Metabolic and proliferative effects of insulin signaling contributing to the liver regeneration

Thesis

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جامعة القاهرة / فرع بني سويف
الدراسات العليا

محضر
اجتماع لجنة الحكم على الرسالة المقدمة من
الطبيب / جورج نزيه بشارة مرقص
توطئة للحصول على درجة الماجستير / الدكتوراه
في الكيمياء الحيوية و البيولوجيا الخلوية

تحت عنوان : باللغة الانجليزية : Metabolic and proliferative effects of insulin signaling contributing to the liver regenerat

باللغة العربية : التأثيرات الأيضية والتكاثرية لاشارات هرمون الانسولين المساهمة في تجديد خلايا الكبد

بناء على موافقة الجامعة بتاريخ ٢٠١٢/٥/٢٠ تم تشكيل لجنة الفحص والمناقشة للرسالة المذكورة أعلاه على النحو التالي :

١. P.د/ منى سركيس كراس أستاذ الكيمياء الحيوية والبيولوجيا الخلوية ط القاهرة (من المشرفين)
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بعد فحص الرسالة بواسطة كل عضو منفردا وكتابة تقارير منفردة لكل منهم انعقدت اللجنة مجتمعة في يوم الأحد بتاريخ ٢٠١٢/٥/٢٧ بقسم الكيمياء الحيوية مدرج الكيمياء الكبرى بكلية الطب – جامعة القاهرة وذلك لمناقشة الطالب في جلسة علنية في موضوع الرسالة والنتائج التي توصل اليها وكذلك الأسس العلمية التي قام عليها البحث .

قرار اللجنة : قبول الرسالة لدرجة الدكتوراة في الكيمياء الحيوية والبيولوجيا الخلوية حيث ان الرسالة حديثة المراجع ، لغتها سليمة المعاني والعناوين تتبع بها المنهج العلمي الحديث ، بها طرق عملية حديثة يمكن تطبيقها عليا .
لذا توصي اللجنة بقبولها لدرجة الدكتوراة في الكيمياء الحيوية الطبية والبيولوجيا الخلوية

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Abstract

The present work is designed to study the expression level of both IRS-1 and IRS-2 in primary mouse hepatocytes (PMH), and investigate their kinetics of phosphorylation in response to addition of variable doses of insulin. The present study also involves determination of subcellular localization of both IRS-1 and IRS-2 in PMH using confocal microscopy. Downregulation of IRS-2 in PMH using siRNA against IRS-2 delivered into hepatocytes by means of adenovirus infection was done. The effect of this downregulation on the expression of IRS-1 protein and the phosphorylation of both IRS-1 and IRS-2 was studied.

Results reveal higher expression level of IRS-2 than IRS-1 in PMH. On addition of insulin IRS-2 shows faster kinetics of phosphorylation than IRS-1 but the degree of phosphorylation of IRS-1 is higher than IRS-2. Results also show that IRS-2 is mainly localized in cell membrane while IRS-1 is homogenously distributed all over the cytoplasm and in nuclei of PMH. Addition of insulin induces nuclear translocation of IRS-1 and to lesser extent IRS-2. Adenoviral infection of PMH may cause endoplasmic reticulum and cellular stress and could affect the expression of IRS proteins in these cells.

Key Words

- Insulin Signaling
- Liver Regeneration
- IRS-1
- IRS-2

Contents

List of Abbreviation

4E-BP-1	4E binding protein-1
AFX	fork head transcription factor
AGC	Protein kinase A, G and C
ANOVA	analysis of variance
aPKC	atypical Protein Kinase C
APS	Ammoniumperoxidesulfate
AS160	Akt substrate of 160 kDa
BKG	Background
BSA	Bovine serum albumin
cAMP	cyclic Adenosine Monophosphate
Cb1	Cannabinoid Receptor-1
CCl_4	Carbon Tetrachloride
CDC42	Cell-division cycle 42
CHO	Chinese hamster ovary
c-met	mesenchymal-epithelial transition factor
CMV	Cytomegalovirus
C-myc	myelocytomatose
CPE	Cytopathic Effect
DAPI	4',6-Diamidine-2'-phenylindole-dihydrochloride
DMEM	Dubeco's modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid

