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In human T cells mifepristone antagonizes glucocorticoid non-genomic rapid responses in terms of Na^+/H^+-exchange 1 activity, but not ezrin/radixin/moesin phosphorylation

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ABSTRACT

Glucocorticoids (GCs) and progesterone have been employed as immunosuppressive agents during pregnancy for many years. Intracellular acidification by GCs is due to a rapid non-genomic inhibition of membrane Na^+/H^+-exchange 1 (NHE1) activity and is followed by immunosuppression of PHA-stimulated proliferation. NHE1 is tethered to the cortical actin cytoskeleton through ezrin/radixin/moesin (ERM) proteins within lipid rafts; these regulate cell shape, migration and resistance to apoptosis. We explored whether mifepristone (RU486), an antagonist of GCs in T cells, is able to completely block rapid non-genomic responses, namely NHE1 activity and the phosphorylation C-terminal residues of ERM proteins at threonine (cp-ERM).

GCs stimulate a rapid non-genomic cp-ERM response in cells within 5 min. RU486 antagonized the GC-induced rapid decrease in NHE1 activity, and arrested PHA-stimulated T cells at G0/G1 phase but had no effect on the rapid increase in cp-ERM, which persisted for 24 h. However, the cp-ERM response was blocked by staurosporine in both resting and GC stimulated cells. The results of RU486 antagonized the GC induced rapid decrease in NHE1 ion transport activity, but not the increase cp-ERM. This suggests that RU486 in T cells exerts its antagonistic effects at NHE1 containing plasma membrane sites and not where cp-ERM links lipid rafts to cortical cytoskeletons.

1. Introduction

Glucocorticoid hormones have already been known for many decades and have been shown to regulate multiple physiological processes in target cells. It is wide recognized that glucocorticoid hormones are able to pass through the cell membrane and then interact with intracellular receptors, which results in regulation of gene expression via various different mechanisms. However, it has also been shown that glucocorticoids are able to induce rapid non-genomic epigenetic effects [1,2]. These effects are happened within minutes, and are too fast to involve any sort of genomic effect. Similar non-genomic effects have been noted with other types of steroid hormones such as estradiol (E_2), and aldosterone [3,4].

Immune responses are repressed at an anti-inflammatory level during pregnancy among women. This is a result of the effects of pregnancy-associated hormones such as E_2, progesterone, and glucocorticoids [5]. In our previous studies, it was found that progesterone induces T cell non-genomic rapid acidification and this is not related to Na^+/H^+ exchange 1 (NHE1), but rather to an intracellular calcium increase [6]. In contrast, the acidification induced by dexamethasone or hydrocortisone is related to an inhibition of NHE1 activity in both T cells and dendritic cells [1,7]. In addition, glucocorticoids (GCs) do not stimulate intracellular calcium

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changes in T cells and as a result it would seem that the rapid acidification by GCs is not related to an increase in intracellular calcium, which is the case with progesterone [8].

NHE1 is widely distributed in cells throughout the body and is involved in the exchange of intracellular protons for extracellular Na^+ and in Na^+ influx, which is able to bring about an expansion in cell volume. Human NHE1 is an 815 amino acid glycoprotein and consisted of 12 transmembrane ion translocation domains [9–11]. NHE1 mediates an increase in cell volume to maintain intracellular osmolarity, control cell growth, control differentiation, control proliferation and affect intracellular alkalinization, which inhibits cell apoptosis [12–14]. Neither hydrocortisone nor dexamethasone alone are able to stimulate T-cell proliferation [8]; however, non-genomic inhibition at the plasma membrane of NHE1 activity by steroids, progesterone and GCs has been suggested to be one of the most immediate mechanisms triggered in T cells and this leads to a suppression of PHA-stimulated T-cell proliferation [1,15,16].

NHE1 is also able to interact with other complex molecules that acts as the anchors of the cytoskeleton, which in turn affects cell migration and shape remodeling [17,18]. The cytosolic tail domain of NHE1 acts as a scaffold that binds with ezrin/radixin/moesin (ERM) proteins and phosphatidylinositol 4,5-bisphosphate; this establishes a signaling complex that culminates in Akt activation and acts in opposition to apoptotic stress [19,20]. ERM is also able to associate with other membrane proteins, such as EGFR, PDGFR, and β2AR, and affect basal membrane polarization [21]. Thus, NHE1 is associated with multiple diseases including ischemic heart disease [22] and several kinds of cancers [23,24].

ERM proteins are highly homologous proteins that are able to link cargo molecules to the actin cytoskeleton. The activation of ERM requires phosphorylation of the C-terminal at threonine, which reduces affinity between the N-terminal and the C-terminal, thus "splitting-out" ERM as an active form. The phosphorylation activated ERM proteins are able to link cell surface proteins, including CD44, CD43 and other, with the intracellular cytoskeleton, especially the F-actin filaments [25,26]. Hence, the phosphorylation of ERM may result in various cytoskeleton rearrangements that affect cell shape, migration, and apoptosis. The present study is the first to explore whether phosphorylation of the C-terminal of ERM to form cp-ERM may be one of the non-genomic rapid responses in T cells brought about by GCs.

