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A novel hypothesis regarding the possible involvement of cytosolic phospholipase 2 in insulin-stimulated proliferation of vascular smooth muscle cells

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Abstract

Insulin (INS) *via* INS receptor acts as a mitogen in vascular smooth muscle cells (VSMCs) through stimulation of multiple signaling mechanisms, including p42/44 mitogen-activated protein kinase (ERK1/2) and phosphatidyl inositol-3 kinase (PI3K). In addition, cytosolic phospholipase 2 ($cPLA_2$) is linked to VSMCs proliferation. However, the upstream mechanisms responsible for activation of $cPLA_2$ are not well defined. Therefore, this investigation used primary cultured rat VSMCs to examine the role of PI3K and ERK1/2 in the INS-dependent phosphorylation of $cPLA_2$ and proliferation induced by INS. Exposure of VSMCs to INS (100 nM) for 10 min increased the phosphorylation of $cPLA_2$ by 1.5-fold ($p < 0.01$), which was blocked by the $cPLA_2$ inhibitor MAFP (10 μM; 15 min). Similarly, the PI3K inhibitor LY294002 (10 μM; 15 min) and ERK1/2 inhibitor PD98059 (20 μM; 15 min) abolished the INS-mediated increase in $cPLA_2$ phosphorylation by 59% ($p < 0.001$), and by 75% ($p < 0.001$), respectively. Further, inhibition of $cPLA_2$ with $cPLA_2$ inhibitor MAFP abolished the INS-stimulated ERK1/2 phosphorylation by 65% ($p < 0.01$). Incubation of rat VSMCs with INS resulted in an increase of VSMCs proliferation by 85% ($p < 0.001$). The effect of INS on VSMCs proliferation was significantly ($p < 0.01$) reduced by pretreatment with MAFP. Thus, we hypothesized that INS stimulates VSMCs proliferation *via* a mechanism involving the PI3K-dependent activation of $cPLA_2$ and release of arachidonic acid (AA), which activates ERK1/2 and further amplifies $cPLA_2$ activity.

Keywords: Vascular smooth muscle cells; INS; Proliferation; $cPLA_2$; ERK1/2; PI3K

1. Introduction

Vascular smooth muscle cells (VSMCs) respond to arterial wall injury by intimal proliferation and play a key role in atherogenesis by proliferating and migrating excessively in response to repeated injury, such as hypertension and atherosclerosis (Mulvany et al., 1978; Folkow, 1982; Beckman et al., 2002). In contrast, fully differentiated, quiescent VSMCs allow arterial vasodilatation and vasoconstriction. Exaggerated and uncontrolled VSMCs proliferation appears therefore to be a common feature of both atherosclerosis and hypertension. This observation has led to a growing interest in the study of drugs capable of interfering with the proliferative process. Cultured VSMCs have proved to be an attractive and useful model for *in vitro* study of the mechanisms of cell proliferation (Campbell et al., 1989; Iouzalen et al., 1999; Isenovic et al., 2008).

Numerous epidemiologic studies indicate that INS resistances as well as hyperinsulinemia, associated with type 2 diabetes, contribute largely to the development of

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Nomenclature

INS	insulin
VSMCs	vascular smooth muscle cells
ERK1/2	p42/44 mitogen-activated protein kinase
PI3K	phosphatidyl inositol-3 kinase
$cPLA_2$	cytosolic phospholipase 2
IRS-1, IRS-2	INS receptor substrate-1 and -2
FCS	fetal calf serum
PD	PD98059
LY	LY294002
MAFP	methylarachidonyl fluorophosphate
BrdU	5-bromo-29-deoxyuridine
AA	arachidonic acid

hypertension and atherosclerotic lesions (Colwell et al., 1992; Colwell and Jokl, 1996; Stout, 1992). INS can inhibit VSMCs migration and growth in the normal vasculature (Stout, 1992) and INS's failure to do so in INS resistant states may contribute to enhanced atherosclerosis/restenosis in these clinical conditions. Therefore it is extremely important to understand the molecular mechanisms which regulate VSMCs proliferation as well as cell growth.

In VSMCs the major pathway responsible for these INS effects involves the tyrosine phosphorylation of INS receptor substrate-1 and -2 (IRS-1, IRS-2) with activation of phosphatidyl inositol-3 kinase (PI3K) and p42/44 mitogen-activated protein kinase (ERK1/2) (Myers et al., 1992; Cusi et al., 2000). Whereas signaling along PI3K pathway is impaired in virtually all states of INS resistance, INS signaling along the pathway leading to mitogenic effects of INS remains intact in INS resistant states and responds normally to INS (Cusi et al., 2000; Montagnani et al., 2002).

