The regulation of NOX4 expression by insulin and its role in the modulation of TGF-β-induced apoptosis in murine hepatocytes

WANG, Wei

Abstract
Nous avons analysé le rôle joué par insulin, IGF-1 et IGF-2 sur le expression de la NADPH oxidase NOX4 dans une model hépatocellulaire et exploré son implication à l'interface des signalisations de IGF-1/2 et TGF-β. Les hépatocytes déficients en récepteur insuline montrent un niveau de NOX4 élevé. IGF-1 et 2 préviennent la stimulation de NOX4 par TGF-β, parallèlement à une inhibition de l'apoptose induites par TGF-β. Ces résultats démontrent que le contenu cellulaire de NOX4 à l'état basal est inhibé par l'insuline et les IGF-1/2, conduisant à une modification de l'apoptose induites par TGF-β. Notre étude démontre l'importance de NOX4 comme médiateur entre l'insuline/IGF-1 et le TGF-β.


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The regulation of NOX4 expression by insulin and its role in the modulation of TGF-β-induced apoptosis in murine hepatocytes

Thèse

Présentée à la Faculté de Médecine de l’Université de Genève
pour obtenir le grade de
DOCTEUR EN MEDECINE (MD)

par
Wei WANG
Pékin, Chine
Thèse n° 10641
GENÈVE

2011
This thesis is dedicated to

my parents, my brother and my husband,

who give me their deepest love and great support.
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<tr>
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<th>Full name</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis signal-regulating kinase-1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCA protein assay</td>
<td>bicinchoninic acid protein assay</td>
</tr>
<tr>
<td>BPB</td>
<td>bromphenol blue</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>cytochrome P-450 2E1</td>
</tr>
<tr>
<td>CRK</td>
<td>the SH2/SH3 domain containing adaptor protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DPI</td>
<td>diphenyleneiodonium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DUOX</td>
<td>dual oxidase</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>eukaryotic Initiation Factor 4E Binding Protein 1</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>elf-4E</td>
<td>eukaryotic Initiation Factor 4E</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>GLUT</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>Grb-2</td>
<td>receptor binding protein 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
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<td>-----------------------------------------------</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>hepatic stellate cell</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>IGF Binding Protein 1</td>
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<td>insulin-like growth factor type 1 receptor</td>
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<td>IGF1RKO</td>
<td>IGF-1R knock-out</td>
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<tr>
<td>IR</td>
<td>insulin receptor</td>
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<td>insulin receptor knock-out</td>
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<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>KATP</td>
<td>ATP-Sensitive Potassium Channels</td>
</tr>
<tr>
<td>LAR</td>
<td>leukocyte common antigen related protein</td>
</tr>
<tr>
<td>LIGFREKO</td>
<td>liver-specific IGF-1 receptor knock-out</td>
</tr>
<tr>
<td>LIRKO</td>
<td>liver-specific insulin receptor knock-out</td>
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<tr>
<td>MAP kinase (MAPK)</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>MAPK kinase kinase</td>
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<tr>
<td>MEK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Na$_3$VO$_4$</td>
<td>sodium orthovanadate</td>
</tr>
<tr>
<td>Nck</td>
<td>SH2/SH3 adaptor protein</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>O$_2$</td>
<td>molecular oxygen</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>PDK</td>
<td>phosphoinositol-dependent kinase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PH domain</td>
<td>pleckstrin homology domain</td>
</tr>
<tr>
<td>PI 3-kinase</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTB</td>
<td>phosphotyrosine binding domain</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue deleted on chromosome 10</td>
</tr>
<tr>
<td>PTPs</td>
<td>protein-tyrosine phosphatases</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>Rheb</td>
<td>Ras homolog enriched in brain</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RS6P</td>
<td>ribosomal S6 protein</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAPK/JNK</td>
<td>Stress-activated protein kinase/c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>Shc</td>
<td>SH2 and collagen homology-containing protein</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β-activated kinase-1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor-necrosis factor alpha</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous Sclerous Complex</td>
</tr>
<tr>
<td>VDCC</td>
<td>voltage-dependent calcium channels</td>
</tr>
<tr>
<td>VLDL</td>
<td>very-low-density lipoprotein</td>
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RESUME (French)
Le foie est un organe complexe qui contrôle d’une part l’homeostasie du glucose et des lipides et d’autre part l’élimination des toxines endogènes et exogènes. Le foie peut être régulé par différentes hormones ; comprenant l’insuline, les IGF-1 et -2 (Insulin-Like Growth Factors 1 et 2) et le TGF-β (Transforming growth factor beta). Lors d’altérations du statut nutritif, l’insuline et les IGF-1/2 modulent un grand nombre de fonctions métaboliques pour adapter l’équilibre hépatocytaire [1-3]. TGF-β inhibe la progression du cycle cellulaire et est un inducteur potentiel de l’apoptose hépatocytaire [1, 4]. La différenciation, prolifération, et la mort cellulaire sont aussi modifiés par une production de ROS (reactive oxygene species) [1, 5-7]. Les ROS peuvent être considérés comme des messagers secondaires qui activent différentes voies de signalisation intracellulaire et qui peuvent interférer avec la signalisation de l’insuline, TGF-β et IGF-1/2 [8, 9]. Les enzymes qui oxdent le NADPH (NOXes) sont la principale source intracellulaire de ROS.


ABSTRACT (English)

Liver is a complex organ controlling both glucose and lipid homeostasis, and endogen and exogen toxin elimination. Liver is subject to regulation by several hormones including insulin, insulin-like growth factors 1 and 2 (IGF-1, IGF-2) and transforming growth factor beta (TGF-β). Insulin, IGF-1 and IGF-2 modulate a wide range of metabolic and growth-promoting functions in order to adapt hepatocyte homeostasis to nutrient status alterations [1-3]. TGF-β inhibits cell cycle progression and is a potent inducer of hepatocyte apoptosis [1, 4]. Cellular homeostasis, differentiation, proliferation and cell death are also modified by endogenously generated reactive oxygen species (ROS) [1, 5-7]. In fact, ROS can serve as second messengers activating multiple intracellular signaling pathways and can interfere with insulin, IGF-1/2 and TGF-β signaling [8, 9]. NADPH oxidase enzymes (NOXes) are prominent sources of intracellular ROS.

The aim of this study was to explore the bilateral relationship between NOX enzymes and insulin, IGF-1/2 and TGF-β-induced cellular events. In particular, we focused our attention on NOX4, and studied the effect of insulin receptor (IR) deletion on NOX4 expression and its link to TGF-β-induced hepatocyte apoptosis. To achieve our goal we used a previously established and well accepted hepatocyte model derived from wild type and IR-deficient mice and compared their response to TGF-β in the presence or absence of IGF-1 and IGF-2.

Our results confirmed the presence of different NOX isoforms in wild type hepatocytes. Further examinations conducted in the IR knock-out (IRKO) hepatocytes revealed that the presence of the IR has a suppressive effect on basal NOX4 mRNA expression. NOX4 protein levels did not tightly follow the pattern of mRNA regulation. In particular, serum withdrawal led to a significant increase in NOX4 protein but not in mRNA levels both in wild type and IRKO hepatocytes. These data point towards a so far uncovered, post-transcriptional regulation of NOX4. When relating NOX4 expression to cell functions we found that higher basal NOX4 mRNA expression in IRKO cells was accompanied by a lower rate of apoptosis. TGF-β-induced apoptosis was similar in WT and IRKO hepatocytes. IGF-1 and IGF-2 abrogated TGF-β induced NOX4 upregulation and apoptosis in IRKO cells and to a lesser extent in WT cells.

In conclusion, our study revealed a two-layer regulatory network governing cellular NOX4 content both at the transcriptional and post-transcriptional levels. Moreover, it demonstrated the importance of intact insulin receptor signaling in modulating basal cellular NOX4 expression and modifying the effect of IGF-1/2 on TGF-β-induced apoptotic response.
PART I: INTRODUCTION
INTRODUCTION

Liver is a complex organ controlling both metabolic processes by regulating glucose and lipid homeostasis, and endogenous and exogenous toxin elimination through the activation of its microsomal enzyme system. Liver is subject to tight regulation by several hormones including insulin, insulin-like growth factors 1 and 2 (IGF-1/2) and transforming growth factor beta (TGF-β).

Insulin, IGF-1 and IGF-2 act through the insulin and IGF receptors (IR, IGF-1R and IGF-2R, respectively) and modulate a wide range of metabolic and growth-promoting functions in order to adapt hepatic homeostasis to changes in whole body nutrient status. Under circumstances of nutrient abundance, insulin/IGF-1R signaling leads to cell growth and division. By contrast, when nutrients are scarce, insulin/IGF-1 signaling is turned off and growth is inhibited [1-3]. The molecular structure and the intracellular signaling pathways of the IR and IGF-1R show a high level of homology, resulting in a certain degree of crossreactivity; however, their effects are not completely overlapping. The differences between their growth promoting and metabolic effects were emphasized by the different phenotypes developed by the IR, IGF-1R, and the IR/IGF-1R double knock-out mice. IR ablation results in embryos that weigh only 10% less than wild type littermates but show a severe metabolic defect and die within days of birth due to diabetic ketoacidosis [10-12]. By contrast, IGF-1R mutations result in serious growth retardation with pups of 45% of normal weight but no primary metabolic abnormalities [12-15]. Combined ablation of IR and IGF-1R results in smaller embryos than either mutation alone (30% of normal), suggesting that the two receptors have partially overlapping growth-promoting functions [12]. The importance of IGF-1 in growth promotion and its intimate relationship to insulin signaling was revealed by the fact that when ~80% of somatic cells lose IR expression, mice develop a phenotype similar to human leprechaunism, with severe postnatal growth retardation and hypoglycemia [12]. The growth retardation appears to be due to a ~50-fold increase in expression of the IGF Binding Protein 1 (IGFBP1), a known modulator of IGFS bioavailability [16, 17]. Taken together, these data indicate that insulin promotes growth by inhibiting hepatic expression of IGFBP1.

Liver specific deletion of the IR or IGF-1R receptors triggered the onset of metabolic disturbances or resulted in alterations in hepatic growth rate. Liver specific ablation of the IR (LIRKO mice) resulted in the progressive development of diabetes, while the lack of the IGF-1R expression (LIGFREKO mice) lead to a decrease in hepatocyte regeneration capacity upon partial hepatectomy [12, 18].
IGF-2 has a very similar structure but a different physiological role from that of IGF-1. In contrast to IGF-1 which exerts its effects after birth, IGF-2 acts as a growth factor mainly during fetal life and development. In line with this function, targeted disruption of the IGF-2 gene in mice leads to low birthweight with poor viability, and those that survive have infantile reproductive systems incompatible with fertility [19, 20]. IGF-2 acts through the IGF-2/mannose-6-phosphate (M6P) receptor. The IGF-2R is a bifunctional glycoprotein which is structurally and immunologically distinct from the IGF-1R and has no measurable affinity for insulin [21, 22]. IGF-2R binds IGF-2 and lysosomal enzymes bearing the M6P recognition marker at distinct binding sites [23]. The IGF-2R mediates the degradation of IGF-2, thus has a negative effect on growth regulation.

Dysregulation of the IR/IGF-R pathway is central to the development of obesity and related pathologies, such as diabetes, cancer and accelerated ageing [1, 24]. At the cellular level these pathologies are also reflected in altered metabolism and the development of cellular redox stress, and accompanied by disturbances in cell cycle progression and an increased rate of apoptosis. In hepatocytes, TGF-β, a product of the activated hepatic stellate cells (HSC), is a potent inducer of both cell cycle arrest and apoptosis [1]. Apart from TGF-β, hepatocyte proliferation, differentiation and death are also modified by the intracellular redox environment, defined by the balance between the rate of production and elimination of the reactive oxygen species (ROS) [25].

Reactive oxygen species can be generated as byproducts in the mitochondria or by peroxisomal enzymes due to elevated nutrient availability, or in the microsomal fraction due to elevated amounts molecules channeled for detoxification [4, 26-28]. In the absence of an appropriate compensatory response from the antioxidant network, ROS will accumulate and will trigger the activation of stress-sensitive intracellular signaling pathways [1, 29]. However, the role of ROS in cellular signaling is a two-sided issue and ROS can be key molecules in mediating the effects of different receptors. Indeed, this phenomenon is well demonstrated in case of the insulin receptor signaling. In fact, chronic exposure to oxidative stress inhibits the insulin-induced metabolic pathways; by contrast, short-term exposure to millimolar concentration of ROS results in insulinomimetic metabolic activities [30]. Similarly, high amount of ROS results in apoptosis, while low concentration of H₂O₂ stimulation leads to cell proliferation [1, 31]. Thus, spatially and timely controlled ROS production within the cell is a critical mediator regulating diverse metabolic and mitogenic cellular functions.

