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Protein kinase C-α and -δ are required for NADPH oxidase activation in WKYMVm-stimulated IMR90 human fibroblasts

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Abstract

The regulation of the activation of non phagocytic NADPH oxidase is poorly understood. Previously we demonstrated that in fibroblasts the exposure to WKYMVm induced $p47^{phox}$ phosphorylation and translocation and that these effects were mediated by ERKs activation. Protein kinase C (PKC) is reported to be involved in regulating the phosphorylation of NADPH oxidase components in polymorphonucleate cells stimulated via FPRL1 receptor, but its involvement in fibroblasts was not demonstrated. Therefore, we investigated in IMR90 cells exposed to WKYMVm the role of PKC isoenzymes in the activation of NADPH oxidase-like enzyme. Preincubation with general pharmacological inhibitors of PKC, before stimulation with WKYMVm, prevented the ERKs activation, $p47^{phox}$ phosphorylation and translocation. The analysis of cellular partitioning of PKC isoenzymes demonstrated that PKCα and PKCδ translocated from the cytosolic to the membrane fraction upon stimulation with WKYMVm. Preincubation with Gö6976 or with rottlerin prevented the phosphorylation and translocation of NADPH oxidase regulatory subunit.

Keywords: NADPH oxidase; Formyl-peptides receptor; WKYMVm; Protein kinase C; ERKs; $p47^{phox}$

Polymorphonucleate (PMN)[1] cells generate superoxide anion ($O_2^{-\cdot}$) through activation of NADPH oxidase when exposed to various stimuli [1,2]. This enzymatic complex catalyzes the one-electron reduction of oxygen that, coupled with the oxidation of NADPH, results in the production of $O_2^{-\cdot}$. Activation of NADPH oxidase involves the translocation of cytosolic subunits and their interaction with the membrane-associated flavocytochrome *b* subunit. Experimental evidence demonstrates that non phagocytic cells also express NADPH oxidase and several proteins homologous to the catalytic membrane subunit $gp91^{phox}$, as well as to the regulatory cytosolic subunit $p47^{phox}$, have been identified in different cell types and tissues [3–18]. Stimuli that activate the phagocytic NADPH oxidase cause extensive phosphorylation of $p47^{phox}$ [19–21] and this is considered the key event in NADPH oxidase activation. In fact, this regulatory subunit is phosphorylated on multiple serine residues that could be targeted by different kinases, thus explaining the tight control of NADPH oxidase.

The peptide *N*-formyl methionyl-leucyl-phenylalanine (N-fMLP) is the prototype activator of PMN chemotactic activity. In these cells the interaction of N-fMLP with its receptor triggers multiple second messengers that results in chemotaxis and in superoxide generation through NADPH oxidase activation. Two functional N-fMLP receptors, FPR and FPRL1, have been identified in human cells [22–25] which bind N-fMLP with high and low affinity,

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[1] *Abbreviations used:* PKC, protein kinase C; PMN, polymorphonucleate; N-fMLP, *N*-formyl methionyl-leucyl-phenylalanine; DAG, diacylglycerol.

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respectively. Even though the two receptors share signal transduction features, binding of formyl-peptides to FPR or FPRL1 activate signal transduction pathways prevalently responsible for chemotactic response or for superoxide generation, respectively [26].

Compared with PMN, much less is understood about the regulation of the activation of non phagocytic NADPH oxidase triggered with N-fMLP. Previously we demonstrated that IMR90 human fibroblasts express on their membrane the low affinity formyl-peptide receptor FPRL1 [27] which shows a higher binding efficiency for WKYMVm (W peptide), a modified peptide isolated by screening synthetic peptide libraries composed of random sequences of hexapeptides [28]. In serum-deprived cells, the exposure to WKYMVm induced both $p47^{phox}$ phosphorylation and translocation, as well as NADPH-dependent superoxide generation [27]. These effects were inhibited by pertussis toxin and were in large part mediated by ERKs, indicating that these kinases are involved in the phosphorylation cascade of $p47^{phox}$ [27].

In PMN cells also other kinase pathways are activated via FPRL1 receptor and involved in NADPH oxidase activation. In fact, phosphorylation of $p47^{phox}$ in intact neutrophils is inhibited by general kinase inhibitors that also inhibit protein kinase C (PKC) [21,29–31].

The involvement of PKCs in the regulation of NADPH oxidase in fibroblasts stimulated with WKYMVm was not demonstrated, as well as the specific PKCs, if any, regulating the phosphorylation and the translocation of $p47^{phox}$ are still unknown.

PKC isoforms can be subdivided among three groups. The conventional PKCs (α, βI, βII, γ) depend on calcium and are activated by diacylglycerol (DAG) or TPA; the novel PKCs (δ, ε, η, θ) are also activated by DAG and TPA but are calcium-independent; the atypical PKCs (ζ, λ) are calcium-independent and do not respond to DAG or TPA. All three groups of PKC isoenzymes participate in signal transduction [32,33] and accumulating evidences suggest that they play unique roles and induce different functional changes within cells.

