Competitive binding of musclin to natriuretic peptide receptor 3 with atrial natriuretic peptide

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Abstract

Musclin is a novel skeletal muscle-derived secretory factor that was isolated by our group. Musclin contains a region homologous to natriuretic peptides (NPs). This study investigated the interaction between musclin and NP receptors (NPRs). Musclin specifically bound to NPR3, but not to NPR1 or NPR2. Musclin and atrial natriuretic peptide (ANP) competed for binding to NPR3. We conducted binding assays using various synthetic musclin peptides and mutant musclin proteins. The first NP-homologous region in musclin (⁸⁸LDRL⁹¹) and the second

homologous region (¹¹⁷MDRI¹²⁰) were responsible cooperatively for high-affinity binding to NPR3. The first NP-homologous region was more importantly associated with binding to NPR3, than the second homologous region. The competitive nature of musclin with ANP for the natriuretic clearance receptor NPR3 was also confirmed *in vivo*. We conclude that musclin binds to NPR3 competitively with ANP and may affect ANP concentrations in a local or systemic manner.

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Introduction

Skeletal muscle is a major energy-expending organ important for regulating glucose, lipid, and protein metabolism. It was demonstrated recently that this tissue produces and secretes biologically active molecules, conceptualized as myokines (Musrò *et al.* 2001, Nishizawa *et al.* 2004, Salih *et al.* 2004, Sell *et al.* 2006). We and others recently identified one of these novel secretory factors, and named musclin/osteocrine respectively (Thomas *et al.* 2003, Nishizawa *et al.* 2004). We demonstrated that musclin mRNA expression was almost exclusive in the skeletal muscle of rodents, and induced in obesity models. We also showed that musclin mRNA expression decreased during fasting and increased upon refeeding (Nishizawa *et al.* 2004, Yasui *et al.* 2007).

The musclin protein contains a region homologous to members of the natriuretic peptide (NP) family, as well as a KKRR putative serine protease cleavage site, also characteristic of NP proteins (Nishizawa *et al.* 2004). These findings suggested that musclin signaling could be related to that of NP family and its receptors. The mammalian NP family comprises the atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP; Potter *et al.* 2006). These proteins function as endocrine/paracrine factors in regulating blood pressure and extracellular fluid volume, fat metabolism, and skeletal

development (Potter *et al.* 2006). To date, three single-membrane-spanning NP receptors (NPRs) have been identified. NPR1 and NPR2 are guanylyl cyclase receptors that regulate cGMP levels, while NPR3 lacks enzymatic activity and may act as a clearance receptor (Potter *et al.* 2006).

The present study investigated interactions between musclin and NPRs, with a special focus on the competition between musclin with ANP for the natriuretic clearance receptor NPR3.

Experimental procedures

Plasmids

Full-length wild-type musclin (WT (30–130); 30–130 amino acid excluding N-terminal signal sequence) or mutant (mutant (80–130); 80–130 amino acid) mouse musclin cDNA was inserted into the ligation independent cloning (LIC)-cloning site of pET32Xa-LIC (Merck KGaA) to produce *Escherichia coli*-derived recombinant proteins. PCR-based point mutations were introduced at amino acid residues Asp89Gly and Arg90Gly to encode mutations in ANP-homologous region 1 (Mutant-1), and at Asp118Gly to encode a mutation in ANP-homologous region 2 (Mutant-2) in full-length (30–130 amino acid) mouse musclin cloned in

pET32Xa-LIC. All three mutations were introduced to encode mutations in both ANP-homologous regions 1 and 2 (Mutant-3). The corresponding mutations in C-terminal half musclin (cWT; mutant (80–130)) were introduced in Mutant-1C (Asp89Gly and Arg90Gly), Mutant-2C (Asp118Gly), and Mutant-3C (all three mutations) respectively.

Full-length (30–130 amino acids) mouse musclin cDNA was cloned into pET15b (Merck KGaA). The residue located in the thrombin cleavage site between the N-terminal His x6 tag and the musclin cDNA was mutated from Leu-Val-Pro-Arg-Gly-Ser to Leu-Val-Pro-Tyr-Gly-Cys (His x6 YC-musclin). The Tyr and Cys are necessary for N-terminal tyrosyliodination and maleimide biotinylation respectively. The N-terminal iodinated one is designated as [¹²⁵I]-Musclin, and the N-terminal biotinylated one through the Cys residue is designated as BC-Musclin.

Full-length (30–130 amino acid) mouse musclin cDNA was cloned into the pFLAG-CMV3 mammalian expression vector (Sigma-Aldrich, Inc.) to encode N-terminal FLAG-tagged mouse musclin (FLAG-WT).