Regulated cellular ROS production can be achieved by a specific family of ROS generating enzymes, termed NADPH oxidases (NOX-es). The best known member of this family is the phagocyte NADPH oxidase, termed gp91phox/NOX2. NOX2 is activated by...
bacterial aggression resulting in the production and release of large amount of ROS essential for bacteria elimination. Recently, several homologues of this phagocyte NOX enzyme were indentified in non-phagocytic cells. To date, the family of NADPH oxidase enzymes comprises of five NOX-es (NOX1-NOX5) and two dual oxidases (DUOX1 and DUOX2). Following their identification several studies addressed their role in different physiological and pathological processes but the function and regulation of various NOX and DUOX enzymes are still scarcely understood. In liver, most notably in hepatocytes, several NOX isoforms have been identified. One particular isoform, NOX4 has been shown to mediate the apoptosis-inducing effect of TGF-β [32]. Moreover, the pro-survival potential of EGF was related to its capacity to abrogate TGF-β-induced upregulation of NOX4 expression [4]. NOX4 has been implicated in insulin receptor signaling in adipocytes [33]. Insulin and the related IGF-1 are potent survival factors; however, this effect has never been investigated in the context of their capacity to regulate NOX4 expression.

Thus, our investigations were aimed at exploring the link between NOX4 and insulin/IGF-1 signaling in hepatocytes with a specific emphasis on its connection to TGF-β-induced apoptosis.

1. DIFFERENCES AND SIMILARITIES BETWEEN INSULIN AND IGFs
1.1 The structures and secretion of insulin and IGFs
1.1a Insulin

Insulin is synthesized as a single molecule (prepro-insulin) composed of 110 amino acids in the pancreas by the beta cells of the islets of Langerhans. Upon insertion into the endoplasmatic reticulum the signal peptide consisting of 24 amino acids is removed by enzymatic cleavage from one end of the chain, resulting in a form termed pro-insulin.

Pro-insulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide, consisting of 33 amino acids. The C peptide is cleaved by the enzymes convertase 1 and 2 in the Golgi apparatus transforming the insulin molecule into the final structure with 2 chains (A and B) connected by two disulfide bonds.

At the final step two more amino acids are removed by the carboxypeptidase E enzyme resulting in the mature peptide of 51 amino acids. When beta cells are appropriately stimulated, insulin is secreted from the cell by exocytosis and diffuses into islet capillary blood with the cleaved but not degraded C peptide.

Figure 1. Intracellular maturation of insulin.
The secretion of insulin from pancreatic beta cells is a complex process involving the integration and interaction of multiple external and internal stimuli. Thus, nutrients (glucose, amino acids and free fatty acids), hormones, neurotransmitters and drugs can all activate or inhibit insulin release [34]. Some neural stimuli (e.g. the sight and taste of food) also promote insulin secretion. However, the primary trigger of insulin secretion is a raise in blood glucose concentration in order to facilitate cellular glucose uptake in insulin-dependant tissues and hasten glucose clearance from blood. According to our current understanding glucose-induced insulin release comprises the following steps:

1. Glucose is transported into pancreatic beta cells through facilitated diffusion by the low affinity GLUT2 glucose transporter.
2. Intracellular glucose is metabolized leading to mitochondrial ATP production.
4. Cell-surface voltage-dependent Ca²⁺ channels are opened, facilitating extracellular Ca²⁺ influx into beta cells.
5. A rise in free cytosolic Ca²⁺ triggers the exocytosis of insulin.
6. Insulin is transported to target cells where it binds to its membrane-inserted receptor and facilitates glucose uptake.

The normal basal serum insulin levels in humans and most mammals is between 5 to 12 mU/L. After meals, insulin levels demonstrate a sharp rise of short duration (30 to 60 minutes postprandially) depending upon the amount and quality of carbohydrates consumed reaching a concentration range of 50-100 mU/L but returning to basal levels in about 180 minutes. Insulin is degraded by hepatocytes through uptake by the surface insulin receptors. It has been estimated that endogenously produced insulin is degraded about 71 minutes after its initial release into circulation [35].

1.1b Insulin like growth factors

Insulin-like growth factors (IGFs) are members of a family of insulin related molecules [36]. IGFs are part of a complex system that cells use to communicate with their environment and to transmit signals to other cells. This complex system, often referred to as the IGF "axis", consists of two cell-surface receptors: IGF-1 and IGF-2 receptors, two ligands: IGF-1 and IGF-2, a family of six high-affinity IGF binding proteins: IGFBP 1-6, as well as associated IGFBP degrading proteases.
**IGF-1** is a small peptide consisting of 70 amino acids with a molecular weight of 7.6kDa [37]. Similar to insulin, IGF-1 has an A and B chain connected by disulphide bonds and a connecting C peptide region with 12 amino acids. This structural similarity to insulin explains the ability of IGF-1 to bind with low affinity to the IR. IGF-1 is secreted by many tissues and the secretory site seems to determine its actions. IGF-1 (13% of total) is secreted by the liver and is transported to other tissues, acting as an endocrine hormone [38]. IGF-1 is also secreted by other tissues, including heart, lung, muscle, testes, stomach, kidney and brain, and can act in a paracrine manner [38-41]. IGF-1 is produced in response to growth hormone (GH) synthesized in the hypothalamus [42]. IGF-1 levels show gender differences (slightly higher in females than in males) and its serum concentrations increase with age from birth through puberty and then decline slowly with age [43].

**IGF-2** is a polypeptide hormone with structural and functional homology with IGF-1 and pro-insulin. IGF-2 is produced in hepatocytes as a 180 amino acid (20 kDa) prepro-hormone and contains a carboxy-terminal peptide of 89 amino acids (E-domain) and a signal peptide of 24 amino acids, both of which are cleaved post-translationally to produce the 67 amino acid (7.5 kDa) monomeric plasma protein. Similar to pro-insulin, IGF-2 is divided into A, B and C domains. A and B domains are bridged by two inter-domain disulphide bonds, with one internal disulphide bond within the A domain. A and B domains are connected by the C-domain, which unlike the insulin C-domain, is not cleaved during structural maturation. Also, the presence of an additional D-domain is unique to IGF-2. Mature IGF-2 displays 47% amino acid sequence identity with insulin and owing to strict conservation of the three interchain disulphide bridges and residues in the hydrophobic core, has a similar 3D-structure [44]. Liver IGF-2 production is stimulated by GH along with the production of IGF-1. In contrast to IGF-1 which exerts its effects after birth, IGF-2 acts as a growth factor mainly during fetal life and development. In adult life, IGF-2 has also been proposed to act as a growth and differentiation factor in the central nervous system, in muscle and colon epithelial cells [45-47]. Moreover, a key role for IGF-2 as a paracrine or autocrine growth factor in certain tumours has been proposed [48]. IGF-2 is capable of binding to the insulin and IGF-1 receptors but it seems that IGF-2 action on embryonic growth is mediated exclusively by the IR [49-51]. In mammals, the activity of IGF-2 is further moderated by IGF-2R, which sequesters IGF-2 for internalization and degradation.

### 1.1c IGF Binding Proteins

The biological availability of IGF-1 and IGF-2 are regulated by binding to the different members of the IGF binding proteins (IGFBPs). In the plasma, 99% of IGFs are complexed to
a family of binding proteins, which modulate the availability of free IGF-1 to the tissues. There are six binding proteins [52], (IGFBP-1-6) ranging in size from 216 to 289 amino acids [53]. IGF Binding Proteins (IGFBPs) are unusually pleiotropic molecules. Like other binding proteins, IGFBPs can prolong the half-life of IGFs via high affinity binding of the ligands. In addition to functioning as simple carrier proteins, serum IGFBPs also serve as regulators of the endocrine and paracrine/autocrine actions of IGF by modulating free IGF amount capable of binding to IGF receptors [54]. Furthermore, IGFBPs can function as growth modulators independent of IGFs. It is stipulated that the binding of IGFBP to its putative receptor on the cell membrane may stimulate the signaling pathway independent of an IGF receptor. In addition, IGFBPs may be transported into the nucleus thus exerting IGF-1 independent effects by transcriptional activation of certain genes [55]. For example, IGFBP-5 stimulates markers of bone formation in osteoblasts [54].

The major IGFBP isoform in the circulation is IGFBP-3 [56]. Indeed, in humans, almost 80% of circulating IGF-1 is carried by IGFBP-3, which is regulated mainly by GH but also to some degree also by IGF-1 itself [57-59]. The other major IGFBP isoform is IGFBP-1. IGFBP-1 is a 25 kDa protein that is produced predominantly by hepatocytes and decidualized ovarian endometrium [60]. Its serum levels are controlled by insulin with the postprandial increase in insulin levels producing a four- to fivefold decrease in IGFBP-1 levels relative to fasting levels [61]. IGFBP-1 levels have been shown to be elevated in Type 1 diabetics, while Type 2 diabetics tend to have low serum IGFBP-1 levels. IGFBP-1 levels are regulated in an opposite manner compared to IGF-1 with high levels in the fetus and newborn, which decline steadily until puberty [62].

Figure 2. The similarities between the structures of insulin, IGF-1 and IGF-2 (from [44, 63, 64]).
1.2 The structure of the insulin and IGF receptors

The insulin and IGF-I receptors belong to the family of Type II tyrosine kinase receptors. Both the IR and the IGF-1R have disulfide-linked heterotetrameric (α2β2) structures consisting of two extracellular α-subunits that contain the ligand-binding domain, and two transmembrane β-subunits with ligand-sensitive tyrosine kinase activity [65]. Upon ligand binding the intracellular kinase domain is activated by *trans*-phosphorylation [66, 67]. The IGF-1R has a very similar organization, with sequence similarity varying from 41% to 84%, depending on the domain. The greatest homology (over 80%) between the two receptors is found in the tyrosine kinase domain while differences in the ligand-binding domain (overall homology 44–60%) account for ligand specificity. The greatest sequence divergence between the two receptors is found in their C termini (44% identity) [66, 68].

The human IR gene maps to chromosome 19 spanning more than 150 kb and consists of 22 exons. The 11 exons encoding the alpha subunit of the receptor are dispersed over greater than 90 kbp, whereas the 11 exons encoding the beta subunit are located together in a region of approximately 30 kbp [69]. Exon 11 is alternatively spliced resulting in two isoforms (A and B) which differ by the absence or presence of a 12-residue amino-acid sequence at the C-terminus of the extracellular α-subunit [69]. It has been shown that the exon 11-containing (A) isoform is enriched in embryonic tissues and that it binds IGF-2 with high affinity [70-72]. The amino acid sequences for the human and mouse pro-receptors exhibit about 95% identity at the amino acid level [73-76]. The receptors are synthesized as single-chain pre-pro-receptors that have a 30-residue signal peptide, which is cleaved co-translationally. The precursor is glycosylated, folded and dimerized under the guidance of the chaperones calnexin and calreticulin before being transported to the Golgi apparatus, where it is processed to yield the mature α2β2-receptor. The structure and overall organization of the IR are shown in Fig. 3.

**Figure 3.** The structure of the insulin receptor (from [77]).
**The human IGF-1R** is the product of a single copy gene spanning over 100 kb of genomic DNA at the end of the long arm of chromosome 15q25–26 [40]. The gene contains 21 exons and its organisation resembles that of the structurally related IR [40, 69]. The IGF-1R gene is expressed by almost all tissues and cell types during embryogenesis and adult life [40, 78]. The similarities and differences between the 3 dimensional structure of the IR and IGF-1R with their respective ligands are shown in Fig. 4.

![Figure 4. Structures of the insulin and IGF1 receptors and their ligands (from [66]).](image)

The three-dimensional structure of the IR (a) and IGF1 receptor (b) as determined by X-ray crystallography. An extended bi-lobed structure comprises the two globular domains (green and orange) with a right-handed β-helix fold that flank the cystein rich (CR) domain (blue). The CR domain comprises an array of disulphide-linked modules that resemble those in the tumour-necrosis factor (TNF) receptor and laminin. A cavity of ~30 Å diameter occupies the centre of the molecule and represents a potential binding pocket. The amino acids that have been determined by alanine-scanning mutagenesis to be important for ligand binding are shown in yellow as van der Waals spheres.

In spite the high degree of sequence and structural homology, and the similarities in their intracellular signaling networks, targeted mutagenesis in mice has shown that the actions of the IR and IGF-1R are quite distinct, with IR mediating mostly metabolic responses and IGF-1R mediating primarily growth [2, 79, 80]. Nevertheless, while the IR can mimic the growth-promoting actions of IGF-1R, IGF-1R cannot fully substitute for the metabolic actions of IR [19, 81, 82]. This is based on three lines of evidence: 1) combined lack of IR and IGF-1R results in greater growth retardation than lack of either receptor alone [81], 2) IR can promote
the growth of mice with combined lack of IGF-1R and IGF-2R [19], and 3) IGF-1 administration cannot fully reverse the metabolic phenotype due to lack of IR [82].

To explain these controversial observations several hypotheses have been advanced [83, 84]. Some have proposed that the different tissue distribution of receptors would influence cellular response [2], while others have argued for a role of hybrid receptors [85], or favored the explanation that the different receptors generate qualitatively different signals, for example, in the subcellular distribution [86] or duration of the stimulus [84]. A complicating factor in the interpretation of these studies is that they were either conducted in nonphysiologic target cells by way of receptor overexpression or were carried out in target cells by inhibition of receptor function with dominant negative constructs.

