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Isolation and detection of cell surface angiotensin II receptor using chemical crosslinking

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Objectives: Cell surface receptors of bioactive peptides act as high value targets for studying their downstream signaling pathways. Despite the number of approaches utilized for deciphering ligand-receptor interaction, capturing cell surface targets of low-molecular weight bioactive peptides still remain to be a challenge. We aimed to isolate angiotensin II type 1 (AT1) receptor using chemical crosslinking using its peptidic ligand.
Methods: The expression of AT1 receptor and changes in angiotensin II-induced intracellular free calcium ($[Ca^{2+}]_i$) level were examined in cultured human aortic smooth muscle cells (HAoSMCs). The binding of fluorescence-labeled angiotensin II (FAM-angiotensin II) to the cell surface AT1 receptor was confirmed using dual staining on confocal microscopy. Biotinylated angiotensin II was overlaid onto the HAoSMCs, and the crosslinked peptide-receptor complexes were isolated using streptavidin beads, which were further immunoblotted using HRP (horseradish peroxidase)-labeled streptavidin and anti-AT1 receptor antibodies.
Results: HAoSMCs expressed AT1 receptor mRNA and exhibited an increase in the $[Ca^{2+}]_i$ level in response to angiotensin II. FAM-angiotensin II overlaid onto the cultured cells colocalized with the immunoreactive AT1 receptor. Biotinylated angiotensin II crosslinked to the cell membrane co-migrated with the immunoreactive AT1 receptor on SDS (sodium dodecyl sulfate)-polyacrylamide gel.
Conclusion: Chemical crosslinking isolated and detected the angiotensin II-bound cell surface AT1 receptor.

Key words: angiotensin II, angiotensin II type 1 receptor, chemical crosslinking, confocal microscopy, human aortic smooth muscle cell

Introduction

Identification of cell surface receptors of biologically active peptides remains to be a challenge as only limited number of functional receptors has been discovered during the past two decades. Many new methodologies have been developed for the isolation, purification, and proteomic characterization of peptidic ligand-receptor interaction[1] and only few have been implemented successfully.[2] However, successful studies on the identification of specific cell surface receptors of low-molecular weight bioactive peptides are limited, which could be attributed to the difficulties associated with keeping the peptide-receptor complex intact during the extraction and purification of the complex, wherein the receptor proteins are embedded in the lipid rich regions of plasma membranes.[3,4]

Chemical crosslinkers can be used to stabilize the transient and labile protein-protein interactions, and have been used to unravel a variety of intracellular interactions of receptor proteins mediating the downstream signaling mechanisms.[1] Chemical crosslinking can help distinguish the binding partners of a specific protein from the non-specifically bound proteins that are usually co-purified with the extracted complexes,[5] and thus, have been implemented successfully in trapping some specific ligand-receptor complexes.[6-8] In this study, we aimed to develop an easy methodology to isolate cell surface receptors of low-molecular weight bioactive peptides and tested whether crosslinking the receptor-ligand binding

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could help in successful isolation of the cell surface receptor of a bioactive peptide. We used angiotensin II peptide as a ligand and human aortic smooth muscle cells (HAoSMCs) expressing angiotensin II type 1 (AT1) receptor.

Materials and Methods

Cell culture

HAoSMCs were purchased from PromoCell (Heidelberg, Germany) and cultured in the supplied medium supplemented with 5% fetal calf serum, 0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, and 5 μg/ml insulin.

Real-time RT-PCR

HAoSMCs were cultured on a 6-well plate, washed twice with phosphate-buffered saline (PBS), and dissolved in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for total RNA extraction. The total RNA was reverse transcribed into cDNA using the PrimeScript 1st-strand cDNA Synthesis Kit (Takara Bio, Shiga).[9] Human AT1 receptor was amplified using the synthetic oligomers as probes (forward 5'-TGCCATCCCAGAAAGTCGG-3' and reverse 5'-CAGCTTTGGGACAATCATCTTGG-3'; Eurofins Genomics, Tokyo), detected and quantified using the C1000 Touch Thermal Cycler and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), as described previously.[10] The amplification mix containing template cDNA and primer was prepared in KAPA SYBR FAST qPCR Master Mix (Kapa Bio Systems, Cape Town, South Africa). After heat denaturation of the template DNA at 95℃ for 3 minutes, the PCR amplification (95℃ for 10 seconds, 55℃ for 10 seconds, 72℃ for 30 seconds) was performed for a total of 40 cycles. To determine the size of the amplified products, real-time PCR products were mixed with Midori Green Direct DNA Stain (Nippon Genetics, Tokyo) according to the manufacturer's recommendation, and visualized using 2% agarose gel electrophoresis and LED transillumination.