The mammalian expression vectors of human NPR Npr1/pCMVXL6, Npr2/pCMVXL4, and Npr3/pCMVXL4 were purchased from OriGene Technologies Inc. (Rockville, MD, USA). The extracellular domain of mouse NPR3 cDNA was cloned from the first strand cDNA of lung prepared from normal C57Bl/6j mice. The extracellular regions of human (aa1–481) and mouse (aa1–477) NPR3 were tandem-inserted with the human IgG-Fc coding sequence (aa240–471 of human IgG1) into pcDNA3.1 (–) for producing Fc fusion constructs (hNPR3-IgGFC and mNPR3-IgGFC respectively; Bennett *et al.* 1991).

Recombinant proteins

Recombinant GST-fusion proteins were produced using *E. coli* Rosseta gami B cells carrying the pET32Xa-LIC musclin fusion vector, and were purified with Ni-agarose. The resultant fusion proteins were cleaved by factor Xa, purified using HiTrap SP cation exchange resin (GE Healthcare Biosciences, Uppsala, Sweden) equilibrated with 25 mM HEPES, pH 7.9, and eluted with increasing concentration of NaCl (0.1–1 M) in the equilibration buffer. The full-length musclin protein (WT (30–130)) was biotinylated with an eightfold molar excess of sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA), and designated as BK-Musclin. The efficacy of biotin incorporation into the musclin molecule was calculated by the HABA method, with 4.8 biotin molecules incorporated into one molecule of musclin. His x6 YC-musclin protein was produced by *E. coli* Rosseta gami B cells carrying the modified pET15b-musclin expression plasmid constructed as above, and purified with Ni-agarose and HiTrap SP cation exchange resin. The purified protein was iodinated by lactoperoxidase with Na¹²⁵I to produce [¹²⁵I]-labeled musclin ([¹²⁵I]-Musclin), or was biotinylated with maleimide-PEO2 biotin (Pierce;

BC-Musclin), followed by TCEP disulfide reduction according to the instructions provided by the manufacture. The efficacy of biotin incorporation was almost 1 biotin per 1 musclin molecule. Partial peptides of 105–130, and 80–112 amino acids of musclin were synthesized by CosmoBio Co. (Tokyo, Japan).

FreeStyle293 cells (Invitrogen) were cultured according to the instructions provided by the manufacturer. The N-terminal FLAG-WT was produced by transfecting FreeStyle293 cells with the pFLAG-CMV3 vector containing full-length mouse musclin cDNA, according to the instructions provided by the manufacturer. Three days after transfection, the culture medium was collected and centrifuged at 440 g for 15 min at 4 °C. The resultant supernatant was adjusted to pH 7.5 with NaOH, and bound to HiTrap SP cation exchange resin (GE Healthcare Biosciences), washed with 25 mM HEPES, pH 7.5 and 50 mM NaCl, and then eluted with 25 mM HEPES, pH 7.5 and 1000 mM NaCl. The eluate was then applied to anti-FLAG agarose conjugate (Sigma-Aldrich) pre-equilibrated with the equilibration buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, one complete protease inhibitor tablet/100 ml buffer (Roche Diagnostics), and 0.1% Tween 20. Following several washes with the equilibration buffer, the desired protein was eluted with 100 µg/ml flag peptide included in the same buffer. The peak fraction monitored at 280 nm was collected and desalted using a PD-10 column (GE Healthcare) pre-equilibrated with sterile PBS.

ANP (rat and mouse), ANP (104–126) Des-[Cys105, Cys121] (L-ANP, rat and mouse), ANP (4–23)-Amide Des-[Gln18, Ser19, Gly20, Leu21, Gly22] (C-ANP, rat and mouse) and biotin-labeled ANP (rat and mouse) were purchased from Phoenix Pharmaceuticals Inc. Burlingame, CA, USA. [¹²⁵I-Tyr28] ANP, rat ([¹²⁵I]-ANP, IM186) was purchased from GE Healthcare Inc. (Potter *et al.* 2006).

The Fc fusion constructs were transfected into FreeStyle293 mammalian expression cells (Invitrogen) according to the instructions provided by the manufacturer. Fc fusion proteins were purified as described (Bennett *et al.* 1991), with minor modifications. After 4 days, the cell supernatants were pooled and purified with HiTrap rProtein A column equilibrated with PBS containing 0.1% NaN₃, and eluted with Gentle Ag/Ab elution buffer, pH 6.9 (Pierce 21027). The peak elution was dialyzed against PBS and concentrated with 10 000 molecular cut-off ultrafiltration membrane (YM-10, Millipore, Bedford, MA, USA).