VSMCs exhibit both 14-kDa and high molecular mass cytosolic phospholipase 2 ($cPLA_2$) activities (Nakano et al., 1990; Sudar et al., 2008; Zakula et al., 2007). But, whether the $cPLA_2$ plays a role in cellular proliferation is not known. The purpose of this study was to directly examine the roles of the 85-kDa $cPLA_2$ and ERK1/2 and PI3K signaling pathways in VSMCs proliferation. Evidence suggestive of a role for $cPLA_2$ in VSMCs proliferation includes reports that inhibition of the enzyme with the potent $cPLA_2$ inhibitor or antisense to $cPLA_2$ blocks serum-stimulated growth in culture (Clark et al., 1990).

However, a direct correlation between INS-induced activation of $cPLA_2$ and growth of VSMCs has not been established. In addition, the mechanisms linking the INS receptor to the activation of $cPLA_2$ are still not clearly defined. Phosphorylation of $cPLA_2$ involves ERK1/2, and agonist-induced phosphorylation and activation of $cPLA_2$ correlates with the activation of the Ras/MAPK pathway in various cell models (Balboa et al., 1997; Tang et al., 1997). Consistent with this mechanism are reports that arachidonic acid (AA) stimulates the activation of ERK1/2 either directly or indirectly (Kramer et al., 1991; Oka and Arita, 1991; LaPointe and Isenovic, 1999). Thus, a positive feedback mechanism appears to exist for activation of $cPLA_2$. However, it is possible that additional kinases may also be involved in the release of AA prior to stimulation of ERK1/2. One such possibility is PI3K, since PI3K has been linked to INS-induced VSMCs growth (Montagnani et al., 2002; Sudar et al., 2008; Zakula et al., 2007; Isenovic et al., 2008) and PI3K participates in the early events leading to activation of ERK1/2 (Lin et al., 1992; Nakamura et al., 1992).

Herein is presented the first direct evidence that $cPLA_2$ activity is associated with VSMCs proliferation. The 85-kDa enzyme appears to serve a selective role in the initial production of AA through direct action or *via* action of metabolites critical for VSMCs proliferation (Silfani and Freeman, 2002; Anderson et al., 1997; Longo et al., 1999).

We hypothesized that INS stimulates VSMCs growth *via* a mechanism involving the PI3K-dependent activation of $cPLA_2$ and release of AA which activates ERK1/2 and further amplifies $cPLA_2$ activity. To test this hypothesis, we investigated the effects of INS on the activation of $cPLA_2$ and ERK1/2, as well as VSMCs proliferation in the presence and absence of inhibitors for PI3K, $cPLA_2$, or ERK1/2 in cultured rat VSMCs.

2. Materials and methods

2.1. Reagents

Cell culture materials and media were obtained from Costar and Life Technologies, respectively. Fetal calf serum (FCS) was from Roche Diagnostic (Gagny, France). Porcine INS was purchased from Galenika (Zemun, Serbia). ERK1/2 specific inhibitor PD98059 (PD), the PI3K inhibitor LY294002 (LY) and irreversible $cPLA_2$ inhibitor, methylarachidonyl fluorophosphate (MAFP) were from Calbiochem (Merck Eurolab; Fontenay-sous-Bois, France). Because of their characteristics PD, LY and MAFP were used to inhibit PI3K or ERK1/2 pathways and $cPLA_2$ activity. All other chemicals were from Sigma Aldrich (St. Quentin Fallavier, France).

2.2. Antibodies

Phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and total ERK1/2, phospho-$cPLA_2$ (Ser^{505}) and total $cPLA_2$ monoclonal antibody, and anti-mouse or anti-rabbit IgG horseradish peroxidase-linked antibodies were purchased from Cell Signaling Technology (Beverly, MA). Briefly, the antibody used against phospho-ERK1/2 detects the enzyme when dually phosphorylated on Thr^{202} and Tyr^{204} that is the activated form of the enzyme (Payne et al., 1991). The antibody used against phospho-$cPLA_2$ detects endogenous levels of $cPLA_2$ only when phosphorylated at $Serine^{505}$.

2.3. Cell cultures

Experiments were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. VSMCs were isolated from the thoracic