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Oxidative stress increases angiotensin receptor type I responsiveness by increasing receptor degree of aggregation using image correlation spectroscopy

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ABSTRACT

Oxidative stress and hyper-functioning of angiotensin II receptor type I (AT_1R) are commonly observed in hypertensive patients but the relationship between oxidative stress and AT_1R function is still unclear. We investigated the effects of H_2O_2 treatment on AT_1R-mediated intracellular calcium $[Ca^{2+}]_i$ signaling and its cell surface distribution pattern in HEK cells stably expressing EGFP-tagged rat AT_1R using image correlation spectroscopy (ICS). Following H_2O_2 treatment (50–800 μM), $[Ca^{2+}]_i$ was significantly increased upon angiotensin II stimulation. Similarly ICS revealed a significant increase in degree of AT_1R aggregation in H_2O_2 treated group during Ang II activation but no difference in cluster density compared with untreated control cells or those with *N*-acetyl cysteine pretreatment. Thus, oxidative stress-induced AT_1R hyper-responsiveness can be attributed by an increase in cell surface receptor aggregation state, possibly stemming in part from oxidant-related increase receptor–receptor interactions.

1. Introduction

Angiotensin II receptor is a G-protein coupled receptor (GPCR) for vasoactive peptide angiotensin II (Ang II) in the renin-angiotensin system (RAS), which plays a crucial role in the control of body water-electrolyte balance and in blood pressure regulation [1]. Two subtypes of Ang II receptor have been characterized, namely, angiotensin II receptor type I (AT_1R) and type II (AT_2R), with the former playing a predominant function in mediating various physiological functions [2]. Human [3] and rat [4,5] AT_1R have been cloned, and based on homology comparison, AT_1R belongs to family A of GPCR superfamily [2]. Upon stimulation by Ang II, $G_{q/11}$ - phospholipase C-inositol triphosphate (IP3) pathway is activated leading to an increase in intracellular calcium level. Subsequently, downstream proteins are activated generating a variety of cellular responses, such as vasoconstriction, increased renal Na^+ reabsorption and aldosterone secretion, all of which ultimately control body water balance and blood pressure [6].

Abnormality of AT_1R signaling can lead to the development of pathological conditions, especially those involving cardiovascular diseases, such as hypertension and atherosclerosis [7]. Hyper-function of AT_1R is commonly observed in hypertension, and thus, AT_1R modulators/antagonists are of potential utility for the treatment of hypertension. Selective AT_1R antagonists, such as losartan, valsartan and candesartan, are in current use as the drugs for hypertensive patient [8]. However, the mechanism by which AT_1R signaling contributes to hypertension remains unclear. Reactive oxygen species (ROS), the product from oxidative stress, was found to play crucial roles in pathological conditions of cardiovascular system [9,10]. Recently, Banday et al. [11,12] have shown that AT_1R content was upregulated at both mRNA and protein levels in oxidant (L-buthionine sulfoximine)-treated rat, resulting in abnormally high blood pressure. Both AT_1R upregulation and hypertension in oxidant-treated rat were abolished by antioxidant, tempol, administration. This evidence clearly shows an interplay between oxidative stress and AT_1R signaling in the development of hypertension. Oxidative stress has also been shown to affect the distribution pattern of mature AT_1R on cell membrane [13]. H_2O_2 induces AT_1R dimer formation, based on Western blotting analysis, in human embryonic kidney (HEK) cells stably expressing AT_1R [13]. Moreover, in monocytes from hypertensive patients, AT_1R is present in a dimeric form, resulting in hyper-responsiveness of AT_1R to Ang II which contributes to the initial state of atherosclerosis [14]. However, the role of oxidative stress on AT_1R distribution pattern and function in intact cell membrane has not yet been studied.

A recent technique used to quantitatively analyze distribution patterns of membrane proteins, especially membrane receptors, is image correlation spectroscopy (ICS) [15,16]. Although ICS has a

Abbreviations: AT_1R, angiotensin II receptor type I; AT_2R, angiotensin II receptor type II; Ang II, angiotensin II; BMP, bone morphogenetic protein; CD, cluster density; DA, degree of aggregation; EGFP, enhanced green fluorescent protein; GPCR, G - protein coupled receptor; HBSS, Hanks' balanced salt solution; HEK cell, human embryonic kidney cell; ICS, image correlation spectroscopy; IP3, inositol triphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; MTT, thiazolyl blue tetrazolium bromide; NAC, *N*-acetyl cysteine; NOX1, NADPH oxidase 1; RAS, renin angiotensin system; ROS, reactive oxygen species

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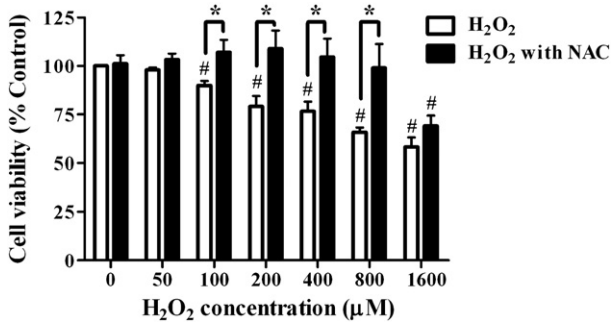

Fig. 1. Effect of H_2O_2 treatment on AT_1R-HEK cell viability. Confluent AT_1R–HEK cells were exposed to H_2O_2 at the indicated concentrations for 3 h and assayed for viability using MTT method. Controls included untreated cells and those prior treated with 4 mM *N*-acetyl cysteine (NAC) for 24 h. * $p<0.05$ compared with NAC-treated groups, and # $p<0.05$ compared with control group, n = 4–10.

limitation for examining biochemical interaction at single molecule level, it does allow examination of the distribution pattern of proteins in native membrane, in which protein conformation, protein–protein interaction and membrane lipids are not perturbed. Thus, ICS provides a good approach to investigate membrane receptor distribution patterns in native cells. For example, Nohe et al. [17] used this technique to demonstrate that bone morphogenetic protein (BMP) receptor rearrangement following its cognate ligand binding is essential for activation of BMP receptor and Smad pathway. Similarly, ligand-induced receptor clustering was also observed for epidermal growth factor [18] and platelet-derived growth factor [19] receptors in order to obtain effective trans-membrane signaling.