Binding assay

BW5147 cells stably expressing NPR3 were generated using retroviral transduction with pMXs-IG-NPR3. BW5147-NPR3 cells (2 × 10⁵) were incubated at 4 °C for 30 min with binding buffer (0.1% BSA/HANKS' balanced salt solution) containing 1 nM biotinylated full-length musclin (BK-Musclin) or 1 µM biotinylated ANP. For competition experiments, BW5147-NPR3 cells (2 × 10⁵) were incubated

at 4 °C for 30 min with binding buffer containing 10 nM biotinylated-musclin (BK-Musclin) with varying concentrations of non-biotinylated recombinant full-length musclin (WT (30–130)), ANP (rat and mouse), insulin or adiponectin. The cells were then washed twice with binding buffer, and incubated at 4 °C for 30 min with binding buffer containing streptavidin–allophycocyanin (GE Healthcare Biosciences). The cells were washed twice, and the fluorescence intensity of the samples was measured using a FACS Calibur instrument (BD Biosciences).

FreeStyle293 cells (1×10^6) transiently expressing human NPR1, NPR2 or NPR3 were incubated on ice for 60 min with 10 nM of ^{125}I -labeled full-length musclin (^{125}I -Musclin) prepared above or 0.1 nM ^{125}I -ANP, in the absence (control) or presence of competitors indicated in the figure legends. The cells were then separated by passing through a dibutylphthalate:dinonylphthalate (1:1) layer, and tubes were frozen in liquid nitrogen. The radioactivity associated with cells in the bottom of the tube was counted using a γ -counter. The radioactivity associated with cells in the absence of competitors (control) was taken as 100% in each cell expressing NPR1, NPR2 or NPR3 respectively.

For the binding studies using IgG-Fc fusion NPR3, black immunoplates were coated at 4 °C overnight with 1.0 $\mu\text{g}/\text{ml}$ anti-human IgG-Fc affinity-purified monoclonal antibody (A80–105A, Bethyl Laboratories, Montgomery, TX, USA) dissolved in 100 μl of 0.1 M NaCO_3 and 0.1 M NaCl (pH 8.3). The plates were then blocked with Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl , and 0.1% NaN_3) containing 25% (V/V) ImmunoBlock reagent (DS Pharma Biomedical Co., Osaka, Japan) at room temperature for 2 h. The mouse or human NPR3-IgGFc-mediated capture binding assay was originally developed and optimized as follows. The optimized reaction mixture contained 0.3 $\mu\text{g}/\text{ml}$ human NPR3-IgGFc protein or 3.0 ng/ml mouse NPR3-IgGFc protein, 0.1 ng/ml N-terminal biotinylated full-length musclin (BC-Musclin) protein, in the absence (control) or presence of the indicated concentrations of unlabeled competitors as described in the figure legends, in a total of 100 μl TBS containing 5% (V/V) ImmunoBlock reagent, and 0.1% Tween 20. The binding reaction was performed at room temperature for 16 h. Following two washes with 250 μl TBS containing 0.1% Tween 20, streptavidin-conjugated β -galactosidase (Roche, 1112481) diluted to 1/10 000 with the same buffer was incubated at room temperature for 1 h. After four further washes with 250 μl TBS containing 0.1% Tween 20, the plates were incubated at room temperature for 3 h with buffer containing 10 mM K_2HPO_4 , 150 mM NaCl , 2 mM MgCl_2 , 0.1% BSA, 0.1% NaN_3 , and 0.5 mM 4-Methylumbelliferyl β -D-galactopyranoside, pH 7.0. Following the addition of 150 μl 0.1 M glycine solution, pH 10.3, fluorescent intensities were measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The fluorescence developed in the absence of any competitors was taken as the control value, and the fluorescence developed without N-terminal biotinylated

full-length musclin (BC-Musclin) protein was taken as the baseline. For the calculation of percent inhibition, the following equation was used; $100 - 100 \times ((\text{fluorescence in the presence of competitor}) - (\text{baseline fluorescence})) / ((\text{control fluorescence}) - (\text{baseline fluorescence}))$.

