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Oxidative stress effect on progesterone-induced blocking factor (PIBF) binding to PIBF-receptor in lymphocytes

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ABSTRACT

Receptor–ligand binding is an essential interaction for biological function. Oxidative stress can modify receptors and/or membrane lipid dynamics, thus altering cell physiological functions. The aim of this study is to analyze how oxidative stress may alter receptor–ligand binding and lipid domain distribution in the case of progesterone-induced blocking factor/progesterone-induced blocking factor-receptor. For membrane fluidity regionalization analysis of MEC-1 lymphocytes, two-photon microscopy was used in individual living cells. Lymphocytes were also double stained with AlexaFluor647/progesterone-induced blocking factor and Laurdan to evaluate -induced blocking factor/progesterone-induced blocking factor-receptor distribution in the different membrane domains, under oxidative stress. A new procedure has been developed which quantitatively analyzes the regionalization of a membrane receptor among the lipid domains of different fluidity in the plasma membrane. We have been able to establish a new tool which detects and evaluates lipid raft clustering from two-photon microscopy images of individual living cells. We show that binding of progesterone-induced blocking factor to progesterone-induced blocking factor-receptor causes a rigidification of plasma membrane which is related to an increase of lipid raft clustering. However, this clustering is inhibited under oxidative stress conditions. In conclusion, oxidative stress decreases membrane fluidity, impairs receptor–ligand binding and reduces lipid raft clustering.

1. Introduction

Progesterone-dependent immunomodulation is one of the mechanisms that enable pregnancy to proceed to term [1]. Immunologic effects of progesterone are mediated by a protein named progesterone-induced blocking factor (PIBF) which induces a Th2 cytokine production by T lymphocytes in vitro and in vivo and modulates peripheral NK activity. Though PIBF production is a characteristic feature of normal pregnancy, this molecule was recently found to be over-expressed in highly proliferating cells and malignant tumors independently from the progesterone receptor status [2,3].

PIBF binds to the glycosylphosphatidylinositol (GPI) anchored PIBF receptor (PIBF-R) which, upon ligand binding, combines with the alpha chain of the IL-4R for signaling. Therefore, engagement of the PIBF-R results in Jak1 phosphorylation which, in turn, activates STAT6 [1]. GPI-anchored proteins are enriched in the leukocyte membrane within glycosphingolipid-cholesterol rafts. These submicron domains need cholesterol for their function and, in line with this, depletion of cholesterol from the cell membrane abolished the STAT6 inducing effect of PIBF. In methyl-β-cyclodextrin (Mβ-CD) treated lymphocytes, neither PIBF nor IL-4 was able to Tyr-phosphorylate STAT6, suggesting that not only the PIBF-receptor but also the α chain of the IL-4 receptor might be raft-associated [4]. Lipid rafts are thought to be platforms within the plasma membrane which aid cell signaling and are rigid compared to other domains in the plasma membrane. These lipid rafts are enriched in sphingolipids and cholesterol and are associated to a subset of membrane proteins with an important role in signal transduction and membrane traffic [5–8].

Lipids within the plasma membrane are one of the preferential targets of reactive oxygen species (ROS) which cause lipid peroxidation. In particular, polyunsaturated phospholipids are an extremely vulnerable target, due to the susceptibility of their chains to lipid peroxidation [9,10]. Recent data have shown that oxidative stress, produced by ROS, can modify the receptors impairing their normal functions [11,12]. This process also disturbs the bilayer structure, modifies membrane properties such as membrane fluidity, alters the physiological functions of cell membranes

Abbreviations: DMSO, dimethylsulphoxide; FBS, fetal bovine serum; GP, generalized polarization; I_d, liquid disordered; I_o, liquid ordered; L_β, gel phase; PIBF, Progesterone-Induced Blocking Factor; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species.

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and contributes to cell membrane damage [9,10]. Altered membrane fluidity might also affect membrane protein function by modifying lipid microenvironment and interactions [13–16]. Several reports have described a decrease of membrane fluidity in different cell membranes as a consequence of lipid peroxidation [17–20]. The immune system is composed by different cell types that have different sensitivities to oxidative damage, depending on their effector functions [20–24].

Recent techniques are able to visualize different lipid domains within the plasma membranes of individual cells [8,25,26]. One of these techniques, known as two-photon microscopy, allows obtaining high resolution images of single cells, in vivo, which enables visualizing the distribution of membrane fluidity in different domains within the plasma membrane. The environment-sensitive probe Laurdan (6-dodecanoyl-2-dimethylamino-naphthalene) is used, which exhibits an emission spectral maximum shift from 440 nm to 490 nm in the transition from gel phase to liquid phase [27,28]. By using a multiphoton confocal microscope it is possible to excite the fluorophore with two photons at double the wavelength causing it to fluoresce [27–33].

Previously we have reported that oxidative stress reduces plasma membrane fluidity of THP-1 induced macrophages [20]. Membrane rigidification may cause an impaired cellular function. In lymphocytes, PIBF-R signaling is essential for pregnancy; an inefficient receptor signaling could impair implantation. The aim of the present study is to analyze lymphocyte membrane fluidity and PIBF/PIBF-R binding distribution in different membrane lipid domains of individual cells, under oxidative stress conditions.