Other issues, such as cross-reactivity of the ligands for their non-cognate receptor and the formation of hybrid receptors also contributed to the difficulties of indentifying the unique characteristics of IR and IGF-1R-mediated signaling. Thus, in order to examine whether these differences relate to intrinsic signaling properties of the intracellular domains of their respective receptors, chimeric receptors were constructed using the extracellular domain of TrkC, the receptor for neurotrophin-3 and the intracellular domain of either the IR or the IGF-1R creating the TrkC-IR (TIR) and TrkCIGF-1R (TIGR) receptors in 3T3-L1 pre-adipocytes. Neither TrkC nor NT-3 are expressed in 3T3-L1 pre-adipocytes, nor is there any biological response to NT-3 in these cells. Thus, the TIR and TIGR chimeras have allowed determining the relative abilities of the intracellular domains of the IR and IGF-1R to stimulate glycogen versus DNA synthesis in response to comparable degrees of receptor activation by a single ligand. While TIR and TIGR cell lines expressing equal numbers of chimeric receptors showed a similar dose-response relationship in NT-3 stimulated DNA synthesis, NT-3 stimulated glycogen synthesis was three times greater in TIR than in TIGR cells providing strong support for the existence of intrinsic differences in the signaling properties of the intracellular domains of the IR versus the IGF-1R.

Another explication for the differences between the IR and IGF-1R was suggested by studies establishing a preferential coupling of the IR receptor to IRS-2 and IGF-1R to IRS-1 [2, 3]. In fact, ablation of IR in hepatocytes resulted in a selective loss of IRS-2 phosphorylation, in the absence of detectable changes in IRS-1 phosphorylation [3, 87]. This conclusion was also reinforced in vivo by data derived from studies of mice with targeted ablation of IRS-1 and IRS-2 [3]. Indeed, the phenotype of IRS-1 knock-out mice suggested that IRS-1 plays a more important role in mediating growth than metabolic responses. By contrast, mice lacking IRS-2 developed lethal diabetic ketoacidosis as a result of combined insulin resistance and insulin deficiency, indicating that IRS-2 plays a crucial role in the
regulation of metabolic homeostasis [88]. Substrate selection, however, is likely to be a more complex event as indicated by studies of mice with combined null mutations of the IR and IRS-1 genes, which develop insulin resistant diabetes with significantly higher frequency than mice heterozygous for each individual mutation [87].

Taken these studies together, it seems that the specificity of IR and IGF-1R is defined at several levels, probably triggered by unique intrinsic features of the each receptor kinase followed by multiple subtle but significant and probably sequentially amplified differences in their signal transmission mechanisms.

IGF-2R, also known as the cation-independent mannose-6-phosphate receptor (M6PR), is a large gene composed of 48 exons over 138 kilobases on human chromosome 6q26 [89]. It is found ubiquitously in human tissues with a truncated soluble form of the receptor present in the circulation [90-92]. The full length, 300-kDa IGF-2R comprises a large N-terminal extracellular region of 15 homologous domains, a single membrane-spanning region and a small cytoplasmic tail. In addition to IGF-2 binding, major IGF-2R functions include sorting newly synthesized lysosomal enzymes and endocytosis of extracellular lysosomal enzymes [90, 93]. To perform these disparate functions, the extracellular region contains separate binding sites for IGF-2 and phosphomannosyl residues [92, 94]. IGF-2R sequesters IGF-2 for internalization and degradation. Thus, IGF-2R is classed as a growth inhibitor, with loss of function causing increased growth [90, 95]. In line with this classification, IGF-2R mutations have been found in several cancers [90, 96]. Moreover, unusually high levels of circulating IGF-2 and simultaneous downregulation of IGF-2R were found to be correlated with the growth of human and murine tumors [90, 97, 98].

The schematic comparison of IR, IGF-1R and IGF-2R is depicted in Fig. 5.
Figure 5. Specific functions of the IR, IGF-1R and IGF-2R (Adapted from [99]).

The IGF-1R receptor is structurally very similar to the IR. It has a high affinity for IGF-1, a slightly lower affinity for IGF-2 and a low affinity for insulin. The primary effect of IGF-1R activation is growth promotion with a lesser effect on the metabolic regulation. The IGF-2R/mannose-6-phosphate receptor consists of a single chain and only binds IGF-2 for internalisation and degradation.

1.3 Intracellular signaling by the insulin and IGF-1 receptors

As described above, insulin and IGF-1 have distinct physiological effects which are partially due to differences in receptor structure and expression, and preferential usage of certain intracellular signal transmitters. However, though the individual members of the signaling cascade are used by the IR and the IGF-1R in an unequal fashion, their signal transduction networks bear a very close resemblance to one another.

Binding of insulin/IGF-1 by the cell surface domains of their respective receptors results in a conformational change in the juxtapositioned cytosolic β subunits, leading to autoactivation of its tyrosine kinase activity and transphosphorylation of the tyrosine residues in one cytosolic β subunit by the other β subunit. The outcome of this trans-phosphorylation is a further increase in kinase activity and additional phosphorylation at the juxta-membrane regions and in the intracellular tail. This phosphorylation cascade produces conformational changes presenting binding sites for the recruitment of IRS proteins and Shc through their phosphotyrosine binding (PTB) or Src homology 2 (SH2) domains [100-102]. The IRS protein family consists of at least four members: IRS-1, -2, -3 and -4. These proteins contain a pleckstrin homology (PH) domain at their N terminus followed by a PTB and a large C-
terminal domain. IRS proteins, following recruitment and phosphorylation by the IR, can on their turn bind to specific SH2 domain-containing proteins including phosphatidylinositol PI3-kinase (IRS1 and IRS-2) and growth factor receptor binding protein 2 (Grb-2) (preferentially IRS-1) [101, 103-105]. The other major direct substrate of the IR, Shc, is involved in the recruitment of Grb2 and the Son of Sevenless (SOS), leading to the high affinity activation of the Ras/Raf/MEK cascade which at the end phosphorylates and activates the p42/44 mitogen activated kinase (p42/p44 MAPK). The p42/44 MAPK pathway is considered primarily to be responsible for insulin’s mitogenic effects. By contrast, most of insulin’s metabolic effects, including stimulation of glucose transport, protein and glycogen synthesis, inhibition of neoglucogenesis and lipolysis are mediated through the PI3-kinase/Akt pathway [103, 106]. PI3-kinase is activated following the binding of the SH2 domain of its p85 regulatory subunit to the tyrosine-phosphorylated IRS-1/2, resulting in the activation of the p100 catalytic subunit [106-108]. This activation triggers the production of phosphorylated phosphatidylinositides (e.g. PI_3,4,5,P3), which then activate the downstream protein serine/threonine kinase Akt/PKB, via 3-phosphoinositide-dependent protein kinase-1 and -2 (PDK1/2) [109, 110]. Activated Akt phosphorylates and inactivates glycogen synthase kinase-3 (GSK-3), thereby leading to a reduced level of phosphorylation of glycogen synthase and to increased glycogen synthase activity [88, 111]. Akt activation also leads to nuclear exclusion (inactivation) of the transcription factor FoxO1 leading to decreased transcription of neo-glucogenic genes and and the suppression of the neo-glucogenic process. Regarding glucose transport, activation of PI3-kinase and its downstream targets, the atypical Protein Kinase C, PKCξ and/or Akt plays a crucial role in the translocation of the insulin-responsive glucose transporter-4 (GLUT-4) [112-114]. Akt-mediated signaling is also crucial for insulin’s protein synthesis enhancing effect through the phosphorylation and inhibition of Tuberous Sclerous Complex 1 and 2 (TSC1/2), triggering the phosphorylation and activation of mammalian Target of Rapamycin (mTOR). mTOR, in turn, phosphorylates and inactivates eukaryotic Initiation Factor 4E Binding Protein 1 (4E-BP1) of the eIF4E/BP1 complex, thereby releasing BP1 from eIF4E and stimulating the initiation of translation [115-117]. Thus, PI3-kinase/Akt-mediated signals play a central role in transmitting insulin’s effect on glycogen and protein synthesis, as well as cellular glucose uptake [70].

Negative regulation of the IR signaling cascade is achieved at the level of receptor tyrosine phosphorylation by different phosphotyrosine phosphatases (PTPs) and at the level of p44/42 MAPK by the serine phosphatase MAPK phsophatase 172 (MKP1/2).

A schematic illustration of the main insulin receptor pathways is shown in Figure 6.
The anti-apoptotic and pro-survival effects of the IGF-1 receptor are mainly regulated through the PI 3-kinase/Akt signaling pathway. A primary target of this pathway is the Bcl-2 family member, Bad [118]. In its non-phosphorylated state, Bad localizes to the mitochondrial membrane where it interacts with Bcl-2 and prevents Bcl-2 from performing its anti-apoptotic functions. Once phosphorylated by Akt, Bad associates with the cytosolic protein 14.3.3 and becomes unable to interfere with Bcl-2 [119]. Akt can also prevent the initiation of the apoptosis-inducing caspase cascade through phosphorylation and inactivation of caspase 9 [120]. In addition, Akt also phosphorylates several pro-apoptotic members of the forkhead (FKH/FoxO1) transcription factor family thus inducing their nuclear exclusion resulting in the inhibition of their activity [121, 122]. In the unphosphorylated state FoxO factors bind to the promoters of various pro-apoptotic target genes, including BCL-6, Bcl-xL, Fas ligand and p53 [123, 124]. Downregulation of p53 is particularly relevant to preventing apoptosis, as p53 can cause a decrease in expression of the IGF-1R and upregulate the pro-apoptotic Bax protein [125]. A further protective pathway activated by Akt involves inhibition of glycogen synthase kinase 3β [126]. In addition to the inhibition of pro-apoptotic molecules, the activity of Akt
also increases the levels of anti-apoptotic proteins including Bcl-2, Bcl-x and the transcription factor NF-κB [122, 127, 128].

Figure 7. Cellular targets of IGF-1R anti-apoptotic and pro-survival effects (Adapted from [129]).

1.4 Liver-specific effects of the insulin and IGF-1 receptors

Insulin is the primary anabolic hormone promoting the storage of energy in the fed state. In response to nutrient secretagogues, insulin is secreted from the pancreatic β cells directly into the portal circulation. High portal insulin levels prime the liver for rapid alterations in hepatic carbohydrate and lipid homeostasis, e.g. i) stimulation of glycogenogenesis, lipogenesis, and lipoprotein synthesis, ii) suppression of neoglucogenesis and glycogenolysis and iii) the secrtaion of the very low density lipoprotein (VLDL) [130-134]. Many of these effects are mediated by regulation of gene expression with insulin stimulating genes that encode glycolytic and lipogenic enzymes and suppressing genes that encode gluconeogenic enzymes [130, 135]. Insulin also stimulates phosphorylation/dephosphorylation of many metabolic enzymes thus modifying their activity, e.g. acetyl-CoA carboxylase, pyruvate dehydrogenase and glycogen synthase kinase-3β [130, 136-140]. Hepatic insulin action is crucial for whole body glucose homeostasis; as long as hepatic insulin action is preserved, mice are protected
from diabetes [12, 141]. Besides its metabolic action, hepatic IR also plays an important role in the degradation and clearance of insulin. Thus, hepatic insulin resistance and the resulting decrease in insulin clearence may in part account for the hyperinsulinemic state found in Type2 diabetes [12].

The direct effect of insulin receptor on hepatocyte metabolism and proliferation was studied in hepatocytes derived from IR knock-out mice (IRKO hepatocytes). IRKO hepatocytes lack the ability to promote glucose oxidation and glycogen synthesis, and to inhibit glucose production. IRKO hepatocytes displayed decreased IRS-2 phosphorylation suggesting that the metabolic effects of insulin in liver are mainly transmitted by IRS-2; a conclusion also supported by independent studies of mice with targeted ablation of the IRS-2 gene [2, 3, 88, 142, 143]. The differences between IR and IGF-1R signaling were highlighted by data showing that IGF-1R overexpression in IRKO hepatocytes restored glycogen synthesis to control levels while overstimulating cell growth. These findings are consistent with a model in which IR mediate metabolic signaling more efficiently than IGF-1R, whereas the latter is a more potent stimulator of cell proliferation [50].

The role of the insulin receptor in cell survival was also demonstrated in the same IRKO hepatocytes [144]. Indeed, the results of Nevada et al. indicated that IR deficiency was associated with accelerated apoptosis in response to withdrawal of growth factors. Immortalized WT and IRKO hepatocytes expressed similar levels of IGF-1R indicating that the lack of IR cannot be fully compensated by IGF-1R-mediated signaling. In line with these observations, insulin-mediated phosphorylation of Akt and p44/42MAPK, two major survival pathways in mammalian cells, was virtually absent in IR-deficient hepatocytes [145, 146]. Moreover, IRKO cells displayed a decrease in the anti-apoptotic Bcl-xL and an increase in the pro-apoptotic nuclear FoxO1 leading to caspase-3 activation, DNA laddering and cell death [144]. The increased apoptosis seems to be linked to the lack of IRS-2-mediated signaling and was shown to be possible to overcome by enhanced signaling through another survival factor, epidermal growth factor (EGF). In IRS-2-deficient hepatocytes EGF stimulated a substantial activation of PI3-kinase/Akt leading to downregulatoin of the proapoptotic genes (Bim and nuclear FoxO1), and the upregulation of the anti-apoptotic Bcl-xL. As expected, these effects were accompanied by attenuated caspase-3 activation and DNA fragmentation [147].