Determination of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) level

Confluent HAoSMCs in non-coated 96 well black/clear bottom plates were deprived of serum for 16 hours, and then incubated in Hank's Balanced Salt Solution (HBSS) with Fluo-4 acetoxymethyl ester (Fluo-4 AM) (Dojindo Molecular Technologies, Kumamoto) at 37℃ for 30 minutes. Fluo-4 AM-loaded cells were washed three times with HBSS and incubated with 10^{-7} M synthetic angiotensin II peptide (Peptide Institute, Osaka). Cell fluorescence was measured at the indicated timepoints at the excitation wavelength of 485 nm and emission wavelength of 535 nm using the POWERSCAN HT Microplate Reader (BioTek Instruments, Winooski, VT, USA).[11,12]

Confocal microscopy

HAoSMCs plated on glass coverslips were deprived of serum for 16 hours before incubating with 10^{-6} M synthetic angiotensin II peptide labelled with 5-carboxyfluorescein at N-terminus (FAM-angiotensin II; AnaSpec, CA, USA) for 60 minutes. Cells were then washed thrice with PBS, blocked for 30 minutes with Blocking One (Nacalai Tesque, Kyoto), fixed with 4% paraformaldehyde for 15 minutes at room temperature, and incubated for 60 minutes with monoclonal anti-AT1 receptor antibody (1:1000; Abcam, Cambridge, UK), and further incubated for 30 minutes with Alexa Fluor 594 goat anti-rabbit IgG[H+L] (1:3000; Life Technologies, Carlsbad, CA, USA). The nuclei were counterstained using DAPI (4',6-diamidino-2-phenylindole) Fluoromount-G (Southern Biotech, Birmingham, AL, USA) and the fluorescence signals were captured using the LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany) as described previously.[2,12]

Chemical crosslinking and immunoblotting

HAoSMCs cultured in 10 cm dishes were deprived of serum for 16 hours, washed twice with PBS (pH 8.0), and incubated with 10^{-6} M biotinylated angiotensin II (AnaSpec, CA, USA) prepared in PBS at 4℃ for 1 hour. Cells were further incubated for 30 minutes at room temperature after the addition of 5 mM bis (sulfosuccinimidyl) suberate (BS3; Thermo Fisher Scientific, Waltham, MA, USA), which is a crosslinking reagent. After terminating the reaction upon addition of 20 mM Tris-HCL (pH 7.4) for 15 minutes at room temperature, cells were scraped to detach and centrifuged at 20,000 × g, 4℃ for 60 minutes. The pellet was dissolved in PBS, centrifuged again at 20,000 × g, 4℃, for 60 minutes, resuspended in 300 μl radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.6/150 mM NaCl/1% Nonidet P-40/0.1% sodium dodecyl sulfate [SDS]), mixed for 10 minutes, sonicated for 10 minutes, and after continuous mixing at 4℃ for 1 hour, centrifuged again at 20,000 × g, 4℃ for 15 minutes to collect the supernatant.

Biotinylated peptide-cell surface protein complexes represent in the solution were immobilized using the commercially available streptavidin-coated paramagnetic

beads (Dynabeads M280 Streptavidin; Invitrogen). About 15 μl of paramagnetic beads were pipetted into a microtube containing 1 mL PBS, vortexed, and placed onto the magnet for 1 minute before discarding the supernatant. The solution containing biotinylated peptide-cell surface protein complexes were added to the beads, mixed continuously for 30 min at room temperature, and placed onto the magnet to remove the supernatant. About 300 μl RIPA buffer was added to the beads in the tube, mixed through inverting 100 times, and placed onto the magnet to remove the supernatant. After repeating this washing procedures for 5 additional times, 300 μl of RIPA buffer was added and mixed at room temperature for 60 minutes. The solution was transferred to a new microtube and placed onto the magnet for 3 minutes to remove the supernatant. The biotinylated peptide-cell surface protein complexes adsorbed onto the magnetic beads were eluted upon addition of 60 μl 0.1% SDS and incubated for 5 minutes at 95℃.

Western blotting

About 10 μl of the eluted sample per lane was loaded on a 5%－20% gradient gel (DRC, Tokyo), electrophoresed at 200 V for 30 minutes using the SDS running buffer (DRC), and transferred at 25 V for 30 minutes onto the Immune-Blot polyvinylidene difluoride (PVDF) membrane (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad Laboratories) using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories) were used as the molecular size marker. PVDF membrane was blocked with Blocking One, washed three times with 2% bovine serum albumin (BSA)/tris buffered saline (TBS) containing 0.05% (w/v) Tween 20 (TBS-T), and reacted at room temperature for 1 hour with horseradish peroxidase (HRP)-labeled Streptavidin (1:10000; SeraCare, Milford, MA, USA) or at 4℃ overnight with monoclonal anti-AT1 receptor antibody (1:1000; Abcam, Cambridge, UK). Bands were detected using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK), photographed using the ImageQuant LAS 4000 (GE Healthcare), and analyzed using ImageJ software.

