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Role of cytosolic and calcium independent phospholipases A_2 in insulin secretion impairment of INS-1E cells infected by *S. aureus*

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ABSTRACT

Cytosolic PLA_2 ($cPLA_2$) and Ca^{2+}-independent PLA_2 ($iPLA_2$) play a significant role in insulin β-cells secretion. Bacterial infections may be responsible of the onset of diabetes. The mechanism by which *Staphylococcus aureus* infection of INS-1 cells alters glucose-induced insulin secretion has been examined. After acute infection, insulin secretion and PLA_2 activities significantly increased. Moreover, increased expressions of phospho-$cPLA_2$, phospho-PKCα and phospho-ERK 1/2 were observed. Chronic infection causes a decrease in insulin release and a significant increase of $iPLA_2$ and COX-2 protein expression. Moreover, insulin secretion in infected cells could be restored using specific *si*RNAs against $iPLA_2$ isoform and specific COX-2 inhibitor.

1. Introduction

Phospholipases A_2 (PLA_2) are a large family of enzymes ubiquitously expressed that catalyze the breakdown of glycerophospholipids, releasing arachidonic acid (AA): cytosolic PLA_2 ($cPLA_2$), Ca^{2+}-independent PLA_2 ($iPLA_2$), and Ca^{2+}-dependent secretory PLA_2 ($sPLA_2$) differ from each other in terms of substrate specificity and Ca^{2+}-requirement [1]. The $cPLA_2$, present in many cell types, including pancreatic β cells ($cPLA_2\beta$), requires phosphorylation at Ser^{505} and binding with Ca^{2+} for its activity. Activation of $cPLA_2\beta$ would cause translocation of the enzyme to the secretory granules and accumulation of AA and lysophospholipids in the membrane, leading to changes in membrane structure or fluidity [2]. It has been demonstrated that $cPLA_2$ β plays a role in the maintenance of insulin stores [3] and its overexpression results in severe impairment of the calcium and secretory responses of β-cells to glucose [4].

The enzyme $iPLA_2\beta$ does not require Ca^{2+} for the catalytic activity and it is inhibited by the suicide substrate bromoenol lactone (BEL) [5]. It has been shown that $iPLA_2\beta$ inhibition prevents cell dysfunction associated with diabetes [6,7].$sPLA_2$ is contained in insulin secretory granules of pancreatic islet β-cells, it is co-secreted with insulin from glucose-stimulated islets [8] and it is expressed in human islets of transplanted pancreas after the recurrence of type 1 diabetes mellitus (T1DM) with insulitis [9,10].

The AA, released from membrane phospholipids by PLA_2 activities, has a significant regulatory and protective action on insulin secretion in pancreatic β cells [11]. These observations are consistent with $cPLA_2$ releasing AA in a controlled fashion from membrane phospholipids during insulin secretion. To date, studies examining the role of $cPLA_2$ and AA in the release of insulin have focused on short-term signaling. However, long-term exposure to high levels of fatty acids, including AA, results in desensitization and suppression of insulin secretion followed by induction of apoptosis [12].

It has been widely discussed at broad spectrum about the correlation between diabetes and the onset of bacterial infections [13,14] but, recently, particular attention has been paid to bacterial infections that may be responsible of the onset of diabetes by reduction of secretion of insulin by pancreatic cells [15,16]. Despite intensive research, a final conclusion concerning the causal role of microbes in the pathogenesis of T1DM has not been made.

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There are several proposed mechanisms of β-cell damage caused by microbes. It has been demonstrated that bacterial infection plays a significant role in bile-induced acute pancreatitis [17–19]. A case of acute cholecystitis and bacteremia with methicillin-resistant *Staphylococcus aureus* (MRSA) in a patient with HIV infection has been reported and highlights the invasive nature of staphylococcal infections [20]. Moreover, a study on obese mongrel dogs demonstrated that *Staphylococcus intermedius* infection leads to impaired insulin secretion [21]. *S. aureus* secretes numerous exotoxins, including a group of polypeptides capable of damaging the host cell plasma membrane. These polypeptides include pore-forming toxins (PFT: α-hemolysin, γ-hemolysin, δ-hemolysin, the Panton Valentine leukocidin, LukED, and LukGH/AB), β-hemolysin, and the phenol soluble modulins [22]. In particular, δ-hemolysin, a small amphipathic peptide with an α-helix structure could bind to the cell surface and aggregate to form transmembrane pores [23,24]. Our previous studies showed the significant role of $cPLA_2$, $iPLA_2$ and PKCα/ERK/MAPK signaling pathways during *E. coli* infection of microvascular endothelial cells [25,26]. The first aim of the present study was to investigate the role of PLA_2 and the involvement of PKCα/ERK1/2 signaling pathways in the response of the cells to *S. aureus* infection; the second was to demonstrate the effects of bacteria infection on insulin release.