IGF-1 plays an essential in liver regeneration in response to liver injury or mass reduction through promoting hepatocyte transition from G1 to M phase of the cell cycle [148]. Quiescent hepatocytes express little IGF-1R but high levels of IGF-1 production upon partial hepatectomy, together with a moderate increase in IGF-1R expression, seems to be sufficient for significant biological IGF-1 action [18]. In line with these in vivo data, the disruption of
IGF-1R gene in hepatocytes in vitro led to delayed cell cycle progression from G1 to S phase and this effect seems to be due to the lack of cyclin D up-regulation [149, 150]. The activation of IGF-1R-dependent signaling pathways may also enhance the action of other mitogens. Consistent with this, IGF-1 has been identified as a comitogen for hepatocyte growth factor (HGF) in murine hepatocellular carcinomas [151], and EGF induction of p42/44 MAPKs in rat hepatocytes has been shown to require IGF-1R transactivation by EGFR [152].

In vivo, liver specific deletion of the IR and IGF-1R resulted in marked phenotypic differences. Liver-specific IR knock-out (LIRKO) mice exhibited age-dependent insulin resistance, glucose intolerance, and a failure of insulin to suppress hepatic glucose production and to regulate metabolic gene expression [130]. In addition, LIRKO mice displayed marked hyperinsulinemia owing to a combination of decreased insulin clearance and increased insulin secretion. Thus, the presence of the IR in liver is critical in regulating glucose homeostasis, facilitating insulin clearance, and maintaining normal hepatic function [11].

Liver is a unique organ concerning its high capacity to regenerate after surgical removal of a large portion of its mass (partial hepatectomy) and IGF-1 seems to play an important role in this process. To gain a better insight into the direct role of IGF-1R in hepatocytes a liver specific IGF-1R-deficient (LIGFREKO) mouse was created [18, 153]. Livers of LIGFREKO males displayed impaired hepatocyte proliferation along with an abrogation of cyclin D1 and A induction [18, 149-151, 154]. The marked decrease in p42/44 MAPK phosphorylation in LIGFREKO livers may also contribute to diminished hepatocyte proliferation. The livers of LIGFREKO mice showed abundant expression of IRS-1, but not of IRS-2, suggesting that the production of IRS-1, but not that of IRS-2, is controlled by IGF-1R-dependent pathways. These data also support the idea that IRS-1 is a major substrate of IGF-1R in liver. As the up-regulation of IRS-1 is strongly associated with liver regeneration [155-157], the abundance of IRS-1 in quiescent LIGFREKO livers may prevent the subsequent induction of IRS-1 production, thereby affecting IRS-1-dependent proliferative responses. Thus, these findings indicate that intact growth hormone/IGF-1/IGF-1R signaling are required for physiological liver regeneration. In this process, IRS-1, rather than IRS-2, seems to be responsible for transduction of the proliferative response downstream from IGF-1R in the regenerating liver, via the activation of p42/44MAPK, cyclin D1 and cyclin A [18, 158].

Liver regeneration in rodents depend on sex [18, 159, 160], with females having a delayed DNA synthesis peak [18, 161]. Interestingly, this sex-related difference in hepatocyte regeneration disappeared in mice lacking hepatic IGF-1R, suggesting that sex-dimorphism in liver regeneration may involve signal transduction via IGF-1R. A similar IGF-1R-dependent
dimorphism has been shown to be related to aging and a resistance to oxidative stress [18, 153, 162, 163].

The summary of the phenotypes of the different whole body and liver specific knock-out mice are provided in Table 1. (Summarized from [10-15, 18, 81, 117, 130, 163]).

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<th>Whole body knock-out</th>
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<td>Insulin+ IGF-1 receptor</td>
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<td><strong>Growth</strong></td>
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<td>abnormalities**</td>
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<td><strong>Abnormalities</strong></td>
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<td>Respiratory failure</td>
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Table 1. Comparison between the general and liver specific IRKO and IGF-1R KO mouse models
2. MODULATION OF THE INSULIN AND IGF-1 RECEPTOR SIGNALING BY REACTIVE OXYGEN SPECIES

2.1 Reactive oxygen species (ROS)
Reactive oxygen species (ROS) are highly diffusible, rapidly generated but also easily degraded molecules or ions formed by the incomplete reduction of oxygen. ROS are ubiquitously present in all cell types. The more reactive the molecule/ion is, the shorter its half-life, as it more readily reacts with neighboring molecules. Chemically ROS can be classified as radicals (containing one or more unpaired electron(s) in their outer molecular orbitals) or non-radicals (not having unpaired electron(s)). Non-radicals can be converted into a radical form. The major types of biologically important cellular ROS are: superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), peroxy radicals (ROO$^-$), organic hydroperoxide (ROOH), hydroxyl radical (HO$^-$) and hypochlorous acid (HOCl) [27].

2.2 Negative effects of ROS
2.2a The concept of oxidative stress
ROS is produced by these different cellular organelles in a continuous manner but are promptly eliminated by anti-oxidant enzymes. In physiological conditions these two processes are in balance. A disturbance in cellular redox balance through uncontrolled, excessive ROS production or decreased ROS elimination leads to the onset of oxidative stress triggering cellular and tissue damages [31, 164]. Due to these damaging actions ROS are considered cytotoxic, and believed to be implicated in the progression of cancer, inflammation, radiation injury and aging [165, 166]. Large amount of ROS can be produced as a by-product through excess and thus uncontrolled activity of mitochondrial, microsomal, peroxisome or endoplasmic reticulum (ER) enzymes [27, 167].

Oxidative stress may cause changes in insulin signal transmission at different levels. Indeed, enhanced ROS accumulation lead to an increase in the inhibitory serine phosphorylation of IRS-1 and IRS-2 resulting in insulin resistance in 3T3-L1 adipocytes [168]. Exposure of different other insulin-responsive cell lines to micromolar concentrations of H$_2$O$_2$ triggered the activation of various stress activated kinases e.g. the c-JunN-terminal kinase (JNK/SAPK), p38MAPK and IκB kinase. The activation of these kinases was accompanied by a down-regulation of the cellular response to insulin manifested in a reduced insulin-stimulated glucose uptake, and attenuated glycogen, lipid and protein synthesis [169]. Persistently elevated ROS can activate redox sensitive transcription factors e.g. NF-κB, the NF-E2 related
factor-2 (Nrf2) and activator protein-1 (AP-1), which may act as molecular switches to turn normal cells into premalignant cells, with subsequent clonal expansion to form solid tumors.

In vivo studies suggested that hyperglycemia-induced oxidative stress occurs before late phase diabetic complications become clinically evident, indicating that oxidative stress plays a crucial role in the pathogenesis of these abnormalities [29]. One major intracellular target of hyperglycemia and oxidative stress is the transcription factor NF-κB [170, 171]. NF-κB plays a critical role in mediating immune and inflammatory responses and apoptosis. The aberrant regulation of NF-κB is associated with a number of chronic diseases including diabetes and atherosclerosis. The best experimental proof of the importance of NF-κB-mediated inflammatory signaling in the development of insulin resistance was provided by the study showing that genetic ablation of NF-κB signaling protected mice against the development of high fat diet induced glucose metabolic disorders [172].

2.2b Hyperglycemia-induced oxidative stress and insulin resistance

Elevation in serum glucose levels increases the input of metabolic substrates into mitochondria leading to an increase in the amount of the electron transfer donors NADH and FADH$_2$. This excess enhances electron flux through the mitochondrial inner membrane electron transport chain (respiratory chain) and generates a higher proton threshold gradient across the mitochondrial membrane. These alterations inhibit the electron transfer between the members of the respiratory chain, notably between coenzyme Q (CoQ) and complex III. Electrons transferred to CoQ are instead released within the mitochondria and used to generate superoxide radicals from molecular oxygen [173]. Mitochondria, therefore, are the principal source of ROS in cells as the result of imperfectly coupled electron transport.

When calorie intake exceeds energy expenditure, the substrat-induced increase in citric acid cycle activity generates an excess of mitochondrial ROS leading to alterations in the cellular redox environment. Increased mitochondrial ROS production plays a critical role in the induction of the apoptotic cascade [1]. Furthermore, hyperglycemia-induced overproduction of superoxide significantly inhibits glucose-6-phosphate dehydrogenase (G-6PDH), the rate-limiting enzyme of the pentose phosphate pathway. The pentose phosphate pathway is induced when intracellular glucose availability exceeds that of the capacity of glycolytic enzymes and is the major source of cellular NADPH. NADPH is the cell’s principal reductant providing reducing equivalents for the glutathione peroxidase (Gpx)–glutathione reductase (GR) system [5]. The glutathione system transforms oxidized glutathione (GSSG) into reduced glutathione (GSH) thus providing protection against intracellular ROS accumulation and the onset of oxidative stress [174]. A decrease in the amount of NADPH
results in a decrease in reduced glutathione levels thus diminishing the cell's capacity to "buffer" oxidant radicals.

Increased serum glucose levels also lead to the formation of advanced glycation end-products (AGEs) triggering the non-enzymatic glycation of structural proteins and enzymes [175]. One of the most important AGE is methylglyoxal. Methylglyoxal is 15 to 20 thousand times more likely to react with other molecules than is glucose. Methylglyoxal often reacts with proteins including mitochondrial proteins, but it can also adhere to DNA inducing mutations [176]. In addition to inducing ROS formation, long-term hyperglycemia also provokes enhanced beta cell insulin secretion and the onset of hyperinsulinemia. Hyperinsulinemia leads to elevation in glucose uptake thus provoking a vicious cycle and increasing the steady-state level of ROS production and triggering the onset of oxidative stress. Indeed, several studies demonstrated that hyperglycemia and diabetes mellitus are associated with an exponential increase in cellular ROS production [29, 177, 178]. The process of the development of glucose-induced oxidative stress is depicted in Figure 8.

Figure 8. The mechanism of hyperglycemia-induced oxidative stress.
Glucose is transported into the cells and is oxidized by glycolitic enzymes leading to the production of NADH and FADH$_2$. The transfer of electrons through the mitochondrial respiratory chain is coupled with ATP synthesis by the ATP synthase which also generates superoxide anions. Elevated glucose levels overwhelm the mitochondrial respiratory chain capacity leading to elevated ROS production. Glucose overload also stimulates insulin production, which, in turn, increases the glucose uptake initiating a vicious cycle.

2.1c Defense against ROS-mediated alterations by IGF-1R signaling

ROS-dependent signals have also been linked to defects in genomic maintenance systems and the aging process; processes that are mainly regulated by IGF-1 signals [179]. The activated IGF-1R transmits a powerful survival signal in several cell lines and is a critical determinant of growth and development. For example, activated IGF-1R rescues mesangial cells from hyperglycemia-induced danger signals that target genomic DNA by suppressing ROS and enhancing DNA repair [180]. Interestingly, the IGF-1R and the adaptor protein p66Shc, a key IGF-1R signaling molecule, have both emerged as major genetic determinants of lifespan and a protection against oxidative stress in mammals [163].

The DNA double helix is a major target for ROS-dependent signals. ROS-induced DNA damage (genotoxic stress) includes a wide range of alterations, from base modifications to single-strand breaks (SSB) and potentially lethal double-strand breaks (DSB) [179, 181]. In mammals, two major cellular responses to genotoxic stress are apoptosis and cellular senescence [182, 183]. Apoptosis eliminates severely damaged cells, whereas senescent cells undergo growth arrest without dying and may acquire altered functions that can disrupt tissue homeostasis [179]. Several lines of evidence suggest that IGF-1R’s antioxidant function plays a key role in protecting genomic DNA thus maintaining cell viability and preserving genomic integrity [180]. The molecular basis for the antioxidant function of the IGF-1R has not been yet been fully uncovered, however, the activated IGF-1R induces a strong oxidant-resistant phenotype that reflects the inhibition of ROS production in cytosolic and mitochondrial compartments [178]. Among the downstream targets of the IGF-1R signaling pathway are the Bcl-2 proteins, Bcl-2, and BclxL, which function as free radical scavengers and also inhibit the mitochondria permeability transition pore [184]. IGF-1R-dependent signals increase the availability of Bcl-2 and BclxL by downregulating the expression of the proapoptotic molecule Bax, and by phosphorylating (inactivating) Bad [119]. The importance of IGF-1-mediated signals was demonstrated by data showing that DNA repair mechanisms are not sufficient to prevent progression to apoptosis in the absence of the IGF-1R antioxidant and prosurvival gene program [180].
2.1d ROS-mediated hepatic injury

Concerning the liver in specific, changes in the redox homeostasis are commonly detected in patients with alcohol abuse, hepatitis C virus infection, iron overload and chronic cholestasis, as well as in liver fibrogenesis [185, 186].