Results

Real-time RT-PCR and the subsequent agarose gel electrophoresis analyses demonstrated the expression level of the AT1 receptor mRNA in HAoSMCs (Figure 1). Addition of angiotensin II led to a significant increase in the level of $[Ca^{2+}]_i$ in HAoSMCs, and this effect was evident with as low as 10^{-7} M concentration (Figure 2). To confirm the binding of angiotensin II to the AT1 receptor present on the cell surface, HAoSMCs were incubated with FAM-angiotensin II and its co-localization with the immunoreactive AT1 receptor was examined. Confocal immunofluorescence microscopy revealed that the immunoreactive AT1 receptor colocalized remarkably with the fluorescence signals elicited by FAM-angiotensin II bound to the cell surface (Figure 3). Altogether, these results indicate the presence of functional AT1 receptor onto the cell surface of HAoSMCs.

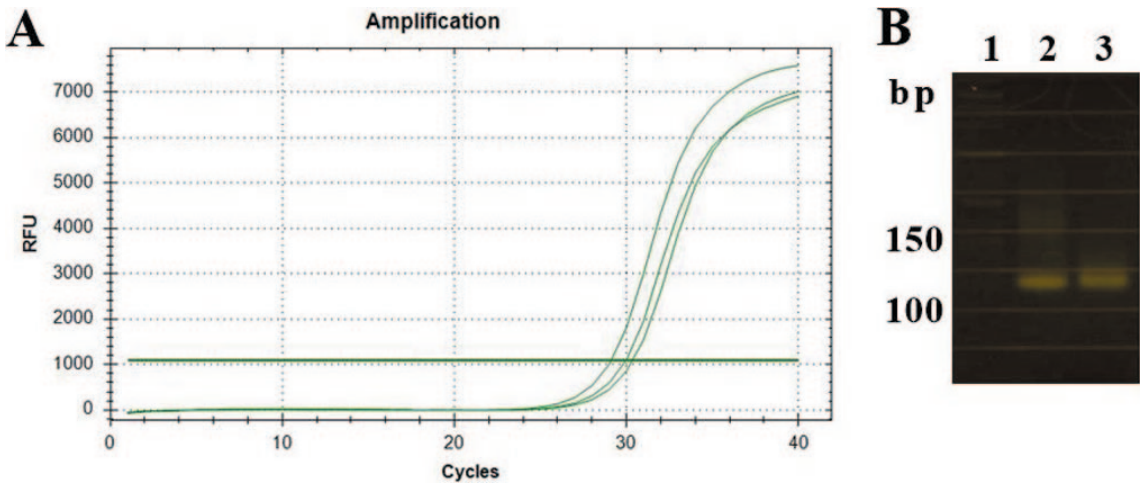

Figure 1. Real-time RT-PCR analysis to assess the expression of AT1 receptor. (**A**) Amplification curves generated through plotting RFU (relative fluorescence unit) vs. cycle number showing the accumulation of mRNA product over the duration of the reaction (n = 3). (**B**) Gel electrophoresis of RT-PCR amplicon of AT1 receptor. Visualization of amplicons generated through the real-time RT-PCR separated on a 2% agarose gel with stained with DNA fluorescent dye. Lane 1: 1 kb DNA ladder; lane 2: β-actin amplicon; lane 3: AT1 receptor amplicon.

To further investigate whether the intact AT1 receptor protein endogenously embedded into the cell membrane of HAoSMCs can be isolated and detected, we applied a chemical crosslinking reagent directly onto the cultured cells. Biotinylated angiotensin II was bound to the cultured HAoSMCs and a membrane impermeable crosslinker, BS3, was simply overlaid in the conditioned media. Cell membrane proteins bound to the biotinylated angiotensin II were then immunoprecipitated using the paramagnetic streptavidin beads, and extracted eluates were immunoblotted using HRP-labeled streptavidin and anti-AT1 receptor antibodies. Addition of the chemical crosslinker in the culture media produced a band when the biotinylated angiotensin II peptide was applied onto the HAoSMCs (Figure 4A, lane 1). This band was hardly visible in the control consisting of the same amount of membrane protein without the addition of angiotensin II (Figure 4A, lanes 2 and 3) or use of the crosslinker (Figure

