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Nanotechnology and Membrane Receptors: Focused on Angiotensin II Receptors

Guangyong Li and Ning Xi

Department of Electrical and Computer Engineering Michigan State University East Lansing, MI 48824 USA

Donna H. Wang^{*}

Department of Medicine Michigan State University East Lansing, MI 48824, USA

Introduction

Cardiovascular as well as other actions of angiotensin II (Ang II) are mediated by type 1 (AT1) and type 2 (AT2) Ang II receptors, which are seven transmembrane glycoproteins with 30% sequence similarity. Most of the known effects of Ang II, e.g., vasoconstriction, stimulation of aldosterone and vasopressin synthesis/release, promotion of salt and water retention, activation of the sympathetic nervous system, and stimulation of cell proliferation/migration and extracellular matrix formation are mediated by activation of the AT1 receptors expressed in cardiovascular, renal, neuronal, endocrine, and other target cells. The AT1 receptor belongs to the G protein-coupled receptor (GPCR) superfamily and is primarily coupled through pertussis toxin-insensitive G proteins to the activation of phospholipase C and calcium signaling. In addition to AT1, the AT2 receptor also contributes to the regulation of blood pressure and renal function. The function of the AT2 receptor has become unraveled over the last few years owing to various sophisticated approaches including gene transfection and deletion. Accumulating evidence suggests that the AT2 receptor counterbalances the effect of the AT1 receptor in vitro as well as in vivo, e.g., it exerts a hypotensive effect—although this function is still controversial. The AT2 receptor is expressed at high density during fetal development but is much less abundant in adult tissues. The re-expression of the AT2 receptor in various disease states suggests a role of this receptor contributing to the development and maintenance of these disease states, albeit its precise action and signaling pathways remain to be elucidated (De Gasparo et al, 2000).

A new Ang II receptor subtype, AT4, has recently been cloned and characterized that preferentially binds Ang II (3–8), a fragment of Ang II now referred to as Ang IV. This receptor site is prominent among brain structures concerned with cognitive processing and motor and sensory functions. Specifically, high densities of AT4 sites have been localized in neocortex, piriform cortex, hippocampus, amygdala, and nucleus basalis of Meynert. The primary functions thus far associated with this Ang IV/AT4 system include memory acquisition and recall, regulation of tissue blood flow, and inhibition of renal tubular sodium reabsorption and cardiac hypertrophy, which appears to oppose the effect of the AT1 receptor. The identification of additional functions awaits further elucidation of this new receptor system.

^{*}Corresponding author: B316 Clinical Center, Department of Medicine, Michigan State University, East Lansing, MI 48824. Dr. DH Wang is an Established Investigator of the American Heart Association. Email Address: Donna.wang@ht.msu.edu, 517-432-0797 (P), 517-432-1326 (F).

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Although the Ang II receptors, especially the AT1 receptor, have been extensively studied for the last two decades, our knowledge of these receptors is still evolving. Further pharmacological and molecular studies will allow for a better and more complete understanding of the role of the renin-angiotensin system in the pathogenesis and treatment of diseases. Understanding the location, structure, and molecular dynamics of these receptors is of fundamental importance to elucidate their function. To gain insights into how these biomolecules operate, advanced technologies are required for gaining information at the level of single molecules. The recent development of atomic force microscopy (AFM) (Binnig et al, 1986) opens a new way for studying the functionalities of cell membrane proteins *in situ* at the single molecule level.