Animal studies

Male C57BL/6J mice (CLEA Japan, Tokyo, Japan), between 10 and 13 weeks old, were anesthetized with pentobarbital sodium (65 mg/kg i.p.) and their body was heated to 40 °C for 30 min. The right jugular vein was then catheterized, and 45 μl blood was collected from the tail vein and placed in iced tubes containing EDTA and aprotinin. Immediately, saline, C-ANP (4–23; Bachem, Bubendorf, Switzerland) or recombinant musclin from *E. coli* was administered as a bolus injection (100 $\mu\text{g}/\text{kg}$ body weight) followed by constant infusion (10 $\mu\text{g}/\text{min}$ per kg body weight) for 1 h, after which a further 45 μl blood was collected from the tail vein. Plasma samples were stored at -80 °C until RIA for ANP. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

Measurement of plasma ANP

Plasma was assayed for ANP levels using the α -rat ANF RIA kit (Bachem) according to the protocol supplied by the manufacturer. C-ANP (4–23) at concentrations up to 10 $\mu\text{g}/\text{ml}$ was completely devoid of cross-reactivity with the antibody.

Statistical analysis

Data were expressed as mean $\pm$ S.D., except for experiments *in vivo* (expressed as means $\pm$ S.E.M. in Fig. 5). Unpaired Student's *t*-test was used to evaluate differences between two groups, unless otherwise specified. A *P* value < 0.05 denoted a statistically significant difference. IC_{50} values were calculated by probit analysis, and expressed with 95% confidence interval in the figure legends.

Results

We previously identified musclin as a novel secretory factor isolated from mouse skeletal muscle (Nishizawa *et al.* 2004). The musclin amino acid sequence is highly conserved among mouse, rat, and human, with an N-terminal signal sequence and two C-terminal regions highly homologous with ANP (Nishizawa *et al.* 2004). Furthermore, musclin contains putative serine protease cleavage sites proximally upstream of the ANP-homologous regions (Fig. 1). However, in contrast to ANP, musclin has no conserved cysteine residue by which ANP forms a ring structure to bind its receptor, NPR1. Based on these structural differences, we hypothesized that musclin might bind specifically to the natriuretic

Figure 1 Schematic presentation of the deletion peptides of musclin used in this study. The full-length and deletion peptides of musclin used throughout this study are summarized in schematic presentation (not to scale). Putative full-length musclin (WT (30–130)) lacking possible signal sequence (1–29 amino acids) and C-terminal half musclin (cWT (80–130)) corresponding to a possible cleavage product at the first dibasic region were prepared as *E. coli* recombinant proteins. Partial peptides of 105–130 and 80–112 amino acids of musclin were chemically synthesized. WT (30–130) was labeled with biotin sulfo-NHS-LC-biotin to incorporate biotin molecules into native lysine residue and was designated as BK-Musclin. N-terminal His x6-tagged putative mature musclin (30–130 amino acids) produced in *E. coli* was labeled with ¹²⁵I or maleimide-PEO2 biotin to produce [¹²⁵I]-Musclin or N-terminal-biotinylated full-length musclin (BC-Musclin). SS, possible signal sequence; DB, dibasic peptides; ANP, atrial natriuretic peptide; HR, homologous region.

clearance receptor NPR3, particularly given that a mutant ANP lacking the ring structure can also bind NPR3 (Maack *et al.* 1987, Potter *et al.* 2006). The full-length and deletion peptides of musclin used throughout this study are summarized in schematic presentation (Fig. 1).

To elucidate whether musclin can specifically bind to any NPR among the NPR family, [¹²⁵I]-labeled mouse musclin ([¹²⁵I]-Musclin; N-terminal His x6 tagged WT (30–130)) binding to FreeStyle293 cells transiently expressing human NPR1, 2 or 3 was examined (Fig. 2a). Specific binding of [¹²⁵I]-Musclin that comprises the binding that could be inhibited with excess amount of non-labeled musclin was observed only with cells expressing hNPR3, and not with those expressing hNPR1 or hNPR2 (Fig. 2a). By contrast, [¹²⁵I]-labeled ANP bound specifically to each of the NPR-transfected cells (Fig. 2b). Similar to the case of C-ANP and L-ANP, which are known to bind specifically to NPR3, musclin slightly inhibited ANP binding to NPR1, did not

inhibit ANP binding to NPR2 at all, and specifically inhibited ANP binding to NPR3 (Fig. 2b). Specific binding of [¹²⁵I]-Musclin or [¹²⁵I]-labeled ANP was negligibly-small in non-transfected cells (data not shown). These results clearly indicated that musclin binds to NPR3 in a competitive fashion with ANP.