Progesterone is able to interact with its receptors on the endometrium in order to prepare for zygote implantation. Mifepristone (RU486) is an intracellular receptor antagonist of progesterone and glucocorticoids that brings about endometrial break down and an interruption of early pregnancy [27]. In addition to the above effect, mifepristone has been used as an alternative treatment for Cushing syndrome since 1985 [28,29]. Furthermore, mifepristone has been suggested as a palliative therapy for patients with a variety of advanced cancer types [30,31]. In our previous reports, both progesterone and glucocorticoids were shown to be able to inhibit NHE1 activity bring about progesterone related rapid responses such as intracellular calcium elevation and acidification, both of which are able to be antagonized by mifepristone [1,8,15]. When mifepristone is used at ≧10 μM, it is also able to inhibit phytohemagglutinin (PHA)-induced T cell proliferation [32], and antagonize the inhibitory effects of glucocorticoids on PHA-stimulated cell proliferation [33]. However, it remains unclear as to whether mifepristone in T cells is able to antagonize the glucocorticoid related rapid non-genomic inhibition of NHE1 activity and affect any rapid non-genomic response related to ERM phosphorylation by GCs that may occur. Therefore, the aims of this study were, firstly, to clarify the role of GCs (hydrocortisone and dexamethasone) on rapid changes in ERM phosphorylation and, secondly, to identify whether mifepristone can antagonize glucocorticoid effects in human T cells via the non-genomic rapid responses, including both NHE1 activity and ERM phosphorylation. The antagonistic effects of mifepristone on immunosuppressive effects were also investigated and this was carried by flow cytometry, which examined the changes in cell cycle distribution in T cells after PHA-stimulation by GCs.

2. Experimental

2.1. Chemicals

BCECF/AM, nigericin and valinomycin were purchased from Molecular Probes (Eugene, OR, USA). Goat anti-rabbit polyclonal antibodies conjugated to HRP was obtained from Jackson (Bar Harbor, Maine, USA). Phytohemagglutinin (PHA), RPMI 1640 medium (RPMI), Hank's balanced salt solution (HBSS) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY, USA). Genistein, staurosporine, hydrocortisone, dexamethasone, mifepristone (17 β-hydroxy-11β-(4-dimethylamino-phenol) 17α-(prop-1-ynyl)estra-4,9diene-3-one; RU486), bovine serum albumin (BSA), dimethylsulfoxide, ethanol, formaldehyde, propidium iodide, DNase-free RNase, and Histopaque-1077 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PHA was dissolved in distilled water. The culture media were supplemented with 10% FCS (v/v). All sera were pretreated with dextran-charcoal to remove small molecules including steroids and thyroid hormone [8].

2.2. T cell preparation

Heparinized peripheral blood samples were obtained from healthy male volunteers (age: 20–25 years old). Before blood collection, all volunteers gave written informed consent. T cells were isolated from these blood samples by the histopaque-1077 gradient-density method as previously described [34]. The results showed that the T cell suspension contained almost 100% CD3-positive cells.

2.3. Immunoblotting

Cell lysates were prepared by resuspending a cell pellet containing $5–10 \times 10^6$ cells in lysis buffer (50 mM of Tris–Cl, 150 mM of NaCl, 10% glycerol, 1.5 mM of $MgCl_2$, 1 mM of EDTA, 50 mM of NaF, 1% Triton X-100, 1 mM of Na_3VO_4, 1 mM of PMSF and 25 μg/ml of aprotinin), which was then followed by centrifugation at 12,000 rpm at 4 °C for 10 min. Protein concentration was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were subjected to 10% SDS–PAGE. The proteins were transferred onto nitrocellulose blotting membrane (Hoefer Scientific Instruments, San Francisco, CA, USA) and the membranes were probed with the following antibodies, rabbit anti-ERM and rabbit anti-phospho-ERM, phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558), (Cell signaling, Beverly, MA, USA). This was followed by treatment with goat anti-rabbit polyclonal antibodies conjugated to HRP. The immunoblots were visualized by ECL (PerkinElmer Life Sciences, Inc., Wellesley, MA, USA).

2.4. Measurement of the pH_i

T-cell suspensions (2×10^7 cells/ml) were incubated at 37 °C for 30 min with BCECF/AM (3 μM) in HBSS containing 5 mM glucose and 0.2% BSA; then the cells were washed three times with HBSS and resuspended in RPMI 1640 containing 10% FCS. For pH_i measurement, 1×10^6 cells were washed twice with HBSS, suspended in 2.5 ml of the same solution, transferred to a plastic cuvette at

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