The atomic force microscope was developed initially as an instrument mainly used for surface science research. Research efforts in the past few years have indicated that AFM is also a potentially powerful tool for biochemical and biological research (Henderson, 1994). This rapid expansion of AFM studies in biology/biotechnology results from the fact that AFM techniques offer several unique advantages. First, they require little sample preparation, with native biomolecules usually being imaged directly. Second, they are less destructive than other techniques (e.g. electron microscopy) commonly employed in biology. And third, they can operate in several environments, including air, vacuum, and liquid and even when the cells are still alive (You et al, 1996). Recent progress in the spatial resolution of AFM technology has made topographical image of single protein a routine work (Baker et al, 2000, Fotiadis, 2002). The unique capability of the AFM to directly observe single proteins in their native environments provides insights into the interaction of proteins that form functionality assemblies. However, it is still impossible to recognize specific proteins like receptors only from the topographical information. Because the interaction between ligands and receptors is highly specific and possesses a high degree of spatial and orientational specificity, the technique to functionalize an AFM tip with certain molecules has opened a promising way to recognize individual specific molecules. It has been proven that individual receptors can be recognized by an AFM tip functionalized with antibodies through a force mapping technique (Willemsen et al, 1998, Ludwig et al, 1997), directly from a phase image in tapping mode (Raab et al, 1999, Stroh et al, 2004). These results, however, were obtained by imaging processed samples putting on substrate surfaces. In practice, it is still very difficult to image single receptors in their original biological environments such as cell membranes due to the topographical interference and softness of the membrane. Fortunately, this problem has been solved by the use of recently developed lift-up interleaved scan tapping phase imaging mode (Li et al 2005).

In this section, a detailed process for investigation of AT1 by AFM with functionalized tip is introduced. The same technique can apply to other type of receptors without significant changes. Given that it is hard to obtain clear image at molecular level when imaging living cells by AFM, some perspective methods to improve the imaging resolution are also discussed.

The AT1 Receptor and Its Antibody

The amino acid sequences encoded by the open reading frame of AT1 cDNA and genomic DNA of all mammalian and rodent species consist of 359 amino acid residues (Murphy and et al, 1991). Structural predictions suggest an extracellular NH₂-terminus followed by seven helical transmembrane-spanning domains, which are connected by three extracellular and three intracellular loops, linked to the carboxyl-terminus. Three consensus sites for N-glycosylation can be found in Asn4 at the NH₂-terminus and in Asn176 and Asn188 within the second extracellular loop.

AT1 antibody (Santa Cruz Biotechnology, Inc. CA) is an affinity purified rabbit polyclonal antibody raised against a peptide mapping near the amino terminus of the AT1 receptor of human origin (identical to corresponding rat sequence). It was chosen as the functionalization agent because it binds to the amino terminus of the AT1 receptor which is outside of the cell membrane. After functionalized the AFM tip with this antibody, the antibody would bind to the AT1 receptor during AFM image scanning.

Tip Functionalization and Surface Modification

By immobilizing samples on a very flat surface such as mica, individual bio-molecules can be observed by AFM. For example, the major intrinsic proteins have been immobilized on a freshly cleaved mica surface by incubating with carboxypeptidase at room temperature overnight (Fotiadis et al, 2002). The mica surface was silanized in a solution of 2% 3-aminopropyl-trithoxysilane in toluene for 2 hours (Ros et al, 1999) or by exposing it to the vapor of 3-aminopropyl-trithoxysilane for several minutes (Baker et al, 2000).

Functionalization of AFM tips by chemically and biologically coating with molecules, e.g. biotin-avidin pairs (Florin et al, 1994, Lee et al, 1994) and antigen-antibody pairs (Hinterdofer et al, 1996, Ros et al, 1998), has opened a new research area for studying interactions of molecules at the molecular level. Chemical coating of probes is mainly done by silanization or by functionalized with thiols and is often the first step before biological functionalization. Many protocols have been used for attaching proteins to an AFM tip. There are two main ways to functionalize the AFM tip with antibodies. One is to directly coat the antibody on a silanized tip, and the other is to tether the antibody on a tip by a linker. The direct coating method is simple and results in high lateral resolution. The tethering method involves much more complicated steps, but it gives better antigen recognition given that the interaction between antibody and antigen is highly specific which involves a high degree of spatial and orientational specificity. The drawback of the tethering method is that the lateral resolution is low. The detailed steps of these two methods are discussed below.