To elucidate the binding affinity of musclin to NPR3 more precisely, the binding of biotinylated recombinant mouse musclin (BK-Musclin; WT (30–130) biotinylated at Lys residues) to BW5147 cells stably expressing mouse NPR3 was evaluated by FACS analysis. The fluorescent intensity of mNPR3 expressing cells increased with the amount of biotinylated musclin added to the reaction (Fig. 3a), and a negligibly-small increase was observed in parent BW5147 cells not expressing mNPR3 (data not shown), suggesting that musclin could bind to NPR3 expressed on the cell surface. Next, unlabeled musclin (WT (30–130)) or ANP were added into the reaction to examine the competitive nature of the

Figure 2 Specific binding of musclin to NPR3. (a) FreeStyle293 cells transiently expressing hNpr1/pCMV6-XL6, hNpr2 or hNpr3/pCMV6-XL4 were incubated with 10 nM $[^{125}\text{I}]$ -labeled musclin ($[^{125}\text{I}]$ -Musclin) in the absence (Control) or presence of 100 $\mu\text{g/ml}$ full-length musclin (WT (30–130)). The radioactivity associated with cells in the absence of competitors (controls) was taken as 100% in each cells expressing NPR1, NPR2 or NPR3 respectively. The specific binding that corresponds to the difference of $[^{125}\text{I}]$ -Musclin binding to control cells minus in the presence of excess non-labeled musclin (WT (30–130)) was observed only in the cells expressing hNpr3. (b) FreeStyle293 cells transiently expressing hNpr1/pCMV6-XL6, hNpr2 or hNpr3/pCMV6-XL4 were incubated with 0.1 nM $[^{125}\text{I}]$ -ANP, in the absence (control) or presence of 100 $\mu\text{g/ml}$ ANP, circular ANP (C-ANP), linear ANP (L-ANP), and WT (30–130) of musclin. The radioactivity associated with cells in the absence of competitors (controls) was taken as 100% in each cells expressing NPR1, NPR2 or NPR3 respectively. The specific bindings that correspond to the difference of $[^{125}\text{I}]$ -ANP binding to control cells minus in the presence of excess non-labeled ANP was observed in any cells expressing hNpr1, 2 or 3, but not observed in non-transfected cells (data not shown). $[^{125}\text{I}]$ -ligands associated with cells were separated by passing through the dibutylphthalate:dinonylphthalate (1:1) layer, and counted using a γ counter. Data are mean $\pm$ s.d. ($n=3-4$). * $P<0.01$. ** $P<0.001$. *** $P<0.0001$. Similar results were obtained in another independent experiment.

musclin/NPR3 interaction. Musclin, 8.8-fold more strongly than ANP, competitively displaced the binding between biotinylated musclin and the NPR3-expressing cells (Fig. 3b). By contrast, structurally different secretory factors, insulin, and adiponectin, had no such effect (Fig. 3b).

Next, the musclin region responsible for the NPR3 binding was investigated by the binding of N-terminal biotinylated musclin (BC-Musclin; N-terminal His x6 tagged WT (30–130) biotinylated at the N-terminal added Cys residue. Refer to Fig. 1) to an IgG-Fc fusion protein of

human NPR3 (hNPR3-IgGFc), and then adding partial peptides of musclin (Fig. 4a). Musclin binding to NPR3 was also confirmed in these binding experiments using hNPR3-IgGFc, since recombinant musclin (WT (30–130), closed circles) and ANP (gray circles) competed with the N-terminal biotinylated musclin (BC-Musclin) binding to hNPR3-IgGFc (Fig. 4a). The cWT (80–130), closed triangles), which corresponds to the region downstream of the first putative dibasic cleavage site $^{76}\text{KKKR}^{79}$, inhibited musclin binding similar to WT (30–130; closed circles). Next, we investigated which part of ANP-homologous regions downstream of $^{76}\text{KKKR}^{79}$ is more important for musclin binding to NPR3, with two overlapping synthetic peptides encompassing 105–130 (open triangles) and 80–112 (open squares). Peptide 105–130 (open triangles) showed no obvious binding inhibition, but peptide 80–112 (open squares) significantly inhibited the binding in a concentration-dependent manner (Fig. 4a). These results suggested that the first ANP-homologous region of musclin from $^{76}\text{KKKR}^{79}$ to $^{110}\text{KKR}^{113}$ is more important for binding to NPR3. In addition, and it is noteworthy that the mouse full-length musclin expressed in mammalian cells (Flag-WT, closed squares) inhibited this binding 3.4-fold more strongly than the *E. coli*-derived, recombinant full-length musclin (WT (30–130), closed circles) or 2.4-fold than ANP (gray circles; Fig. 4a), which suggests that native musclin may have more potent binding affinity to NPR3, than ANP.