Dock	Dedicator of cytokinesis
DOK	Downstream of tyrosine kinase
EDG	Endothelial differentiation gene
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene glycol tetraacetic acid
eIF	Eukaryotic Initiation Factor
EmGFP	Emerald Green Fluorescent Protein
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FCS	Fetal cow serum
FFA	Free Fatty Acids
FH	Forkhead family
FITC	Fluorescein isothiocyanate
FKHR	Fork Head like protein receptor
FOX	Forkhead class of transcription factors
Gab	Grb2-associated binder
GFP	Green Fluorescence Protein
GLUT	Glucose Transporter
gp 96	Glucose regulated protein 94 or Tumor rejection antigen
Grb-2	Growth factor receptor binding protein 2
GSK	Glycogen synthase kinase
HATs	Histone acetyltransferases
HBS	HEPES Buffered Saline
HCS	High Content Screening

HD	High Density
HDACs	Histone deacetylases
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)1-piperazineethanesulfonic acid
HGF	Hepatocyte Growth Factor
HMS	Hepatic microvascular subunits
HRP	Horseradish Peroxidase
HSP70	Heat Shock Protein 70
ICAM	Intercellular Adhesion Molecule
IF	Immunofluorescence
IGF	Insulin-like Growth Factor
IGF1R	Insulin growth factor-1 receptor
IL	Interleukin
IP	Immunoprecipitation
IR	Insulin receptor
IRE	Insulin response element
IRR	IR-related receptor
IRS	Insulin receptor substrate
ITRs	Inverted Terminal Repeats
IκB	Inhibitor of κB
JNK	c-jun-N-terminal kinase
kDa	kilo Dalton
KRLB	Kinase regulatory loop binding
LAL	Liver-associated lymphocytes
LAU	Light Arbitrary Unit
LB	Luria Broth Base medium

LD	Low Density
LGL	Large granular lymphocytes
MAP	Mitogen-activated Protein
MAPK	Mitogen Activated Protein Kinase
MEF	mouse embryo fibroblasts
MEK1	MAPK and ERK kinase 1
miRISC	miRNA-containing RNA induced silencing complex
miRNAs	MicroRNAs
MOI	Multiplicity of Infection
mRNA	messenger RNA
mSOS	Mammalian Son of Sevenless
mTOR	Mammalian target of rapamycin
MWCO	Molecular weight cut-off
NEAA	Non-essential aminoacids
NF-κB	Nuclear Factor-κB
NK	Natural Killer Cells
OD	Optical Density
O-GlcNAc	O-linked β-N-acetylglucosamine
p70S6K	p70 ribosomal S6 kinase
p90RSK	p90 ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PDGF	Platelet-derived Growth Factor
PDK	Phosphoinositide-dependent kinase
PEI	poly ethyleneimine
PEPCK	Phosphoenol pyruvate carboxy kinase
PFU	Plaque Forming Unit

PH	Pleckstrin Homology
PHLPP	PH-domain leucine-rich repeat protein phosphatase (PHLPP)
PI3-K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidyl inositol trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PMH	Primary Mouse Hepatocyte
PP2A	protein phosphatase-2A
PTB	Phosphotyrosine Binding
PTEN	Phosphatase and tensin homologue
PTGS	Post-transcriptional gene silencing
PTP1B	Protein tyrosine phosphatase 1B
Puc 19	plasmid university of California
PVDF	Polyvinylidene membrane
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RTKs	Receptor tyrosine kinases
S1P	Sphingosine 1-phosphate
SD	Standard Deviation
SDS-PAGE	Sodium Dodecylsulfate -Polyacrelamide gel electrophoresis
SE	Standard Error
SGK	Serum and glucocorticoid-inducible kinase
SH2	src homology 2
SHIP2	SH2-containing inositol 5′-phosphatase-2
SHP-2	SH2-containing protein-tyrosine phosphatase-2
shRNA	short hairpin RNA

siRNA	small interference RNA
Smad	Drosophila protein (mothers against decapentaplegic)
SOC	Super Optimal Broth with Catabolite repression medium
SOCS	Suppressor of cytokine signaling
SOP	Standard Operation Procedures
SREBP	Sterol regulatory element binding protein
STAT	Signal Transducer and Activator of Transcription
TBS-T buffer	Tris buffered saline and tween 20
$TCID_{50}$	Tissue Culture Infectious Dose 50
TEMED	N, N, N, N-Tetramethylethylendiamin
TGF	Transforming Growth Factor
TNFα	Tumor Necrosis Factor α
TRB3	Tribbles-3
TSC	Tuberous sclerosis complex
UDP	Uridine Diphosphate
upaR	urokinase type plasminogen activator protein
VLB	Virion Lysis Buffer
WB	Western blotting

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Introduction and Aim of the Work