The purpose of this study was to investigate the role of PKC isozymes in regulating the phosphorylation and the translocation of $p47^{phox}$ in serum-starved IMR90 cells stimulated by WKYMVm. Our results indicate that the calcium-dependent PKCα isoform and the rottlerin-sensitive PKCδ isoenzyme are required for nonphagocytic NADPH oxidase activity, given their involvement in the regulation of phosphorylation and translocation of $p47^{phox}$.

Materials and methods

Reagents and cell culture treatments

The WKYMVm peptide was synthesized and HPLC purified by PRIMM (Milan, Italy). SDS–PAGE reagents were from Bio-Rad (Richmond, CA, USA). Protein A/G plus-Agarose, anti-ERK2, anti-active phosphorylated ERK1/2, anti-PKCα, anti-PKCβI, anti-PKCβI I, anti-PKCδ, anti-PKCε, anti-PKCλ, anti-PKCζ and anti-$p47^{phox}$ antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Protein A–horseradish peroxidase, anti-mouse Ig–horseradish peroxidase were from Amersham Pharmacia Biotech. PD098059, GF109203X, Gö6983, Gö6976 and BAPTA-AM were purchased from Calbiochem (La Jolla, CA, USA). Anti-phospho serine antibody and rottlerin were obtained from Sigma (St. Louis, MO, USA). PepTag® assay for non-radioactive detection of Protein kinase C was from Promega (Madison, WI, USA).

Human fibroblasts IMR90 were purchased from ATCC (Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. They were grown until they reached 90% confluence and then starved in serum-free DMEM. After 48 h, cells were stimulated by adding WKYMVm to final concentrations indicated in the figures for 5 min. In other experiments, starved cells were preincubated with rottlerin or BAPTA-AM for 60 min or GF109203X, Gö6983, Gö6976 for 10 min to final concentrations indicated in the figures before stimulation with WKYMVm.

Protein kinase C activity assay

The assay of non-radioactive detection of PKC was performed accordingly to manufacturer's instructions. Briefly, 1×10^7 IMR90 cells were serum-starved for 48 h and incubate with 10 μM WKYMVm for 1 and 5 min. Cells were washed with cold PBS, resuspended in 0.5 ml of cold PKC extraction buffer (25 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and homogenized. After centrifugation for 5 min at 14,000*g*, the supernatants were purified on 1 ml column of DEAE cellulose pre-equilibrated in PKC extraction buffer and the PKC-containing fractions were eluted using 5 ml of PKC extraction buffer containing 200 mM NaCl. Ten microliters of samples were assembled in a PKC assay in the presence of PKC reaction buffer (20 mM Tris–HCl, pH 7.4, 1.3 mM $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP), 2 μg of the fluorescent peptide PepTag (PLSRTLSVAAK), 0.2 mg/ml phosphatidylserine. Samples were incubated at 30 °C for 30 min, the reactions were stopped at 95 °C for 10 min, the phosphorylated and nonphosphorylated forms of the PepTag petide were separated in a 0.8% agarose gel and photographed on a transilluminator. The positive control reaction contained 10 ng of Protein kinase C.

Assay of superoxide production

The method used to determine $O_2^{-\cdot}$ produced by IMR90 cells was previously described [27]. Membranes and cytosol were isolated from serum-starved fibroblasts stimulated with 10 μM WKYMVm in the presence or absence of 10 μM Gö6983 for 10 min or 10 μM GF109203X for 10 min. NADPH-dependent superoxide production was determined as the superoxide dismutase-sensitive rate of reduction of cytochrome *c*. Briefly, combinations of 10 μg of membrane and 200 μg of cytosol proteins in PBS were incubated for 5 min at room temperature in the presence of 15 μM GTPγ-S, 100 μM cytochrome *c* and 10 μM FAD in a total volume of 1 ml. NADPH (100 μM) was then added and the production of superoxide was monitored at 550 nm. The specificity of cytochrome *c* reduction was controlled by the addition in control samples of 200 U/ml SOD. Rates of $O_2^{-\cdot}$ production were calculated from the linear segment of the increase in absorbance at 550 nm and translated into nanomoles of $O_2^{-\cdot}$ by the extinction coefficient of cytochrome *c*, $\Delta E_{550}/\Delta t = 21.1\ mM^{-1}\ cm^{-1}$, considering that 1 mole of $O_2^{-\cdot}$ reduces 1 mole of cytochrome *c*. The Student's *t* test was used to compare individual treatments with their respective control value and, in the legend for figures, * indicates significant differences at the $P < 0.01$ probability level, as compared with the values obtained from growth-arrested fibroblasts.

Western blot analysis

Growth-arrested fibroblasts were exposed to 10 μM WKYMVm for 5 min at 37 °C in the presence or absence of the appropriate amounts of inhibitors. Cells were rinsed with phosphate-buffered saline (PBS) buffer