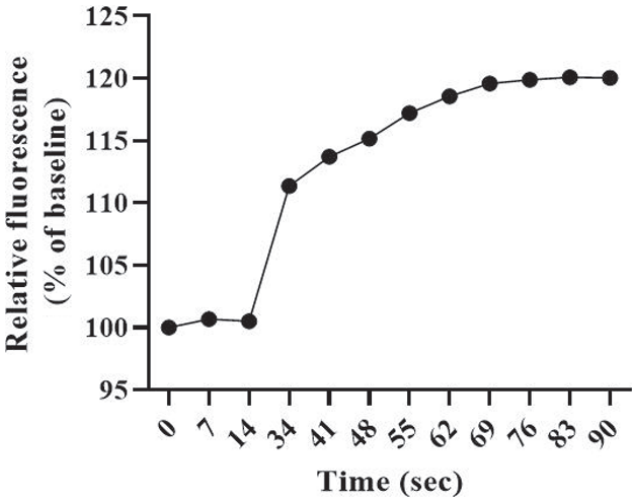

Figure 2. Stimulation of $[Ca^{2+}]_i$ upon addition of synthetic angiotensin II peptide. HAoSMCs loaded with Fluo-4/AM were stimulated with 10^{-7} M angiotensin II and the fluorescence intensities were monitored. Data points represent the mean value of triplicate experiments.

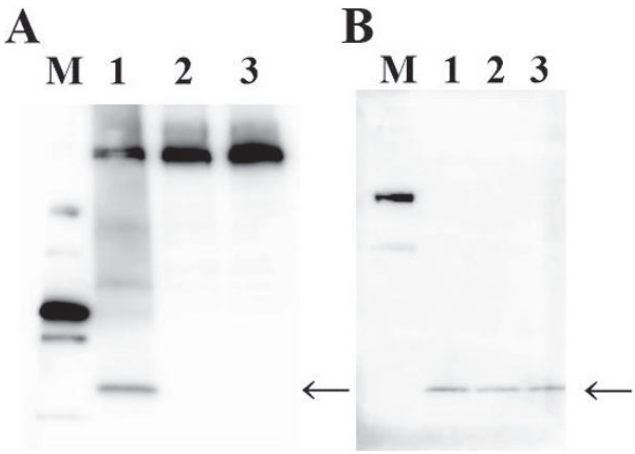

Figure 4. Detection of interaction between angiotensin II and AT1 receptor. HAoSMCs, deprived of serum for 16 hours, were incubated with (lane 1) or without (lanes 2 and 3) biotinylated angiotensin II (10^{-6} M) for 1 hour, overlaid with (lanes 1 and 2) or without (lane 3) the crosslinking reagent, BS3, for 30 minutes, and then, the extracted proteins from the scraped cells were isolated using the paramagnetic streptavidin beads. Eluates were then loaded on the SDS-polyacrylamide gel, which were electrophoresed and immunoblotted using (**A**) HRP-labeled streptavidin or (**B**) monoclonal anti-AT1 antibody. Arrows indicate the position of immunoreactive positive bands. Lane M corresponds to the Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories).

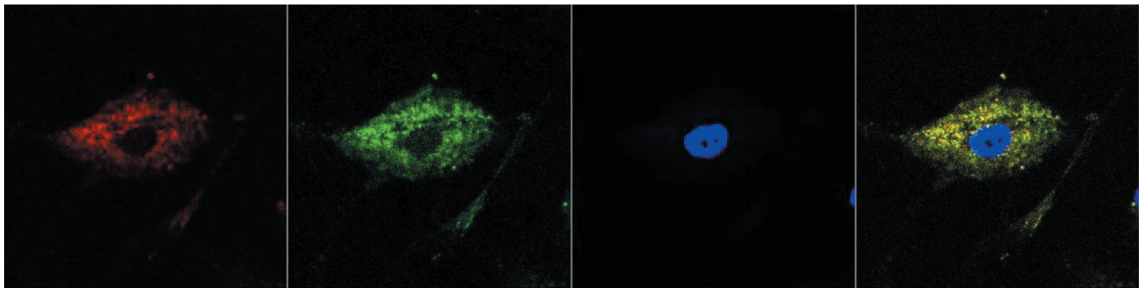

Figure 3. Confocal laser-scanning microscopy images of fluorescent angiotensin II overlaid onto the cultured HAoSMCs and immunofluorescence staining of the AT1 receptor. FAM-angiotensin II (10^{-6} M) was overlaid for 60 min onto the HAoSMCs, which were fixed and stained with specific antibody against the AT1 receptor (1:1000). The green signals correspond to the FAM-angiotensin II peptide present on the cell surface and the red signals represent the immunoreactive AT1 receptor, labelled using the Alexa Fluor 594 goat anti-rabbit IgG [H+L] (1:3000). The nuclei were counterstained with DAPI. Overlay resulted in yellow signals, which indicates co-localization.