2. Material and methods

2.1. Bacterial strains

S. aureus ATCC 33591 (methicillin-resistant strain) were grown in tryptic soy broth overnight at 37 °C. Bacteria were harvested by centrifugation for 10 min at 4300×*g* at 4 °C and washed twice in HBSS. The density of bacteria was measured by enumerating the number of CFU on LB agar plates (Difco).

2.2. Cell cultures

Rat insulinoma (INS-1E) β-cell line was kindly provided by Dr. C. B. Wollheim, (Médical Universitaire, Genève, Switzerland). INS-1E cells, widely used as a pancreatic β cell model [27,28], were cultured in RPMI-1640 medium containing as described [29].

2.3. Invasion assay

Cell monolayers (grown in 6-well tissue culture plate at a density of 8×10^5 cells/well) were infected with *S. aureus* (10^7 CFU/well) for 2 h, 4 h, 6 h and 8 h in a serum free medium. At the end of the incubation times, invasion was determined as described [25].

2.4. Electron microscopy

For transmission electron microscopy (TEM), cells were embedded in Durcupan ACM (Fluka Chemika-Biochemika). Ultrathin sections were double stained with uranyl acetate and lead citrate. Observations were carried out using a Hitachi H-7000 transmission electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

2.5. Insulin secretion assay

Glucose-induced insulin secretion was evaluated as previously described [29]. INS-1E cells (8×10^5 cells/well) were seeded in 6-well plates and incubated for 6 h in a serum free medium containing *S. aureus* (10^7 CFU/well) in presence or absence of 20 nM wortmannin plus 10 μM LY294002 (WTM/LY) or 20 μM PD98059 (kinase inhibitors) or 5 μM NS-398, COX-2 specific inhibitor.

At the end of the incubation period, gentamicin at 100 μg/ml was added and left for 1 h to kill extracellular bacteria. Cells were then washed three times with PBS and cultures were randomly divided into two groups. The first group (short-term infection) was stopped at this point (after incubation with *S. aureus* for 6 h) miming an acute infection. The second group (long-term infection), after 6 h of infection with *S. aureus*, was further incubated for another 72 h, in presence or absence of above mentioned inhibitors, in bacteria-free medium, containing 5 mM glucose in order to allow bacterial proliferation inside the cells and to mimic a chronic infection. Cells from the two groups were then incubated for 1 h at 37 °C in Krebs–Ringer-HEPES buffer (KRHB) [29] containing 2.7 mM glucose (starvation). Thereafter, cells were incubated for 2 h in the same buffer containing different concentrations of glucose (2.7 mM, 5.5 mM, 11.1 mM, 16.6 mM and 22.2 mM). Insulin levels in the culture media were measured by ELISA kit (Millipore).

2.6. Immunoblotting

The lysates of INS-1E cells were prepared for Western blotting as previously described [30–32]. Membranes were incubated with primary antibodies against total $cPLA_2$, $iPLA_2$, total PKCα, ERK 1/2, phospho-$cPLA_2$, phospho-PKCα, phospho-ERK 1/2, COX-1, COX-2 and α-actin, and then incubated with secondary antibodies for 1 h at room temperature.

2.7. Phospholipases A_2 activity assay

INS-1E cells were pre-incubated for 1 h in RPMI 1640 medium containing 5 mM glucose, supplemented or not with either 50 μM AACOCF3 or 2.5 μM BEL or 5 mM EDTA, or 20 nM wortmannin plus 10 μM LY294002 (WTM/LY) or 20 μM PD98059. The cells were then re-fed with fresh culture medium containing the inhibitors, in the presence or in the absence of *S. aureus* (10^7 CFU/well) for 6 h. Cells were then divided into two groups and processed as described in order to mime an acute and a chronic infection. Cells were lysed [31], and PLA_2s activity assays were performed by ELISA kit (Cayman Chemical Co.).

2.8. Transfection of siRNAs

The $cPLA_2$ and $iPLA_2$ knock-down in INS-1E cells was carried out by using rat ON-TARGET plus SMART pool *si*RNA duplex (Dharmacon), transfected by Lipofectamine RNAiMax (Life Technologies). Two sets of oligonucleotides were used: the first direct against $cPLA_2$ (Gene Bank NM_133551) and the second one direct against $iPLA_2$ (Gene Bank NM_001005560). After transfection with $iPLA_2$-*si*RNA or $cPLA_2$-*si*RNA, the cells were infected for 6 h with *S. aureus* (10^7 CFU/well). The cells were then divided in two groups, as described, and insulin release was determined.

2.9. Statistical analysis

Data is reported as mean ± standard deviation (SD). Statistical significance between two groups was analyzed by Student's *t*-test. One-way analysis of variance (ANOVA), followed by Tukey's post-hoc test, was used to compare the means for the multiple groups. The P value <0.05 was considered statistically significant.

3. Results

3.1. Invasion of INS-1E by S. aureus

In Fig. 1, panel A, the percentage of invasion at 4 h and 6 h increased by 1.3- and 1.8-fold respectively in comparison with