Exposure to alcohol leads to increased hepatic ROS generation by activation of microsomal enzymes [187, 188]. ROS may activate signaling cascades leading to hepatic fibrosis and may have direct toxicity on hepatocytes [189, 190]. However, while a role of ROS in alcohol toxicity is widely accepted, there is disagreement concerning whether the predominant source is a cytochrome P-450 (CYP2E1) [26, 191, 192] or an NADPH oxidase [188, 193]. Indeed, in alcohol-induced liver disease (ALD), a macrophage NADPH oxidase appears to be a key oxidant, as mice lacking p47phox, a cytoplasmic component required for the activation of the NADPH oxidase complex, do not develop alcoholic liver disease [188]. Liver resident macrophages, called Kupffer cells, account for 70-80% of total body macrophages and provide the first line of defense for hepatocytes. Kupffer cells offer defensive mechanisms against invading microorganisms and function as the major site for endotoxin clearance [26, 164, 194]. In chronic alcoholic hepatitis, Kupffer cells have enhanced sensitivity to endotoxin and become chronically activated [195]. Once activated, Kupffer cells release a number of biologically active compounds including proteases, cytokines and growth factors (i.e. TNF-α, TGF-β, IL-1 and IL-6), proinflammatory lipid mediators and most importantly ROS. The massive release of these inflammatory molecules, along with lysosomal enzymes induces hepatocyte necrosis and liver inflammation [196, 197]. Liver fibrosis is the common consequence of chronic liver injury and is closely related to the formation of ROS [198, 199]. The role of NADPH oxidase in this process was suggested by data demonstrating that p47phox-deficient mice show preservation of the hepatic architecture and less fibrosis following bile duct ligation [200]. ROS-mediated cellular damage also plays a role in the development of hepatic cancer in a variety of rodent models including mice overexpressing c-myc and TGF-α [201] and exposure to diverse carcinogens [202, 203]. In this context, the NADPH oxidase is considered to play an important role [204, 205]. Moreover, increased NADPH oxidase activity has also been implicated in the progression of nonalcoholic fatty liver disease (NAFLD) [206].

As mentioned earlier, redox stress can also develop when the rate of ROS elimination is diminished and cannot match that of production. Indeed, the lack of superoxide dismutase (SOD), a potent anti-oxidant enzyme resulted in hepatocyte damage as demonstrated by the phenotype of the SOD2-deficient mice [207]. The significance of the maintenance of proper cellular redox balance in human liver physiology was underlined by findings which demonstrated reduced activities of several anti-oxidant enzymes (e.g. SOD and catalase) in
cirrhotic tissues compared to healthy liver [208]. The summary of ROS-mediated liver injury is depicted in Figure 9.

Figure 9. ROS-induced liver damage in obesity and insulin resistant states (From [209]).
Increased fatty acid concentration and cellular oxidation will lead to elevated ROS production in the mitochondrial respiratory chain (MRC), in the peroxisomes by the acetyl-coA oxidase (AOX) and in the endoplasmatic reticulum by the cytochrome enzymes (CYP). Increased ROS production will lead to hepatic inflammation, fibrosis and hepatocyte death.

2.3 Positive effects of ROS
2.2a Intracellular signaling through ROS generation
Recently, the role of ROS production in cellular homeostasis has been given a new consideration. In fact, keeping a balanced cellular redox environment plays a critical role in maintaining several physiological functions, most notably the regulation of metabolism, and cell proliferation and differentiation [1, 173]. This role is achieved by a controlled, low level generation of ROS which can then serve as second messengers activating multiple intracellular
signaling pathways that have key roles in cell biology and metabolism [25, 210]. As a proof of this concept, ROS are known to be generated in response to a variety of hormones, growth factors and cytokines, e.g. platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and angiotensin II [211] [212, 213] and have been demonstrated to be capable of modifying the activity of signaling proteins such as protein kinases, phosphatases and ion and/or proton channels [214-216].

Reversible tyrosine phosphorylation plays an essential role in the regulation of transmission of the insulin signal at receptor and postreceptor sites in its intracellular pathway. The protein-tyrosine phosphatases (PTPs) are integral to the negative regulation of insulin signaling. Insulin-stimulated ROS generation can inhibit these negative regulators by oxidative biochemical alterations and thus facilitate the insulin signaling cascade. Activation of both the insulin and the IGF-1R involves phosphorylation of three tyrosine residues in the autocatalytic region of the β-domain, and the control of the phosphorylation state of these residues is critical for the propagation of the signal. The phosphatases PTP1B and SHP-1 dephosphorylate these three phosphotyrosines in vitro [217-219] and in vivo [220]. Understanding the subcellular localization of IR dephosphorylation might provide clues as to how the oxidative regulation of PTP activity may enhance receptor signal transduction. One of the sites of interaction between PTPs and the IR is the cell surface, where the receptor is kept inactive in a tonically dephosphorylated state. Here ROS-mediated PTP inhibition may facilitate the initial receptor activation process. However, the major interaction appears to occur after internalization at sites involving endosomes and the endoplasmic reticulum. IR activation triggers its internalization through an endosomal compartment accompanied by dynamic changes in receptor phosphorylation and activation state [221]. Within minutes after internalization, the IR is dephosphorylated through preferential interaction with PTP1B residing in the endoplasmic reticulum, rather than with a soluble, cytoplasmic form [222, 223]. Similarly, work with the EGF receptor has demonstrated it to undergo dephosphorylation at specific sites on the surface of the endoplasmic reticulum after ligand-mediated activation and endocytosis [224, 225]. Increasing PTP activation with the inhibitor diphenyleneiodonium (DPI) also suppresses IR activation and several aspects of the downstream insulin signaling cascade [226-228].

ROS production after insulin stimulation generates only a fraction of the ROS concentration observed in phagocytic cells and follows a brief time course, on the order of minutes [229]. These features apparently account for the signaling role of insulin-induced ROS compared with the chronic exposure to ROS in patients with hyperglycemia that is associated with organ dysfunction and the onset of diabetic complications [8, 173]. The low levels of ROS in insulin signaling imply that there must be specific cellular protein targets that are
particularly susceptible to oxidative modification. Importantly, using novel protein labeling techniques, Rhee et al have shown that even in a cellular milieu containing millimolar concentrations of slowly reactive thiols like glutathione, only a limited set of proteins are rapidly oxidized by growth factor–stimulated ROS, including PTP1B and a few other proteins with reactive cysteines, including protein disulfide isomerases, thioredoxin reductase, and creatine kinase [230-232]. The biochemical evidence, therefore, also supports the notion of a discrete network of “redox circuitry” with temporal and spatial influences that are likely to correspond to other regulatory aspects of the insulin action pathway [230, 233-235].

Several phosphatases regulating the insulin / IGF-1 signaling pathways are susceptible to redox modulation. The enzymes and their functions are summarized in Table 2.

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Enzyme</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTP1B</td>
<td>Protein-tyrosine phosphatase 1B</td>
<td>Insulin receptor Tyrosine kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
<td>Akt/PKB PI3K</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein serine/threonine phosphatase 2A</td>
<td>Akt (Ser 473)</td>
</tr>
<tr>
<td>MKP-1/DUSP1</td>
<td>MAP kinase phosphatase-1 Dual-specificity phosphatase 1</td>
<td>P44/42 MAPK</td>
</tr>
</tbody>
</table>

Table 2. Cellular targets of insulin-induced ROS (From [8]).

3. NADPH OXIDASE ENZYMES IN LIVER FUNCTION

The main source of receptor-induced, controlled ROS production has been increasingly attributed to different non-phagocytic NOX isoforms. In liver, several different NOX isoforms have been described though their exact functions in physiological and pathological processes are far from being elucidated. A summary of different NOX enzyme isoforms and of our current knowledge of their role in liver homeostasis is provided in the next two chapters.

3.1 NADPH oxidase enzymes (NOXes)

NADPH oxidase enzymes, NOX-es generate ROS in response to specified external stimuli. The first identified member of this family was the phagocyte NADPH oxidase, later termed NOX2. Currently, seven NOX isoforms, two organizer subunits (p47phox, NOXO1), two
activator subunits (p67phox, NOXA1), and two DUOX-specific maturation factors (DUOXA1 and DUOXA2) have been identified [27].

All NOX family members are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide. In accordance with this preserved function, there are conserved structural properties of NOX enzymes that are common to all family members. Starting from the COOH terminus, these conserved structural features include 1) an NADPH-binding site, 2) a FAD-binding region, 3) six conserved transmembrane domains, and 4) four highly conserved heme-binding histidines. Given the additional NH2-terminal transmembrane domain, the histidines are in the fourth and sixth transmembrane domains in DUOX proteins. Additional features, such as EF hands, an additional NH2-terminal transmembrane domain, and/or a peroxidase homology domain, are limited to some of the family members [27]. Both the phagocytic and nonphagocytic oxidases are multimeric enzymes composed of plasma membrane-associated proteins as well as cytosolic factors [236]. In the phagocytic NADPH oxidase, the plasma membrane-associated proteins gp91phox and p22phox compose the flavocytochrome b558 complex, which forms the catalytic subunit of the oxidase. The cytosolic factors p47phox and p67phox are phosphorylated by agonists such as AKT, ERK1/2 and p38MAPK and translocate to the cell membrane to form the active NADPH oxidase [237].

The characteristics of different NOX isoforms and their general structure are provided in Table 3 and Figure 10.
<table>
<thead>
<tr>
<th>High-Level Expression</th>
<th>Intermediate- to Low-Level Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOX1</td>
<td>Colon</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle, endothelium, fibroblast, uterus, placenta, prostate, osteoclast, retinal pericyte</td>
</tr>
<tr>
<td>NOX2</td>
<td>Phagocyte</td>
</tr>
<tr>
<td></td>
<td>B lymphocyte, neuron, cardiomyocyte, skeletal muscle, hepatocyte, endothelium, hematopoietic stem cell, smooth muscle</td>
</tr>
<tr>
<td>NOX3</td>
<td>Inner ear</td>
</tr>
<tr>
<td></td>
<td>Fetal kidney, fetal spleen, skull bone, brain, liver, lung</td>
</tr>
<tr>
<td>NOX4</td>
<td>Kidney</td>
</tr>
<tr>
<td>Blood vessel</td>
<td>Osteoclast, endothelium, smooth muscle, hematopoietic stem cell, fibroblast, adipocyte keratinocyte, melanoma cell, neuron, islets</td>
</tr>
<tr>
<td>NOX5</td>
<td>Lymphoid tissue</td>
</tr>
<tr>
<td>Testis</td>
<td>Endothelium, smooth muscle, pancreas, fibroblast, placenta, ovary, uterus, stomach, fetal tissue, breast, brain</td>
</tr>
<tr>
<td>DUOX1</td>
<td>Thyroid</td>
</tr>
<tr>
<td></td>
<td>Airway epithelia, tongue epithelium, cerebellum, testis, vascular smooth muscle cell</td>
</tr>
<tr>
<td>DUOX2</td>
<td>Thyroid</td>
</tr>
<tr>
<td></td>
<td>Salivary and rectal glands, gastrointestinal epithelia airway epithelia, uterus, gall bladder, islets</td>
</tr>
</tbody>
</table>

Table 3. NOX isoforms adapted from [27].
3.2 NOX isoforms in the liver

Liver is a complex organ, comprising several cell types, each displaying a distinct pattern of NOX enzyme expression.

3.2a Hepatocytes

In total liver NOX2 and NOX4 mRNAs have been described [27, 238, 239]. Our own analysis suggested that liver has the NOX4 mRNA content about 20% of kidney [240]. Rat hepatocytes express mRNAs of NOX1, NOX2, NOX4, DUOX1 and DUOX2 [241]. Human hepatocytes
alos express NOX1, NOX2, NOX4 [32, 242, 243]. The main function attributed to NOX-derived ROS in hepatocytes is apoptosis [241, 244]. Indeed, two independent studies demonstrated that i) TGFβ-induced hepatocyte apoptosis occurs through Smad3 dependent activation of ROS with subsequent activation of the caspases and ii) TGF-β-induced upregulation of NOX4 is required for its pro-apoptotic activity in hepatocytes [32, 245].

3.2b Hepatic stellate cells
In response to hepatocyte injury a specific set of cells, termed hepatic stellate cells (HSCs) transform from star-shaped vitamin A-rich to myoblast-like, vitamin A-deficient cells and engage in both the secretion and degradation of extracellular matrix [246, 247]. HSCs express key components of the phagocytic NADPH oxidase (p22\textsubscript{phox}, gp91\textsubscript{phox}, p47\textsubscript{phox}, and p67\textsubscript{phox}) at both the messenger RNA and protein levels [248]. However, in freshly isolated human HSCs, the situation seems to be more complex: neither isoform was found in healthy controls, while NOX1, but not NOX2 expression was upregulated in samples from patients with hepatic fibrosis [200]. Proposed functions of NOX-derived ROS in HSCs include the induction of cell proliferation [248-250] and the production of collagen [250, 251].

3.2c Kupffer cells
Kupffer cells are specialized tissue macrophages within the liver. Kupffer cells express the phagocyte NADPH oxidase NOX2 and its subunits [27, 204, 252]. NOX2-derived ROS in Kupffer cells play an important role in clearing pathogens derived from the gut [253]. HSCs are also involved in the regulation of gene expression, in particular in the NFκB-dependent production of TNF-α [254]. Finally, NOX2-derived ROS have been suggested to be essential mediators in antigen presentation by Kupffer cells [255].