Several direct coating methods are available, and most of them have been based on silanizing a solid surface. Here is an example that has been used by us for directly attaching antibodies to an AFM tip. The silicon nitride tips were treated with 10% nitric acid solution which was left in a silicone bath for 20 minutes at 80 °C. This causes the formation of surface hydroxyl groups on the SiN tips. The tips were then thoroughly rinsed with distilled water, placed into 2% APMDEOS solution in toluene, and kept in a desiccator purged with argon gas for 5 hours. This treatment provides reactive primary amine groups on the nitride surface. The tips were washed thoroughly with PBS and placed into a solution of 2 µg/ml Anti-AT1 IgG for 10 minutes. The antibody-conjugated tips were then washed thoroughly with PBS and distilled water to remove loosely attached antibodies. These tips should be used immediately before being dried.

Functionalization of the AFM tip with antibody using the tethering method involves much more steps than the direct coating method. It usually needs a spacer to covalently bind the protein in order to orient the protein to expose specific site(s) of the protein. Polyethyleneglycol (PEG) is a common spacer to be used. A terminal thiol group can be first attached to PEG, which then will be attached to a gold-coated silicon nitride tip. An amine group at the other end of the PEG molecule attaches proteins (antibodies for example) via a covalent bond (Hinterdofer et al, 1996).

Tip functionalization with antibody can be completed using the following steps: The first step is to modify the antibody with N-Succinimidyl 3-(Acetylthio) propionate (SATP); the second step is to modify AFM tips with APTES (aminopropyltriethoxysilane); the third step is to tether the cross-linkers (spacer) on tips; and the final step is to link the SATP-Labeled antibody to

tips. The detailed protocol can be found in (Stroh et al, 2004). After tip functionalization, the tips can be stored in PBS buffer at 4°C for two weeks.

Single Molecules Recognition Using Functionalized Tip

Although high-resolution images can provide some detailed conformal information of molecules, they may not provide information related to any specific protein. Because the interaction between ligands and receptors is highly specific and possesses a high degree of spatial and orientational specificity, the technique to functionalize an AFM tip with specific molecules make investigation of single specific molecule possible. Rupture forces representing biomolecular specific interactions can also be exploited as a contrast parameter to create images in which the individual biomolecules can be recognized. It has been proven that single receptors can be recognized by an AFM tip functionalized with antibody through a force mapping technique (Willemsen et al, 1998, Ludwig et al, 1997). In this study, the functionalized tip was raster-scanned over the surface while a force-distance curve was generated for every pixel. From the force distance curve, the surface parameters such as stiffness and adhesion force was extracted either by online in real time or by off-line analysis. The individual receptors were recognized through these so called adhesion mode AFM. However, the adhesion AFM image obtained by this method has low lateral resolution and the work is extremely time-consuming.

Another way to recognize specific proteins like receptors is by the use of tapping-mode phase imaging. It can differentiate between areas with different properties regardless of their topographical nature (Cleveland et al, 1998, Tamayo and Garcia, 1998). The phase angle is defined as the phase lag of the cantilever oscillation relative to the signal sent to the piezo driving the cantilever. Theoretical simulations and experiments of the cantilever dynamics in air have shown that phase contrast arises from differences in the energy dissipation between the tip and the sample. The phase shift is related analytically to the energy dissipated in the tip sample interaction by following equation (Cleveland et al, 1998, Tamayo and Garcia, 1998)

$$\sin \psi = \left(\frac{\omega}{\omega_0} \frac{A}{A_0} \right) + \frac{QE_D}{\pi k A A_0}$$

where ψ is the phase angle; ω/ω_0 is the working frequency/resonance frequency; A/A_0 is the set-point amplitude/free amplitude; Q is the quality factor; E_D is the energy dissipation; and k is the cantilever spring constant. The phase shift due to the tip-sample interaction, which involves energy dissipation, is the displacement of the non-contact solution to higher phase shifts and the intermittent contact solution to lower phase shift values. The more dissipative features will appear lighter in the non-contact regime, whereas they will appear darker in the intermittent-contact regime (James et al, 2001).