To elucidate the contribution of two ANP-homologous regions more precisely, we next constructed mutants of musclin with amino acid changes in two ANP-homologous regions (Fig. 1). The competitive binding of mutants with full-length musclin (WT (30–130), closed circles) for the mNPR3-IgGFc fusion protein was examined (Fig. 4b). Mutant-1 (closed squares) has two amino acid changes at Asp89Gly and at Arg90Gly in ANP-homologous region 1 (aa94–98), while Mutant-2 (open circles) has a single amino acid change at Asp118Gly in ANP-homologous region 2 (aa113–128). Prior to this experiment, we established that mouse musclin bound more strongly to mouse NPR3 (mNPR3-IgGFc) than to human NPR3 (hNPR3-IgGFc) in the IgG-Fc fusion system (data not shown), and used mNPR3-IgGFc in this experiment. Although musclin (WT (30–130), closed circles) potently inhibited N-terminal biotinylated musclin binding with 0.2 nM of IC_{50} value, Mutant-1 (closed squares) and Mutant-2 (open circles) exhibited more than 75-fold and 4.7-fold reduction in inhibitory potency as compared with full-length musclin (WT (30–130)). These observations shown in Fig. 4b indicated that the mutation in ANP-homologous region 1 reduced the binding affinity of musclin for NPR3 10-fold more than those in ANP-homologous region 2. Mutant-3 (open squares) that has both mutations in ANP-homologous regions 1 and 2 virtually abolished the binding to NPR3. The result suggested that NP-homologous region 1 and 2 are cooperatively responsible for high-affinity binding to NPR3.

Figure 3 Binding of musclin to cellular NPR3. (a) BW5147-mNPR3 cells stably expressing mNPR3 were incubated without (gray lines) or with 1 nM (broken lines) and 1 μM (solid lines) of biotinylated full-length musclin (BK-Musclin). Fluorescence intensity (FL4) was analyzed by FACS. Percent of relative cell numbers, top as 100%, were represented against fluorescent intensity. (b) BW5147-mNPR3 cells were incubated with 10 nM BK-Musclin and varying concentrations of non-biotinylated ligands, including full-length musclin (WT (30–130); closed circles), ANP (open circles), insulin (closed triangles), and adiponectin (open triangles). Mean fluorescence intensity at indicated concentrations of non-biotinylated ligand was analyzed by FACS. IC₅₀ value with (95% confidence interval) by full-length musclin (WT (30–130)) was 11.5 nM (6.3–19.7 nM), and by ANP was 102 nM (38.9–224 nM) respectively. Similar results were obtained in three independent experiments.

These reduced binding affinities of musclin to NPR3-IgGfC were recapitulated by introducing the same mutations in cWT (80–130; open circles), which corresponds to cWT-Mutant-1 (closed squares, 200-fold), cWT-Mutant-2 (open circles, 3-fold) and cWT-Mutant-3 (open squares, diminished); Fig. 4c).

Finally, because NPR3 is a clearance receptor for NPs, we investigated the effects of musclin on plasma endogenous ANP in C57BL/6J mice (Fig. 5). Maack *et al.* (1987) reported that constant infusion of C-ANP, a synthetic NPR3 ligand, increased plasma ANP by decreasing the clearance rate of

ANP (Almeida *et al.* 1989). As expected, constant infusion of recombinant full-length musclin (WT (30–130)) in mice significantly increased plasma ANP to a degree similar to that observed with C-ANP (Fig. 5). These results suggest that infused musclin competed for the binding of circulating ANP for NPR3 *in vivo* as well.

Discussion

The present study demonstrated several features of musclin binding to NPR3: 1) musclin selectively binds to NPR3 with high affinity among the NPR family; 2) musclin binds to NPR3 with higher affinity than ANP; 3) the first of two ANP-homologous regions in the cWT is important for binding to NPR3; 4) mammalian-expressed musclin shows higher affinity binding to NPR3 than *E. coli*-derived musclin; and, 5) systemic infusion of recombinant musclin increases plasma ANP concentrations, possibly via competing for the natriuretic clearance receptor NPR3.

We originally identified musclin as a novel secretory factor from skeletal muscle (Nishizawa *et al.* 2004). The present study demonstrated that musclin specifically and selectively binds to NPR3, among the NPR family. As described previously, the NP family member most homologous to musclin, ANP, binds to all of the NPR family members, NPR1, NPR2, and NPR3. Consistent with these findings, only the ANP/NPR3 binding was competed for by musclin, with similar strength to C-ANP and L-ANP, two ligands specific for NPR3. L-ANP is artificially linearized, resulting in no Cys–Cys loop structure used by ANP to bind NPR1. NPR3 itself has no guanylyl cyclase domain required for signal transduction, and was thus proposed to function as a clearance receptor for the NP molecules (Yasui *et al.* 2007). Musclin has no cysteine residues, suggesting that it could not form a disulfide-bonded intermolecular loop structure, as in ANP. It also has three dibasic putative cleavage sites. Collectively, these data suggest that musclin exists as a linear peptide, with potential only to bind NPR3.