3.3 The role of NOX4 in TGF-β receptor signaling in hepatocytes
TGF-β is a homodimeric polypeptide with a molecular weight of 25 kDa. It is involved in many biological and pathological processes including embryonic development, tissue remodelling, inflammation, angiogenesis, atherosclerosis, fibrosis and carcinogenesis [165]. Hepatocytes from normal and cirrhotic livers did not express TGF-β 1. By contrast, the cytoplasm of hepatocytes in neoplastic nodules showed intense staining for TGF-β RI mRNA and protein. TGF-β and related factors induce their response by assembling a heterotetrameric complex comprised of two type I / type II receptor pairs. Type I and type II receptors have the same overall domain structure, including a cysteine rich extracellular domain, a single transmembrane helix, and an intracellular serine-threonine kinase domain. TGF-β type II
receptor is mostly expressed in hepatocytes [256]. For TGF-β receptors, ligand binding involves two steps: initial binding is to TGF-β RII, which induces dimerization of TGF-β RII with TGF-β RI, resulting in phosphorylation and activation of the TGF-β RI and subsequent activation of downstream pathways [257, 258]. Type I receptor phosphorylates the transcription factors Smad2 and 3 which then translocate from the cytoplasm to the nucleus, where they regulate the transcription of target genes [4]. In addition, Smad2/3 bind to transcriptional co-activators and co-repressors, which induce the acetylation and de-acetylation of histones, respectively, and play important roles in transcriptional regulation [259].

In hepatocytes, TGF-β mediates apoptotic signals through transcriptional induction of NOX4 resulting in increased ROS formation, a loss of the mitochondrial transmembrane potential, cytochrome c release and caspase 3 activation triggering hepatocyte cell death [210]. EGFR activation abrogates TGF-β-induced apoptosis through the prevention of NOX4 mRNA upregulation and the induction of survival pathways by the PI3-kinase/Akt pathway [28]. TGF-β also exerts a growth inhibitory effect on fetal hepatocytes, arresting cells in G1 phase through up-regulation NOX4 transcription [4, 260]. The signaling pathway leading to TGF-β-induced apoptosis and the central role of NOX4 in it is depicted in Figure 11.

![Diagram](image)

**Figure 11. Regulation of apoptosis by TGF-β and EGF in foetal hepatocytes (from [4]).**

TGF-β binds to the Type II receptor situated in the plasma membrane triggering the dimerization and activation of the Type I receptor. Activation of the Type I receptor leads to Smad2/3 phosphorylation and nuclear
translocation leading to the transcriptional activation of target genes. Parallel to the activation of the Smad cascade
TGF-β stimulation induces NOX4 transcription leading to subsequent ROS production, mitochondrial membrane
depolarization, cytochrome C release, caspase 3 activation and ultimately apoptosis. EGF hampers TGF-β-
induced apoptosis by preventing up-regulation of NOX4 transcription through a PI 3-kinase-dependent pathway.

In summary, alterations in hepatocyte cellular redox homeostasis both *in vitro* and *in vivo* were shown to be associated with differences in mitogenic capacity and the susceptibility of apoptotic injury. In this context, the role of NOX4 is of critical importance as NOX4 plays a key role in TGF-β-mediated apoptosis induction. Moreover, modulation of NOX4 levels was shown to be closely related to the capacity of EGF to prevent TGF-β-induced hepatocyte death. Insulin and IGF-1 play a central role in hepatocyte mitogenesis and are important factors in hepatocyte survival. Moreover, as demonstrated in the previously, NOX4 is an important regulator of insulin signaling in other insulin-sensitive cells, e.g. adipocytes.

These data prompted us to explore the relationship between the anti-apoptotic effects of insulin /IGF-1 and their capacity to abrogate TGF-β-induced NOX4 transcription.
PART II: THE THESIS IN A NUTSHELL
THE THESIS IN A NUTSHELL

1. State of research
1.1. ROS production is crucial in the signal transduction of the insulin and IGF-1 receptors
1.2. TGF-β-induced upregulation of NOX4 and the following ROS production mediates apoptosis in hepatocytes
1.3. EGF inhibits TGF-β-induced NOX4 expression and apoptosis through PI3-kinase
1.4. Insulin and IGF-1 stimulate PI3-kinase and the mitogen activated protein kinase (MAPK)
1.5. Insulin and IGF-1 have anti-apoptotic effects

2. State of our own research
NOX4 expression is downregulated by insulin during adipocyte differentiation

3. Hypothesis
Insulin and IGF-1-induced downregulation of NOX4 expression plays a role in the prevention of TGF-β-induced apoptosis in hepatocytes

4. Summary of the scientific approach
Comparing NOX4 expression between wild type and insulin receptor knock-out hepatocytes and exploring the relationship between NOX4 expression, TGF-β-induced apoptosis and the anti-apoptotic effects of insulin and IGF-1

5. Expected results
Increased TGF-β-induced apoptosis in the insulin receptor knock-out hepatocytes due to the lack of down-regulation of NOX4 expression by the insulin/IGF-1 receptors
Existing data

EGF → PI3-K → SURVIVAL

TGF-β

Our hypothesis

Insulin/IGF-1 → PI3-K → MAPK

NOX4

H₂O₂

caspase 3 activation

APOPTOSIS
PART III: EXPERIMENTAL RESULTS
1. MATERIALS AND METHODS

1.1 Mouse hepatocyte culture and treatment

SV-40 transformed hepatocytes were derived from WT and IR knock-out mice and were a kind present of Prof. D. Accili (Columbia University, New York, NY) [3]. Cells were maintained in DMEM completed with 4% fetal calf serum, 200 nM dexamethasone, 100 U/ml penicillin, and 10 µg/ml streptomycin at 33°C in 5% CO₂. Cells were used at 70-80% confluency and were serum deprived for 16 hours before treatment by insulin (100nM) (Hoechst AG, Frankfurt, Germany), IGF-1 and IGF-2 (10nM) (RnDSystems, Abingdon, UK), TGF-β (1ng/ml) (Calbiochem, Merck Chemicals Ltd, Nottingham, UK). When indicated, the MAPK inhibitor PD98059 (50µM) or the PI3-kinase inhibitor wortmannin (0.5µM) (Cell Signaling, Allschwil, Switzerland) were applied for 1 hour prior to stimulation.

1.2 RNA isolation, RT-PCR and real-time PCR

Total RNA was prepared by homogenizing cells in TRIZOL Reagent (Invitrogen, Basel, Switzerland) and was purified by using RNase free DNAse in combination with the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland). cDNA was synthesized from 2 µg of DNA-free RNA by Superscript II Reverse Transcriptase (Invitrogen, Basel, Switzerland). PCR products were visualized by ethidium bromide stained agarose-gel electrophoresis. The primers used for conventional RT-PCR are listed in Table 3. Primers and probes for real-time PCR were designed by Primer Express software (Applied Biosystems, Rotkreuz, Switzerland) and are listed in Table 4. Real-time PCR reactions were carried out using the Sybergreen detection method in an ABI 7500 instrument (Applied Biosystems). The results were quantified by the ΔΔCt method using cyclophillin A as the standard internal non-variable gene to compensate for differences in RNA input and efficiency of cDNA synthesis. Results were expressed as arbitrary units compared to the average expression levels in WT control cells.

Table 3. RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
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<tbody>
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<td>Gene</td>
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</tr>
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<tr>
<td>Cyclophillin</td>
<td>caaatgctggacaaacaaacaa</td>
<td>gcatecacegcatctagtct</td>
</tr>
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</table>

Table 4. Real-time PCR primers

1.3 Western blot analysis

Cells were homogenized in RIPA buffer (150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% Deoxycholic sodium salt, 50 mM Tris HCL pH 7.4, 2 mM EDTA, 2 mM Na$_3$VO$_4$, 10 mM NaF, one tablet of Complete Inhibitor Cocktail (Roche Diagnostics, Rotkreuz, Switzerland) in 30ml). Protein concentration was measured by bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Lysates were dissolved in Laemmli buffer (10 mM sodium phosphate - pH 7.0, 0.1% glycerol, 2% SDS, 100 mM DTT and a trace of BPB) and were resolved on a 5-20% gradient polyacrylamide gel. Gels were transferred onto nitrocellulose membranes (GE Healthcare, Otelfingen, Switzerland). Non-specific binding of the antibody was prevented by blocking the membranes with 0.001% polyvinyl alcohol (PVA) followed by incubation with the respective primary antibodies overnight at 4°C. Antibodies are listed in Table 5. Blots were then washed 3 times for 20 minutes at room temperature with TBS supplemented with 0.1% Tween20, and subsequently incubated with the applicable secondary horseradish peroxidase (HRP)-conjugated antibody (Sigma-Aldrich, Buch, Switzerland). Signals were revealed by enhanced chemiluminescence (ECL) and were recorded in ChemiDoc™ XRS system (Bio-Rad Laboratories, Reinach, Switzerland). Quantification of the detected bands was performed
by using the Quantity One program (Bio-Rad Laboratories, Reinach, Switzerland). Protein expression was related to the amount of actin as a non-variable reference protein and was expressed as arbitrary units compared to the average expression in wild type control cells.

Table 5. List of antibodies

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cat. #</th>
<th>Concentration</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS1</td>
<td>#2382</td>
<td>1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>IRS2</td>
<td>#4502</td>
<td>1:1000</td>
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1.4 Detection of apoptosis by Fluorescence Activated Cell Sorting (FACS)

Cells were starved overnight and subsequently stimulated with TGF-β (1ng/ml) for 24 hours. When indicated, cells were preincubated with IGF-1 (10 nM) or IGF-2 (10 nM) for one hour prior the addition of TGF-β. Cells were trypsinised and resuspended in 1x binding buffer (supplied in the kit) and stained with annexin V-Cy3 and Sytox green dye for 5-10 minutes at room temperature in the dark (Annexin V-Cy3 apoptosis detection kit plus, Biovision, Lausen, Switzerland). Stained cells (at least 100000 cells) were analyzed by FACS using FL1 channel for Sytox green dye (Excitation wavelength (Ex) = 488nm and Emission wavelength (Em) = 530nm) and FL2 channel for Annexin V-Cy3 (Ex = 543nm and Em = 570nm). The cell population was separated into three groups, live cells with no or low amount of fluorescence, apoptic cells with red fluorescence and necrotic cells with green fluorescence in three different zones.

1.5. Statistical analysis

Data are expressed as mean ± SEM. Results were analyzed by using ANOVA or Student’s t test, or in case of non-parametric data distribution by Mann-Whitney test using the Sigma Stat software (version 2.0 – SPSS, Chicago, IL). p values less than 0.05 were considered significant.
2. RESULTS

2.1 Molecular characterization of the IR and IGF-1 receptor signaling in wild type and IR-deficient hepatocytes

To characterize our hepatocyte model cells with respect to their main insulin signaling pathways, we compared the expression levels of the IR, its major substrates, IRS-1 and IRS-2, and the IGF-1R by Western blot and real-time PCR analyses (Fig. 1). Wild type cells showed high levels of IR expression which was abolished in the KO cells. The two major hepatocyte IRS isoforms, IRS-1 and IRS-2 displayed differential regulation in response to the lack or presence of the IR, confirming results of previous studies [79, 105]. In fact, IRS-1 showed decreased, while IRS-2 showed increased expression in the IRKO cells. By contrast, IGF-1R expression levels were similar in WT and IRKO cells.

Figure 1. Expression of molecules related to insulin signaling in WT and IRKO hepatocytes

Insulin receptor (IR), insulin receptor substrate 1 and 2 (IRS-1, IRS-2) and insulin like growth factor 1 receptor (IGF-1R) expression was determined by Western blot and real-time PCR analyses. Protein and mRNA levels are expressed as arbitrary units compared to the average levels of WT cells and depicted in the graphs as mean±S.E.M. Data are derived from three independent experiments using duplicates or triplicates in each experiment. *=p≤0.05, **=p≤0.01 and ***=p≤0.001 IRKO vs. WT cells. A representative Western blot image is shown for each protein.
2.2 Expression of NOX/DUOX enzyme isoforms in wild type hepatocytes and modification by the deletion of the insulin receptor

Isolated fetal rat primary hepatocytes have been described to express different isoforms of the NADPH oxidase (NOX) family enzymes [4]. Our cells displayed a similar pattern of NOX expression showing NOX1, NOX2 and NOX4 but not NOX3 expression (Fig. 2 A, left panel). We also extended our investigations to the homologue dual-oxidase (DUOX) family and found both DUOX1 and DUOX2 to be present (Fig. 2 A, left panel). Moreover, all known regulatory subunits of NOX enzymes were identified (Fig. 2 A, right panel). All identified NOX isoforms with the exception of DUOX1 were detected in mouse liver as well, providing evidence for a physiological setup in our cells.

In order to characterize the physiological role of IR in NOX4 expression we compared their mRNA levels between WT and IRKO hepatocytes. The lack of IR resulted in enhanced NOX4 expression (Fig. 2 B). By contrast, p22phox levels remained unaltered (data not shown). The physiological relevance of the IR in the regulation of NOX4 expression was confirmed by quantification of their mRNA in the livers of the liver-specific insulin receptor knock-out (LIRKO) mice showing similar regulation upon the deletion of the IR (Fig. 2 C).
Figure 2. NOX enzyme expression profile in wild type and IRKO hepatocytes and mouse liver (A) NOX/DUOX isoform expression in wild type hepatocytes detected by RT-PCR. RT+ and – signify PCR reaction carried out with or without a preceding reverse transcription. H2O represents PCR reaction devoid of cDNA. Positive controls were as follows: NOX1, NOXO1, NOXA1, DUOX2 and p22phox: colon, NOX2: spleen, NOX4: kidney, NOX3: brain, DUOX1: cerebellum. (B) NOX4 mRNA expression quantified by real-time PCR. Results are expressed as arbitrary units compared to the average expression in WT cells. Error bars represent mean±S.E.M. (C) NOX4 mRNA expression in the livers of LIRKO mice. Data are derived from six mice/group. *= p≤ 0.05, **= p≤ 0.01 and ***= p≤ 0.001 IRKO vs. WT hepatocytes, or LIRKO mice vs. WT mice.