4A, lane 3). We next performed western blot analysis using the same eluates, and the membranes were blotted using anti-AT1 receptor antibody. Immunoreactive AT1 receptor was detected at the migration position using HRP-streptavidin (Figure 4B). Signal intensity was greater in eluates obtained using the crosslinker and peptide ligand than it was in those in the negative controls.

Discussion

In the present study, we demonstrated that a low-molecular weight bioactive peptide, angiotensin II, chemically crosslinked to its cell surface receptor in growing cells can be isolated and detected after stringent solubilization and purification procedures. AT1 receptor is a G-protein coupled receptor (GPCR), which is embedded within the plasma membrane comprising abundant proteins and lipids. We directly applied membrane impermeable chemical crosslinker, BS3, onto the culture media, after which the biotinylated angiotensin II peptide was bound to the receptors present on the cell surface of HAoSMCs. Prior to the experiment for assessing the ability of BS3 to crosslink and isolate the AT1 receptor, we confirmed the expression of the AT1 receptor in HAoSMCs using RT-PCR analysis and the typical intracellular signaling downstream to the AT1 receptor through monitoring the response of $[Ca^{2+}]_i$ to angiotensin II. Biotinylated angiotensin II crosslinked to the cell surface proteins was immunoprecipitated using the paramagnetic streptavidin beads, and after efficient solubilization with stringent chemical detergent, RIPA buffer, to avoid protein degradation and interference with protein immunoreactivity, the comigration of immunoreactive AT1 receptor and its biotinylated peptidic ligand was demonstrated through performing western blot analysis. The results demonstrated that the chemical crosslinker, BS3, helped to isolate and detect the seven-transmembrane receptor, AT1 receptor, using its low-molecular weight peptidic ligand.

The cell membrane permeable chemical crosslinker, formaldehyde, has been used in a previous study to elucidate the receptor interactome that enabled us to unravel the dynamic changes in protein-protein association with receptor interaction at various stages.[13] Another membrane permeable chemical crosslinker, dithiobis (succinimidyl propionate) (DSP), has been used to crosslink the receptors and their associated proteins that interact with each other during the process of receptor internalization.[14-18]

Conversely, the cell membrane impermeable chemical crosslinker, BS3, has often been used to crosslink the receptors and their interacting proteins either directly[19] or after solubilizing the cell membrane proteins.[20] In the present study, we conducted preliminary experiments using DSP to determine whether it can be used to retrieve the intact cell surface targets using biotinylated peptidic ligands, but the results were discouraging. However, the current results demonstrated that the addition of the membrane impermeable and irreversible crosslinker, BS3, is an effective method for analyzing cell surface receptors and their peptidic counterparts.

Identification of cellular targets of many endogenous bioactive peptides is crucial, while the pathophysiology of many GPCRs remains elusive as their endogenous ligands are yet to be discovered. Many of these 'orphan' GPCRs were characterized using the reverse pharmacology approach,[21,22] but the pairing of GPCRs with their specific endogenous agonists was far less successful in the recent years, especially in the past 2 decades, and thus, more than 100 GPCRs remain uncharacterized,[23] thereby increasing the demand for the development of more efficient ligand/receptor screening strategies. GPCRs are transmembrane proteins that can be activated through extracellular stimuli, and they trigger intracellular signaling that mediate a variety of physiological responses. Therefore, GPCRs are regularly exploited as drug discovery targets.[24,25]

We recently made an advancement in identifying a large number of low-molecular weight native peptides from the human peripheral circulation, and using this native peptide library, we successfully identified three novel endogenous peptides with potent biological activities.[10] Because the results of our peptidomics approach would accelerate the identification of yet unknown bioactive peptides, we designed the present study in an attempt to facilitate the discovery of cell surface targets of endogenous peptides whose receptors are not known yet. These results mark a significant step forward in obtaining more suitable samples needed for conducting mass spectrometry analysis to identify the cell surface target proteins of bioactive factors.

In conclusion, chemical crosslinking of biotinylated angiotensin II bound to the intact surface of cultured cells assisted in successful isolation and detection of the cell surface AT1 receptor. This simple and straightforward methodology can be applied to screen the cell surface target proteins of bioactive peptides.

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Conflicts of Interest: None

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