2. Materials and methods

2.1. Cell line culture

MEC-1 lymphocytes were established and characterized from peripheral blood of a patient with B-chronic lymphocytic leukemia [34]. MEC-1 cells were cultured in RPMI medium 1640, 10% fetal bovine serum (FBS) with GlutaMax and without phenol red (Invitrogen). Cells were maintained at 37 °C in 5% CO_2. All experiments were performed within the cell passages 20–50.

2.2. Analysis of cell viability under oxidative stress conditions

We determined the highest H_2O_2 concentration allowing acceptable levels of viability. 200 μl of MEC-1 lymphocytes at 5×10^6 cells/ml was seeded in each well of 96 microwell plates for viability analysis. Cells were then centrifuged at 300 ×*g* for 5 min and washed three times with PBS. H_2O_2 in fresh medium without FBS was added to final concentrations ranging from 0.1 mM to 10 mM. After 4 h at 37 °C and 5% CO_2 cells were centrifuged, washed and viability was assessed by MTT assay (EZ4U Cell Proliferation Assay, Biomedica Gruppe, Wein, Austria). This assay measures the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to formazan (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) catalyzed by mitochondrial dehydrogenase in functional mitochondria. Results showed that the H_2O_2 concentrations below 5.0 mM for 4 h maintained cell viability above 80%.

2.3. Oxidative stress induction in MEC-1 cells for two-photon microscopy analysis

For oxidative stress induction, MEC-1 cells were centrifuged at 300 ×*g* for 5 min. Cell pellet was then resuspended in RPMI phenol and serum free medium at a final concentration of 10^6 cells/ml. Cells were then incubated during 2 h at 0.5, 1 or 2 mM H_2O_2 at 37 °C in a 5% CO_2 atmosphere. After oxidative induction, cells were washed three times with the same RPMI medium and thereafter cells were stained with Laurdan as described in section below.

2.4. Laurdan-staining of lymphocytes membranes

Lymphocytes were labeled with Laurdan (6-dodecanoyl-2-dimethylamino-naphthalene). Laurdan was dissolved to a concentration of 2 mM in dimethylsulfoxide (DMSO) and stored at −20 °C at the dark. For membrane labeling, cells were incubated in medium without FBS at a final concentration of 5 μM Laurdan for 30–60 min at 37 °C and 5% CO_2 with agitation. In the Laurdan-stained lymphocyte suspension the final concentration of DMSO was less than 0.25% (v/v). This cell suspension was used to evaluate membrane fluidity by two-photon microscopy.

2.5. Two-photon microscopy technique

Membrane fluidity distribution in stained lymphocytes was evaluated as previously described [20], with a multiphoton scanning confocal microscope Leica TCS-SP5 (Leica Microsystems, Heidelberg GmbH) at the Servei de Microscòpia (UAB). Images were obtained with a 63× oil immersion objective lens and a 1.4 numerical aperture, at a resolution of 512 × 512 pixels and at a scan speed of 400 Hz, by using LEICA LAS AF software. After fluorescence excitation with the multiphoton laser at 800 nm, the two-photon microscope captures two simultaneous emission images, with wavelengths ranging from 400–460 nm and 470–530 nm.

Emission intensities from every image pixel were introduced into the generalized polarization (GP) Eq. (1) providing a final GP value, which is a measure of membrane fluidity:

$$GP = \frac{I_{(400-460)} - G \times I_{(470-530)}}{I_{(400-460)} + G \times I_{(470-530)}} \quad (1)$$

where G is a correction factor for the microscope being used, which was calculated by using Eq. (2):

$$G = \frac{GP_{theo} + GP_{theo} \times GP_{exp} - 1 - GP_{exp}}{GP_{theo} \times GP_{exp} - GP_{theo} + GP_{exp} - 1} \quad (2)$$

where GP_{theo} is the GP theoretical value of a standard solution of 5 μM Laurdan in DMSO, which has a known value ($GP_{theo} = 0.207$), whereas GP_{exp} is the GP value of the same solution measured in our confocal microscope [35]. GP values range from −1, corresponding to the highest fluidity, to +1 for the lowest fluidity. GP images, therefore, show membrane fluidity distributions across the cell membrane providing an excellent tool for membrane dynamics visualization [20,36].

2.6. Image analysis

In order to obtain a GP value for each pixel, a WiT 8.3 imaging software (Dalsa Digital Imaging, Canada) was used and adapted for two-photon images. All calculations were carried out in floating point format and all images were first converted to 8-bit unsigned format [36]. Images were then processed with a custom made sub-program. Background values were set to zero: Eq. (1) denominator was converted to a binary image with background values set to zero and nonbackground values set to one, then the binary image was multiplied by the GP image [32]. Images were then pseudo colored by means of Adobe Photoshop 7.0. All GP images were corrected using the G factor previously obtained from a 5 μM Laurdan solution for each experiment [36]. GP distributions were obtained from the histograms of the GP images.

2.7. Detection of PIBF receptors in lymphocytes by conventional confocal microscopy

MEC-1 lymphocytes were first placed under oxidative stress as described above. After the 2 h incubation with hydrogen peroxide, cells were washed and incubated with soluble PIBF bound to AlexaFluor647 fluorochrome. Fifty microliters of PIBF-AlexaFluor647,