2.3 Intracellular pathways involved in the regulation of NOX4 expression
Our previous results demonstrated that the IR exerts a basal inhibitory effect on NOX4 transcription. The two major signaling pathways activated by the insulin receptor are the mitogen activated kinase (MAPK) and phosphoinositide 3-kinase (PI3-kinase) pathways. To better characterize the effects of insulin on NOX enzyme expression we stimulated WT and IRKO hepatocytes by insulin in the presence or absence of different inhibitors affecting
selectively these two pathways. In order to elucidate the involvement of these pathways in the insulin receptor-mediated effects on NOX expression, we treated WT and IRKO hepatocytes overnight with selective MAPK and PI3-kinase inhibitors (PD98059 and wortmannin, respectively). In WT cells none of the inhibitors showed any effects on NOX4 expression (Fig.3 A, “WT”). By contrast, in IRKO cells, increased NOX4 expression was to a certain extent abrogated by both inhibitors (Fig.3 A, “IRKO”). These results suggest that the lack of IR signaling in IRKO cells leads to the basal activation of both the MAPK and the PI3-kinase pathways most likely through other serum factors (e.g. IGF-1). The activation of both pathways seems to be partially and equally responsible for the increase in basal NOX4 mRNA expression.

Our next experiments were aimed at dissecting role of the IR signaling and the serum factors in the observed changes in NOX4 expression. To achieve this goal we deprived cells of serum and stimulated them by insulin in the absence and presence of the MAPK or PI3-kinase inhibitors. The results obtained confirmed the importance of correct basal insulin receptor signaling as well as the essential modulatory function of other serum derived factors. In IRKO cells, in the presence of serum NOX4 mRNA levels were increased. Serum deprivation led to a further increase in NOX4 expression that was inhibited by insulin stimulation. The inhibitory effect of insulin on NOX4 expression was selectively blocked by the MAPK inhibitor PD98059 while the PI3-kinase inhibitor wortmannin was without any effect (Fig. 3D).

**Figure 3.**

![Figure 3](image_url)

**Figure 3. Regulation of NOX4 expression in wild type and IRKO hepatocytes by insulin and other serum-derived factors.**

(A and B) Basal NOX4 mRNA expression in the presence or absence of the MAPK inhibitor PD98059 (PD) or the PI3-kinase inhibitor wortmannin (Wort) as determined by real-time PCR. Inhibitors were administered
overnight (16 hours). **(C and D)** NOX4 mRNA expression determined by real-time PCR. Serum deprivation was for overnight, insulin stimulation for six hours. PD98059 (PD) or wortmannin (Wort) were administered one hour prior to insulin stimulation. Data are shown as arbitrary units compared to the average expression levels in non-serum-deprived WT cells. Data are derived from three independent experiments using duplicates or triplicates in each experiment. Error bars represent mean±S.E.M. * = p ≤ 0.05, ** = p ≤ 0.01 and *** = p ≤ 0.001 IRKO hepatocytes vs. WT hepatocytes; # = p ≤ 0.05, ## = p ≤ 0.01 control (cells cultured with normal medium) vs. serum starved cells; † = p ≤ 0.05 insulin treated vs. serum starved cells; & = p ≤ 0.05, && = p ≤ 0.01 insulin vs. insulin and inhibitor treated cells.

2.4 Effects of TGF-β, IGF-1 and IGF-2 on NOX4 expression

Several studies established the crucial role that TGF-β plays in mediating hepatocyte G1 cell cycle arrest and ultimately apoptosis [28, 165, 261-263]. TGF-β-induced apoptosis has been linked to increased NOX4 expression [28, 264]. The signaling mediated by the IR/IGF-1R pathways promote cell growth and differentiation. Our previous data indicated that the presence of intact insulin receptor signaling is an important factor in the regulation of NOX enzyme expression. In order to explore the possible interaction between the TGF-β receptor and the IR/IGF-1R signaling pathways we compared NOX4 expression in TGF-β-stimulated cells in the presence or absence of IGF-1 or IGF-2 in WT and IRKO hepatocytes. Our data revealed a suppressive effect for both IGF-1 and IGF-2 on NOX4 expressions when administered alone and that this inhibition was more pronounced in IRKO than in WT cells (Fig. 4). TGF-β stimulation led to an upregulation of NOX4 mRNA in WT but not in IRKO cells. These data confirm the previously reported upregulatory effect of TGF-β on NOX4 expression [4] but also add a new dimension by demonstrating that this effect was abrogated upon the lack of the IR. IGF-1 and IGF-2 could not prevent TGF-β-induced NOX4 upregulation though they were able to partially block this effect in IRKO cells. These data are in concordance with the results of our previous experiments and confirm the importance of the basal regulatory effect of the insulin receptor on NOX4 expression and the increased sensitivity towards IGF-1 and IGF-2 stimulation in the IRKO cells.
Figure 4. Regulation of NOX4 expression by TGF-β, IGF-1 and IGF-2 in WT and IRKO hepatocytes.

NOX2 and NOX4 mRNA levels upon stimulation with IGF-1, IGF-2 or TGF-β alone or in combination as analyzed by real-time PCR. Data are derived from three independent experiments using duplicates in each experiment. Error bars represent mean±S.E.M. * = p ≤ 0.05, ** = p ≤ 0.01 and *** = p ≤ 0.001 IRKO vs. wild type hepatocytes; += p ≤ 0.05, +++ = p ≤ 0.01 and ++++ = p ≤ 0.001 IGF-1, IGF-2 or TGF-β stimulated vs. serum starved cells.

2.5 The relationship between IGF-1/2-induced modification of NOX4 expression and TGF-β-induced apoptosis

TGF-β has an important physiological role in hepatocyte growth and apoptosis partly linked to its capacity to up-regulate NOX4 expression [265, 266]. Our results indicated that IGF-1 and IGF-2 can not counteract this enhancing effect on NOX4 mRNA transcription. Therefore, we proceeded to investigate if IGF-1 and IGF-2 are capable of modifying TGF-β-induced apoptosis. To achieve this goal we stimulated WT and IRKO hepatocytes with TGF-β in the presence or absence of IGF-1 or IGF-2 and after 24 hours of stimulation we determined cellular apoptotic rate. The results of these experiments are depicted in Fig. 5.

24 hour serum deprivation resulted in elevated NOX4 protein levels in both WT and IRKO hepatocytes without a concomitant increase in mRNA levels, arguing for a post-transcriptional regulatory mechanism upon serum withdrawal (Fig. 5A and B). 24 hours of TGF-β incubation was not able to further increase NOX4 protein levels inspite of its enhancing effect on NOX4 mRNA transcription (Fig. 5A and B). Co-stimulation with IGF-1 or IGF-2 had no effect on NOX4 protein expression (Fig. 5A). By contrast, IGF-1 and IGF-2 co-stimulation prevented TGF-β-induced up-regulation of NOX4 mRNA transcription (Fig. 5B).
In the presence of serum, apoptosis was decreased in IRKO cells compared to WT cells (Fig. 5C). Serum withdrawal resulted in increased apoptosis in both WT and IRKO hepatocytes and this was further augmented upon TGF-β stimulation. IGF-1 and IGF-2 co-stimulation lead to slightly decreased apoptotic rate in WT cells. By contrast, in IRKO cells IGF-1 stimulation abrogated TGF-β-induced apoptosis. A similar, but less significant effect was observed when IRKO cells were co-stimulated with IGF-2 (Fig. 5C).

Figure 5.

(A and B) NOX4 protein (A) and mRNA (B) expression in wild type and IRKO hepatocytes upon stimulation by TGF-β in the absence or presence of IGF-1 or IGF-2. In case of co-stimulation IGF-1 and IGF-2 were administered 1 hour prior to the addition of TGF-β. Protein amount was determined by Western blot analysis and
mRNA expression by real-time PCR. (C) Apoptosis in WT and IRKO cells in the same conditions as mentioned above. Apoptotic cell rate was determined by FACS analysis. Graphs represent data derived from 2-3 independent experiments performed in duplicates or triplicates and expressed as arbitrary units related to the mean of wild type cells cultured in the presence of serum (mRNA and protein), or as percentage of gated cells (apoptosis). Bars represent mean S.E.M. * = p ≤ 0.05, ** = p ≤ 0.01 and *** = p ≤ 0.001 IRKO hepatocytes vs. wild type hepatocytes; + = p ≤ 0.05, ++ = p ≤ 0.01, +++ = p ≤ 0.001 serum starved cells vs. TGF-β, IGF-1 or IGF-2 treated cells; && = p ≤ 0.01 TGF-β treated cells vs. TGF-β+IGF-1 or IGF-2 treated cells; # = p ≤ 0.05, ## = p ≤ 0.01, ### = p ≤ 0.001 cells cultured in the presence of serum vs. serum starved cells.

2.6 Intracellular signaling pathways related to the regulation of NOX4 expression

A previous study suggested that the prevention of TGF-β-induced NOX4 mRNA expression is abrogated by EGF through activation of the PI3-kinase pathway [4, 267]. In the first part of our studies we established that basal NOX4 mRNA expression is regulated through the MAPK pathway. The MAPK pathway is also involved in the regulation of cell cycle progression. Therefore, our next set of experiments were aimed at examining the two main intracellular signaling pathways in relation to the observed changes in NOX4 expression and cell cycle regulation upon TGF-β, IGF-1 and IGF-2 stimulation. In the presence of serum IRKO cells displayed significantly higher phosphorylation of MAPK, in line with their elevated NOX4 mRNA and protein levels and their increased rate of cell division (Fig. 6). Serum deprivation resulted in a significant decrease in MAPK phosphorylation in both wild type and IRKO cells, though the relatively higher rate of phosphorylation persisted in the IR deficient cells. TGF-β stimulation resulted in a low rate increase in MAPK phosphorylation in IRKO but not in wild type cells. IGF-1 and IGF-2 co-stimulation along with TGF-β resulted in a further significant increase in MAPK phosphorylation in wild type cells. However, in concordance with previous results demonstrating that IGF-2 signaling requires the presence of the insulin receptor, enhanced MAPK phosphorylation was only observed with IGF-1 co-stimulation in IRKO cells.

Contrary to the MAPK pathway, Akt phosphorylation did not present changes between the wild type and IRKO cells indicating that PI3-kinase/Akt signaling does not play a determining role in the alteration of NOX4 expression.

Altogether, these data suggest that basal NOX4 expression is indeed regulated by the MAPK pathway, while other, currently not well characterized, pathways are responsible for the control of induced expression.
Fig. 6. MAPK and Akt phosphorylation in wild type and IRKO cells in conditions related to altered NOX4 mRNA expression.

(A and B) Western blot analysis of MAPK (A) and Akt (B) phosphorylation as determined by Western blot analysis. Graphs represent data derived from three independent experiments performed in triplicates and are expressed as arbitrary units relative to the values of wild type cells cultured in the presence of serum. Bars represent mean ± S.E.M. ** = p ≤ 0.01 and *** = p ≤ 0.001 IRKO vs. wild type hepatocytes; + = p ≤ 0.05 serum starved cells vs. TGF-β treated cells; & = p ≤ 0.05, && = p ≤ 0.01 and &&& = p ≤ 0.001 TGF-β treated cells vs. TGF-β+IGF-1 or IGF-2 treated cells; # = p ≤ 0.05, ## = p ≤ 0.01, ### = p ≤ 0.001 cells cultured in the presence of serum vs. serum starved cells.
3. DISCUSSION

Liver, an organ consisting of a variety of cell types with different physiological importance, expresses different NADPH oxidase isoforms (NOX-es) characteristic to each cell type. The three most relevant cell types are the Kupffer cells, hepatic stellate cells (HSCs) and the hepatocytes. In these cells, NOX enzymes are functionally expressed both in the phagocytic form, termed gp91phox/NOX2, and in other non-phagocytic isoforms [200].

Kupffer cells are the resident macrophages of the liver [188], which mainly produce ROS through NOX2 and its subunits [27, 204, 252]. Kupffer cells exerts an important role in host defence and inflammatory processes [31, 268]. Hepatic stellate cells, the main fibrogenic cell type, express NOX2 and all of its regulatory subunits (p22phox, p47phox and p67phox) at both the messenger RNA and protein levels [248]. In these cells, NOX2 plays an important role in cell signalling regulation, the induction of cell proliferation and collagen production [27, 185, 200]. In hepatocytes, the dominant cell type of the liver, mRNAs of NOX1, NOX2, NOX4, DUOX1, and DUOX2 were identified [241]. The main function attributed to NOX-derived ROS in hepatocytes is the regulation of apoptosis and in this context current investigations suggested NOX4 as a major isoforms being involved [27].

NOX4 has been demonstrated to be involved in mediating insulin signaling in 3T3L1 adipocytes [33]. Its expression has also been shown to be to down-regulated during adipogenesis, a process governed in part by the insulin/insulin-like growth factor-1 pathway [240]. Thus, in adipocytes, NOX4 seems to play a double-faced role: on one hand it is a mediator of insulin signaling, on the other hand its expression is inhibited by insulin. In fact, this scenario revokes the role and regulation of one of the major insulin receptor mediating molecule, the insulin receptor substrate 2, IRS2 [87, 269]. The insulin-induced regulation of NOX4 mRNA expression is even more of an intriguing subject in the light of the fact that NOX4 activity was demonstrated to be regulated at the transcriptional level [33, 240, 270].