The specific binding of musclin was similarly observed on stably NPR3-transfected BW5147 cells and transiently NPR3-transfected HEK293 cells, and not observed on NPR1- nor NPR2-transfected HEK293 cells. Furthermore, musclin inhibited labeled musclin binding more strongly than ANP, which has been considered to be the highest affinity ligand of NPR3 among NPs. Binding was observed using an IgGfC-fusion protein, independent of labeling methods. Musclin binding to NPR3 was inhibited by unlabeled recombinant full-length musclin produced in *E. coli* and by ANP, in NPR3 expressed on the cell surface or fused with IgG-Fc. The N-terminal Flag-WT expressed in mammalian cells inhibited musclin binding to NPR3 more strongly than *E. coli*-produced musclin and ANP. This strong inhibition of mammalian-expressed musclin binding was also observed on cells expressing NPR3 (data not shown). The musclin protein expressed in mammalian cells has a molecular size of 20 kDa,

Figure 4 Characterization of musclin binding to NPR3. (a) *E. coli*-derived full-length musclin (WT (30–130)), C-terminal half musclin (cWT (80–130)), mammalian-derived N-terminal FLAG-tagged full-length musclin (Flag-WT), ANP (atrial natriuretic peptide), and peptides corresponding to aa105–130, and 80–112 of musclin were incubated with N-terminal-biotinylated full-length musclin (BC-Musclin) and IgG-Fc fusion human NPR3 (hNPR3-IgGFc) in anti-IgG-Fc antibody pre-coated plates. The fluorescent intensity associated with bound BC-Musclin, which was developed with streptavidin- β -galactosidase were measured. The results are expressed as a percentage of the control (without non-labeled competitor), taken as 100%, and baseline (without IgG-Fc fusion human NPR3) taken as 0%. Data are mean $\pm$ s.d. ($n=3$). IC_{50} value with (95% confidence interval) by each peptide was as follows, WT (30–130); 0.14 nM (0.12–0.19 nM), cWT (80–130); 0.10 nM (0.088–0.12 nM), Flag-WT; 0.044 nM (0.035–0.056 nM), ANP; 0.11 nM (0.088–0.13 nM), and aa80–112; 0.39 nM (0.34–0.45 nM). (b) Full-length musclin (WT (30–130)), Mutant-1, Mutant-2, and Mutant-3 or (c) C-terminal half musclin (cWT (80–130)), cWT-Mutant-1, cWT-Mutant-2, and cWT-Mutant-3, which have corresponding mutations in C-terminal half musclin (cWT (80–130)), as described in detail in Fig. 1, were incubated with 0.1 ng/ml N-terminal-biotinylated full-length musclin (BC-Musclin) and 3 ng/ml IgG-Fc fusion mouse NPR3 (mNPR3-IgGFc). The fluorescent intensity associated with bound BC-Musclin was measured and the results are expressed as a percentage inhibition of the control (without non-labeled competitor), taken as 0% inhibition, and baseline (without mNPR3-IgGFc) taken as 100% inhibition. Data are average values of $n=2$. IC_{50} value with (95% confidence interval) by each peptide was as follows, WT (30–130); 0.20 nM (0.066–0.60 nM), Mutant-1; 15.2 nM (4.6–50 nM), Mutant-2; 0.95 nM (0.72–1.2 nM), cWT (80–130); 0.062 nM (0.033–0.12 nM), cWT-Mutant-1; 13 nM (7.7–20 nM), and cWT-Mutant-2; 0.19 nM (0.12–0.31 nM). Essentially same result was obtained in another independent experiment.

which is larger than the 11 kDa predicted from the amino acids sequence (Nishizawa *et al.* 2004). Taken together, these data indicate that the higher-order structure of musclin, including the sugar-chain modifications, is important for the binding to NPR3.