Introduction

Insulin receptor substrate (IRS) proteins play a central role in maintaining basic cellular functions such as growth and metabolism. In the presence of insulin, activation of its receptor tyrosine kinase creates binding sites for intracellular substrates such as IRS family of proteins and induces their phosphorylation ***(Pirola et. al., 2004)***. IRS molecules act as docking proteins between the insulin receptor and a complex network of intracellular signaling molecules containing Src Homology 2 (SH2) domains. This is indispensable both for amplification and transmission of the signal transduction ***(Sesti et. al., 2001)***. Phosphorylated IRS proteins are linked to the activation of two main signaling pathways; the PI3K-AKT/protein kinase B (PKB) pathway, which is responsible for most of the metabolic actions of insulin, and the Ras-mitogen activated protein kinase (MAPK) pathway, which regulates the expression of several genes and cooperates with the PI3-K pathway to control cell growth and differentiation ***(Giovannone et. al., 2000)***.

Six IRS members have been identified that are considered similar. They include an NH_2 terminal pleckstrin homology (PH) domain and a phosphotyrosine binding (PTB) domain (which bind the insulin receptor IR). The COOH terminal region contains multiple potential tyrosine phosphorylation motifs. Despite their similarity, IRS proteins differ in some respects like tissue distribution and mediation of different biological signals. The most important members of IRS family of proteins are IRS-1 and IRS-2 ***(Dearth et. al., 2007)***. IRS-1 null mice are stunted in growth but do not develop diabetes ***(Khamzina et. al., 2003)***. IRS-1 metabolic effects could be observed in muscle and adipose tissue and play less role in the liver metabolism. In contrast, IRS-2 play a main role in the liver and IRS-2

knockout mice display dysregulated lipolysis, peripheral glucose uptake and hepatic gluconeogenesis. Further, only IRS-2 knockout mice develop diabetes ***(Withers et. al., 1998)***.

The liver is the major organ involved in the glucose homeostasis. Hepatic glucose metabolism includes the formation of glycogen as well as the generation of glucose from non-sugar carbon substrates and from glycogen. Insulin is an essential factor in these processes and maintains together with glucagon the glucose homeostasis under physiological conditions ***(Raddatz and Ramadori, 2007)***. Dysregulated insulin action in the liver contributes to many metabolic disorders like dyslipidemia, hypertension, female infertility and glucose intolerance that might progress to type 2 diabetes ***(Lin and Sun, 2010)***.

Moreover, the liver is the primary site of insulin clearance, removing approximately 50% of portal insulin as a mainly receptor mediated process ***(Kotronen et. al., 2007)***. By mediating insulin degradation, receptor endocytosis ends the ability of the hormone to signal and plays a key role in the regulation of insulin activity.

The enormous regeneration capacity of the liver is unique among mammals. In rats, only five to seven days after two third partial hepatectomy, the liver recovers to its original weight and nearly reconstitutes its hepatocyte mass ***(Higgins & Anderson, 1931)***. During the intensive phase of proliferation, hepatocytes continue to ensure their essential metabolic functions such as glucose regulation, degradation of toxic compounds or synthesis of proteins. Insulin is an essential factor in these processes stimulating the hepatocyte to store glucose, also insulin signaling regulates the expression and activity of metabolic genes resulting in both proliferative and anti-apoptotic responses, mediating a complex regulated

network, which substantially contributes to successful liver regeneration ***(Jeschke et al., 2005)***. Loss of insulin signaling in hepatocytes leads to progressive hepatic dysfunction ***(Michael et al., 2000)***. On the other hand the overexpression or activation of components of signaling pathway is believed to contribute to tumorigenesis, tumor progression and disease metastasis ***(Whittaker et. al., 2010)***. Consistent with this, IRS proteins are elevated and active in many human tumors ***(Dearth et. al., 2007)*** including hepatocellular carcinoma ***(Tanaka et. al., 2002)***.

Understanding the molecular mechanisms of insulin signaling may contribute significantly to new treatment not only for diabetes mellitus ***(White, 2003)*** but also for diseases in which there are abnormal liver proliferation e.g. hepatocellular carcinoma ***(Whittaker et. al., 2010)***.

Aim of The Work:

The present work is designed to study the expression level of both IRS-1 and IRS-2 in primary mouse hepatocytes (PMH), and investigate their kinetics of phosphorylation in response to addition of variable doses of insulin. This to be compared with their expression level and phosphorylation kinetics in PMH in which IRS-2 is down regulated using siRNA delivered into PMH by adenovirus. The present study also involves determination of subcellular localization of both IRS-1 and IRS-2 in PMH using confocal microscopy.

Review of Literature