Liver metabolism, cell cycle and apoptosis are regulated by insulin, IGF-1 and TGF-β, respectively; all known to affect NOX4 expression/activity. Therefore, our study examined the potential role of NOX4 as a cross-talk point of these different intracellular signaling networks. To reach this goal we i) compared the regulation of NOX4 expression in wild type and insulin receptor-deficient hepatocytes, ii) uncovered some of the intracellular pathways responsible for NOX4 regulation and iii) explored the link between modulation of TGF-β signaling and NOX4 expression.
3.1. Regulation of basal expression of NOX4 by the presence of the insulin receptor

Fetal rat primary hepatocytes express different NOX family enzymes [4]. In our experiments we identified the same NOX/DUOX isoforms in our hepatocyte models that were detected in mouse liver arguing for the physiological set-up of our experimental model. To elucidate the role of the insulin receptor in the expression of NOX4 we compared NOX4 expression levels of wild type and insulin-receptor deficient hepatocytes. We found that NOX4 mRNA and protein expression was up-regulated in IRKO hepatocytes. The physiological relevance of this regulation was confirmed in vivo as livers of the liver specific insulin receptor knock-out mice showed similar mRNA regulation.

The up-regulation of NOX4 expression in the IRKO cells was suggestive of a negative regulatory function of the insulin receptor on NOX4 mRNA transcription. In hepatocytes, several enzymes are known to be repressed by insulin, e.g. the phosphoenol-pyruvate kinase (PEPCK), an enzyme involved in gluconeogenesis. Through its inhibitory action on PEPCK expression insulin plays a key role in suppressing hepatic neoglucogenesis in nutrition abondant states. Along the same line, the constant inhibition of NOX4 expression implies an important role of insulin in the regulation of the basal cellular redox state as well as a role in the adaptation to nutrition/energy depletion. Taken these data together with other studies indicating the presence of NOX4 in the endoplasmatic reticulum [27, 240] it is feasible to hypothetize a role for NOX4 acting as a “redox chaperone” and participate in the repair mechanism of proteins requiring proper redox modifications for correct folding and function.

Comparing NOX4 mRNA and protein levels we uncovered a second mechanism of regulation for cellular NOX4 content, notably the regulation by altered protein turnover. Indeed, overnight serum deprivation in wild type cells resulted in increased protein amount without a concomitant increase in mRNA transcription suggesting a post-transcriptional control mechanism. Insulin-induced protein regulation might involve two different pathways: controlling translation through the eukaryotic initiation factor 1 (eIF1) or increasing ubiquitin-mediated proteasomal degradation as in case of eIF4E-binding proteins (4E-BPs) and IRS-2, respectively [271, 272]. Further experiments will be required to properly address the question which mechanism is responsible for the observed NOX4 protein upregulation. In any case, an increase in NOX4 protein content upon serum depletion (stress induction) would corroborate our suggestion noted in the previous paragraph concerning a possible “redox chaperone” role for NOX4. The important regulatory role of the insulin receptor in the regulation of basal NOX4 expression underlines the potential interest of NOX4 in insulin resistant states where hepatocyte proliferation is often altered [130].
Paradoxically, however, serum deprivation in IRKO but not in WT cells lead to further enhancement in NOX4 mRNA transcription. In theory, if serum factors other than insulin (e.g. IGF-1) are to play an important role in NOX4 mRNA suppression, then serum removal should lead to similar responses in WT and IRKO cells due to the complete abrogation of IR/IGF-1R stimulation. These unexpected data could inspire two different interpretations; one suggesting that the permanent lack of insulin receptor expression in IRKO cells would lead to intracellular molecular changes rendering the cells more responsive to IGF-1. In this case serum IGF-1/IGF-2 would have a greater suppressive effect on NOX4 expression and their removal during serum depletion would lead to further enhancement in NOX4 transcription. The other possible explanation would suggest a different rate of production of autocrin survival factors, most notably EGF during starvation, thus the increase in NOX4 expression would reflect not the “release” of inhibition but an actual stimulatory effect.

While our experiments did not yet provide detailed answers to all these possibilities they certainly point towards the importance of an intact insulin receptor signaling in the regulation of cellular NOX4 content and the response to different stress conditions.

3.2. Modulation of TGF-β-induced NOX4 up-regulation and apoptosis by IGF-1 / IGF-2
TGF-β is a multifunctional cytokine regulating cell survival, differentiation, migration, adhesion and synthesis of extracellular matrix (ECM) components. Due to this multitude of functions, TGF-β it is involved in many biological and pathological processes including embryonic development, tissue remodeling, inflammation, angiogenesis, atherosclerosis, fibrosis and carcinogenesis. Through these functions, TGF-β plays an important part role in both normal and diseased conditions in the liver but also in other organs [273]. Insulin, IGF-1 and IGF-2 also mediates growth-promoting and anti-apoptotic functions in hepatocytes [3].

A large number of growth factors and cytokines, including TGF-β, insulin and the IGFs are capable of generating ROS [274]. In hepatocytes, ROS generated through activation of NOX4 has been linked to TGF-β-induced apoptosis and EGF-mediated abrogation of NOX4 up-regulation could abolish this effect [4, 262].

Therefore, in order to elucidate the link between insulin/IGF-1-induced NOX4 down-regulation and the diminution of TGF-β-mediated apoptosis of we carried out a series of experiments where WT and IRKO hepatocytes were stimulated by TGF-β in the presence or absence of IGF-1 and IGF-2 and their apoptotic response was compared. Our results demonstrated decreased apoptotic rates in IRKO cells along with increased basal NOX4 mRNA expression. TGF-β stimulation led to an increase in NOX4 mRNA expression both in
WT and IRKO cells and an increase in cellular apoptotic rate which was abrogated upon co-stimulation with IGF-1 or IGF-2. These data are in line with results obtained in relation to another survival promoting factor, EGF. Indeed, Irene Carmona-Cuenca et al. recently demonstrated that the anti-apoptotic/pro-survival effect of EGF is directly linked to its capacity to abrogate TGF-β-induced NOX4 upregulation [4].

3.3. Intracellular pathways mediating regulation of NOX4 expression in response to insulin, IGF-1 and IGF-2 and TGF-β

Cell fate is decided by the balance between death and survival signals. Paradoxically, along with its pro-apoptotic effect, TGF-β also induces survival signals in hepatocytes through the transactivation of the EGF receptor which is required for Akt phosphorylation and cell death rescue [275]. PI 3-K mediates the survival effect of EGF on TGF-β-induced death by acting upstream from the mitochondrial changes preventing to the loss of the anti-apoptotic member of the BcL-2 family, BcL-XL. In support of this hypothesis, inhibiting EGF receptor signaling or PI3-kinase activation in response to TGF-β greatly increased the apoptosis induced by TGF-b in fetal hepatocytes [276].

When looking for an intracellular signaling pathway responsible for the upregulation of NOX4 expression and the following cell cycle progression in IRKO cells we found that basal, insulin receptor-related NOX4 expression is positively associated with increased MAPK phosphorylation.

The family MAP kinase enzymes consists in three subfamilies: the extracellular signal-regulated kinases (ERK1 and ERK2, also termed p42/44 MAPK), the stress-activated protein (SAP) kinases known as c-Jun N-terminal kinase (JNK1, JNK2 and JNK3), and the p38/MAP kinases [277]. ERK-mediated pathways are mostly involved in proliferation and differentiation and generally considered antiapoptotic acting through the regulation of the members of the bcl family [278]. JNK and p38-signaling pathways are activated by stress stimuli, many of which induce apoptosis, but in some cellular systems they have been implicated in proliferation and differentiation as well [279]. ERK5, described as a mediator of Src activation [280], is another member of the MAP kinase super family, but thus far, unlike the first three groups of MAKs, it has not been shown to be activated by TGF-β, or to interfere with Smad signaling. Signaling initiated by each MAPK pathway occurs through sequential activation of a MAPK kinase kinase (MAPKKK) by membrane-associated kinases such as cytokine or growth factor receptors, a MAPK kinase (MAPKK), leading to phosphorylating activation of a MAPK [281]. MAPK activation leads to downstream phosphorylation of nuclear kinases or, most commonly, transcription factors. ERKs are phosphorylated by the MAPKKs MEK1 and MEK2,
themselves substrates of the MAPKKK Raf-1, the latter being activated by the membrane-bound small G-protein Ras, for example, following induction by different stimuli such as insulin, IGF-1 or EGF. JNK family members are the substrates of MKK4 (also known as SEK1) and MKK7. p38-MAPK is phosphorylated by MKK3 and MKK6, themselves the substrates of several MAPKKKs, including, but not restricted to, apoptosis signal-regulating kinase-1 (ASK1), mixed lineage kinases, and TGF-β-activated kinase-1 (TAK1) [282]. Noteworthy, that recent findings suggested the essential role of NOX4-mediated ROS production and p38MAPK activation during cardiac differentiation [283].

Reversible phosphorylation of MAPK proteins is essential for their proper signal transduction. The phosphorylation state is defined by a balance between the rate of phosphorylation and dephosphorylation. As mentioned above, the phosphorylation is governed by an array of specific MAPK kinases while the dephosphorylation process is regulated by MAPK phosphatases terminating their activation [284]. MAPK phosphatases are classified by their substrate specificity: dual-specificity MAPK phosphatases (MKPs), serine/threonine phosphatases (PSPs) and tyrosine phosphatases (PTPs). The p42/44 MAPK pathway is regulated by MKP-1 and MKP2, the PSP PP2A and the PTP SHP-2. In general, the catalytic activity of phosphatases is prone to redox modification due to the presence of redox-sensitive thiol groups in their catalytic center [285]. In particular, the activity of MKP1 has been shown to be regulated by NOX2 in human endothelial cells [286]. Serum-depletion induced p42/44 MAPK desactivation was shown to be linked to increased MKP1 and MKP2 activity [287, 288]. Thus, it is plausible to hypothesize that the increased basal phosphorylation of MAPK in the IRKO cells might be due to a decrease in MKP1 phosphatase activity provoked by the increased NOX4 expression and the following elevation in ROS production. This hypothesis, however should be further examined

In summary, our results provided the first study exploring the relationship between the mitogenic and anti-apoptotic signaling of insulin and IGF-1 and NOX4 expression in hepatocytes. Based upon our data we suggest a two-layered regulatory mechanism: one regulating the “basal” expression of NOX4 which would in consequence modify the cellular response (“induced” expression) to other mitogens and apoptotic agents. We propose that this regulation is mainly related to the MAPK pathway in a bidirectional way: NOX4 expression being suppressed by insulin and IGF-1 through the activation of MAPK and in reverse, NOX4 exerting a positive effect on MAPk signaling most likely through the down-regulation of the phosphatase activity of MKP1.
4. CONCLUSIONS

**Insulin and IGF-1** receptors have crucial roles in hepatocyte metabolism, growth, aging and cell death. Even though they display high structural similarity, their functions and intracellular signaling pathways are not completely overlapping. Indeed, insulin receptors mediate mainly metabolic responses while IGF-1 receptors mediate cell growth. However, both receptors mediate pro-survival effects. One of the common points in their signal transmission is the production of cellular reactive oxygen.

**NOX4** is reactive oxygen species generating enzyme whose function has been related to insulin’s metabolic action in adipocytes and the pro-apoptotic and survival effect of TGF-β and EGF in hepatocytes.

**TGF-β** is secreted by the hepatic stellate cells exerting its growth promoting and apoptotic effects on hepatocytes.

Our study sought a link between IR/IGF-1R and TGF-β receptor signaling, using NOX4 as a point of convergence in their intracellular signaling pathways.

We provided evidence that:

1./ Basal hepatocyte NOX4 expression is negatively regulated by the presence of the insulin receptor at the transcriptional level
2./ Basal NOX4 expression is positively correlated with MAPK phosphorylation
3./ Serum factors, most probably IGF-1 or EGF regulate NOX4 protein amount at the posttranscriptional level
4./ IGF1/2-induced down-regulation of NOX4 mRNA expression correlates with their capacity to abrogate TGF-β-induced apoptosis

In summary, our data provide an intriguing insight into the complexity of the regulation of hepatocyte growth and death through the regulation of basal cellular redox homeostasis linked to NOX4 function. Moreover, they indicate a new direction of investigation concerning the role of insufficient insulin receptor signaling (insulin resistance) in the progression of hepatocyte injury and apoptosis triggering the process of hepatic fibrosis. Evidently, the direct role of NOX4 in hepatocyte function has to be addressed in NOX4-depleted hepatocytes and in the future, in the liver specific NOX4 knock-out mice. Our data, however, provide a solid basis for these studies and encourage further investigations concerning the modulation of NOX4 expression in hepatocyte insulin signaling, cellular redox homeostasis and apoptosis.
PUBLICATIONS


REFERENCES


with insulin receptor suggests structural determinants that define functional specificity. Embo J 5, 2503-2512.


253. LaCourse, R., Ryan, L., and North, R.J. (2002). Expression of NADPH oxidase-dependent resistance to listeriosis in mice occurs during the first 6 to 12 hours of liver infection. Infect Immun 70, 7179-7181.