When scanning the proteins immobilized on mica surface using a tip functionalized with its antibody, the tip-sample interaction force will increase as the tip approaching to the AT1 receptor, thus a significant change of the phase shift will be generated. Since the topographical information is also convoluted to the phase contrast image but with low frequency, a band-pass filter can be used to remove the low frequency topographical information and the high frequency noise. After filtering the phase contrast image, only the receptors' image will be left on the surface. Individual surface receptor has been identified using these techniques (Raab et al, 1999, Stroh et al, 2004).

In Situ Probing AT1 Receptor

Although individual proteins can be recognized efficiently through the tapping-mode phase imaging by a functionalized tip, biomolecules have to be extracted, purified, and attached to a flat and rigid surface using the methods described above. Detecting directly membrane proteins

in their native environments is daunting given the cell membrane surface topographical information will interfere with the antigen-antibody binding which “buries” the signal and make the recognition impossible. Fortunately, these problems can be solved by an interleaved lift-up scanning recently developed to remove the interference from topography. By scanning the same line twice, the topographical image can be obtained from the first scan, and the phase image can be obtained from the second lift-up scan. By choosing a proper lift up height of the AFM tip, the second scan will only contain the force information of the antigen and antibody interaction. After combining the phase image with the topographical image, the individual receptors can be located and labeled on the membrane surface. Using this technique, the AT1 receptor has been successfully recognized from a fixed neuronal cell membrane surface (Li et al, 2005). In this study, an AFM tip was biologically functionalized with the AT1 antibody by a tethering method as discussed previously. A phase contrast image of the neuronal cell membrane was obtained using the functionalized tip and then processed with a band-pass filter (Li et al, 2005). The experimental result shows that single biomolecule such as the AT1 receptor expressed in neuronal cell membrane can be recognized using the biologically functionalized tip through a lift-up interleaved tapping phase imaging mode AFM.

Perspectives and Conclusions

Although high-resolution images can provide some detailed conformational information of molecules, it is essential to recognize and characterize individual molecules such as receptors embedded in cell membranes in living cells. However, studies of living cells using AFM with high resolution have been hampered by cell deformation and tip contamination (Putman et al, 1994). Different approaches have been used to obtain high-resolution images of soft biological materials. At low temperature, cells stiffen and high-resolution imaging become feasible (Prater et al, 1991). Cells also become stiff after chemical fixation (Butt et al, 1990). These circumstances, however, can hardly be called physiological. To reduce cell surface deformation and cantilever contamination in “authentic” physiological conditions, the applied cantilever loading forces needs to be much smaller than what is currently available commercially, which remains a technical challenge. Another solution is to use TMAFM (tapping mode AFM) in liquid, which gives a substantial improvement in imaging quality and stability over standard contact mode. Because of viscoelastic properties of the plasma membrane, the cell may harden when responding to externally applied high frequency vibration and hence is less susceptible to deformation. Moreover, with this imaging mode, the cantilever oscillates at its resonant frequency and is only in intermittent contact with the cell surface. As a result, the destructive shear force is minimized.

In conclusion, the technique using a functionalized tip to measure the interaction force between ligands and receptors by AFM has been discussed for more than a decade, and single-molecule recognition using a functionalized tip from processed samples is achievable now. However, techniques for detecting and characterizing specific individual molecules from a living cell are still developing. Although barriers exist, they should be overcome in the very near future given the rapid advancement of nanotechnology. Once we have reached such breakthrough, further studies of membrane receptor functionalities including trafficking, signaling, and cross-talk would be possible. Given that many diseases have their roots at the molecular scale and are best understood as a malfunctioning biological nanomachines, the prospects of these unique techniques in basic biomedical research or in clinical practice are only limited to our imagination.

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