In this study, we investigated the effect of oxidative stress on cell-surface AT_1R distribution pattern using microscopic approach with image processing technique (ICS). In addition, the effect of H_2O_2 treatment on AT_1R-mediated intracellular calcium signaling was examined.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney cells (HEK293) stably expressing EGFP-tagged rat AT_1R (AT_1R–HEK cells) were kindly provided by Dr.Tamás Balla, National Institutes of Health, USA [20]. AT_1R used for transfection was $AT_{1a}R$ subtype. AT_1R–HEK cells were cultured in Dulbecco's modified Eagle medium (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), at 37 °C in a humidified atmosphere of 5% CO_2. The selection for maintaining EGFP-tagged AT_1R expression was performed every two weeks using medium containing 500 μg/ml Geniticin (Invitrogen).

2.2. Oxidant treatment and cell viability assay

AT_1R–HEK cells were seeded in 96-well plate at a density of 2×10^4 cell/well. Confluent cells were exposed to H_2O_2 (50 to 1600 μM) as oxidant treatment for 3 h at 37 °C in a humidified atmosphere of 5% CO_2. Pre-incubation overnight with 4 mM *N*-acetyl cysteine (NAC) alone was also included as antioxidant control. Then, cell viability was determined using MTT assay [21].

2.3. Intracellular calcium measurement

A fluorometric-based assay was used to determine intracellular calcium level. Confluent AT_1R–HEK cells were trypsinized and washed twice with Hanks' balanced salt solution (HBSS). Then, they were resuspended to obtain a density of 2×10^6 cell/mL and incubated with 6 μg/mL indo1-AM (Invitrogen) at 37 °C for 60 min. Subsequently, cells were washed twice with Ca^{2+} flux buffer (HBSS supplemented with 1% bovine serum albumin (Calbiochem) and 1 mM $CaCl_2$) and resuspended in Ca^{2+} flux buffer to a density of 1×10^6 cell/mL. Intracellular calcium level of un-stimulated baseline and upon Ang II stimulation was measured as a ratio of fluorescent emission (R) at 405 nm and 490 nm, excitation wavelength of 338 nm, using the following formula [22]:

$$\left[Ca^{2+}\right]_i = K_d\left[\frac{(R-R_{\min})}{(R_{\max}-R)}\right]\left[\frac{S_{f2}}{S_{b2}}\right] \quad (1)$$

where K_d is the dissociation constant of indo1-AM and (S_{f2}/S_{b2}) is the fluorescence ratio of emission wavelength at 490 nm in the absence of Ca^{2+} (3 mM of EGTA) to that at Ca^{2+} saturation (5 μM of ionomycin (Calbiochem)). Calcium level upon Ang II stimulation was obtained from the peak of fluorescent signal after Ang II administration.

2.4. Image correlation spectroscopy(ICS) and image processing

EGFP-tagged AT_1R-HEK cells were placed in cover glass bottom black dish (Electron Microscopy Sciences) at a density of 1.5×10^5 cell/plate and visualized using a 488 nm laser line under a 60× oil immersion objective. The image was captured with a 3× digital zoom at a pixel resolution of 0.1 μm/pixel (Olympus, FLUOVIEW® FV1000). Z–stack capture mode was applied at 0.5 μm height for each step. The final image was composed of 30–40 individual planar cross-section images, which were reconstructed to reveal surface receptor distribution. The images, which were captured before and after 1.0 nM Ang II treatment, were processed by ICS [15] to determine receptor distribution parameters,

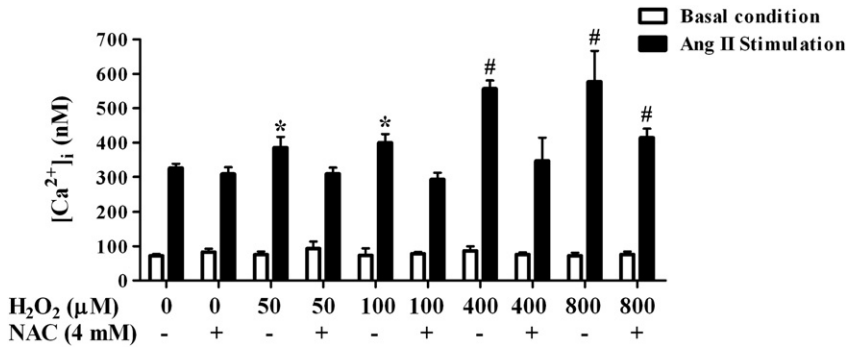

Fig. 2. Effect of H_2O_2 treatment on angiotensin signaling of AT_1R-HEK cells. Confluent AT_1R–HEK cells were exposed to H_2O_2 at the indicated concentrations for 3 h. The basal intracellular Ca^{2+}, $[Ca^{2+}]_i$, and $[Ca^{2+}]_i$ following Ang II stimulation were determined using a fluorometric method. * $p<0.05$ and # $p<0.01$ compared with control group (AngII treatment alone), n = 3–12.