencing the fibrosis through inhibiting or enhancing the serine protease activities and collagen degradations.

In conclusion, naofen C-fragments may play a role as an endogenous serine protease inhibitor while naofen N-fragment may be an enhancer of serine protease and counteract the action of inhibitor, bikunin. It is possible that naofen could be a precursor of active fragments which interacts with serine proteases.

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