In the binding studies using NPR3-IgGFc fusion protein, the cWT mutant showed the same or more potent inhibitory activity than full-length protein. Peptides corresponding to

aa105–130 showed lower inhibitory activities than aa80–114, suggesting that the first of the two ANP-homologous regions in musclin has a more significant role in the binding to NPR3. In addition, we could confirm the relative importance of the first ANP-homologous region using mouse NPR3-IgGFc, which binds with higher affinity to mouse musclin. The two consecutive amino acid mutations of 89DG to 90RG in the first ANP-homologous region (Mutant-1)

Figure 5 Effects of infused musclin on plasma concentrations of ANP. Plasma ANP concentrations were measured in mice before (pre) and after (post) constant infusion of saline, C-ANP or recombinant full-length musclin (WT (30–130)) for 1 h, as described in Experimental Procedures. Results are mean $\pm$ S.E.M. ($n=8$). ** $P<0.01$, *** $P<0.001$ compared with pre-values.

caused a 100-fold reduction in the inhibitory potency compared with full-length musclin. By contrast, the amino acid change of 118DG in the second ANP-homologous region (Mutant-2) resulted in less than a 10-fold reduction in inhibitory activity. These results provide further evidence for the importance of the first ANP-homologous region of musclin in NPR3 receptor recognition. Additionally, the same amino acid mutations in the cWT mutant produced the same observations. Finally, both mutations in ANP homologous regions (Mutant-3) almost abolished inhibitory activity. The same amino acid mutations in cWT mutant led to similar result. These results suggest that the two regions act cooperatively to achieve the high affinity binding to NPR3.

During the course of this work, high affinity binding of musclin/osteocrin to NPR3 overexpressed in HEK293 cells was reported (Moffatt *et al.* 2007). The present study further demonstrates that such binding occurs in different cell lines and also by using purified IgG-Fc-NPR3. Although the previous work (Moffatt *et al.* 2007) concluded that the second ANP-homologous region was more important for binding to NPR3, we show here that the first ANP-homologous region is more significantly involved in the binding to NPR3, based on two independent approaches using four peptides encompassing distinct regions, as well as site-directed mutagenesis in full-length and cWT. We also show that the first and second NP-homologous regions cooperatively play a role in high affinity binding of musclin to NPR3.

We reported previously that musclin gene expression is transcriptionally regulated by nutritional change (Thomas

et al. 2003, Nishizawa *et al.* 2004), and that musclin represses glucose uptake and glycogen synthesis in skeletal muscle cells (Nishizawa *et al.* 2004). We also identified that musclin gene expression in skeletal muscle could be regulated by FOXO1, a key control switch between anabolism and catabolism in skeletal muscle (Thomas *et al.* 2003). However, other studies suggested that NPR3 gene expression is decreased markedly by fasting (Sarzani *et al.* 1995), and therefore, the tissue bioactivity of ANP might be promoted in fasting conditions. Increased ANP bioactivity related to NPR3 expression could be related to increased natriuresis, diuresis, hypotension, and lipolysis under low-caloric diets or fasting conditions (Sigler 1975, Sarzani *et al.* 1995, Dessi-Fulgheri *et al.* 1999, Sengenès *et al.* 2002). ANP also has the switching role in glycolysis from anaerobic to aerobic metabolism (Birkenfeld *et al.* 2005). NPR3 is expressed in various tissues including adipose, heart, lung, kidney, adrenal, and skeletal muscle (Sarzani *et al.* 1995, and unpublished data). The previous work (Moffatt *et al.* 2007), by using osteoblast-lineage specific transgenic mouse model, demonstrated that osteocrin/musclin augmented NP's function locally in bone by blocking the clearance action of NPR3. Although this transgenic mouse approach will not totally represent physiological tissue distributions, it is quite interesting that the phenotype of this mouse is similar to the NPR3 knock-out mice in the aspect of bone overgrowth, presumably due to the blockage of NPR3. The present study also suggests a possible role for musclin in regulating blood ANP concentrations. Although we have not yet successfully demonstrated the presence of mature peptide or processing fragments of musclin in plasma, the increased and decreased tendency of systolic blood pressure in musclin transgenic mice and null mice respectively, are observed in our preliminary experiments (data not shown). Although the detailed phenotype analysis of these genetically modified mice are needed, we observed the tendency of decreased glucose tolerance by infusing the recombinant musclin in normal C57BL/6J mice, in our preliminary experiments (data not shown). We assume therefore that musclin could play both a paracrine role in regulating the local bioactivity of ANP, by binding to NPR3 expressed in skeletal muscle, and possibly an endocrine role in regulating the systemic availability of ANP for other organs. The exact physiological significance of musclin in the regulation of systemic and local ANP bioactivities is now under investigation.

In conclusion, the present study demonstrated that musclin binds to NPR3 with high affinity and specificity via two intermolecular ANP-homologous regions acting cooperatively. In addition, musclin may regulate the bioactivity of ANP locally and/or systemically.

Declaration of interest

There is no conflict of interest for all the authors to the study.

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