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Growth hormone modulation of EGF-induced PI3K-Akt pathway in mice liver

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ABSTRACT

The epidermal growth factor (EGF) activates the phosphatidylinositol 3-kinase (PI3K)-Akt cascade among other signaling pathways. This route is involved in cell proliferation and survival, therefore, its dysregulation can promote cancer. Considering the relevance of the PI3K-Akt signaling in cell survival and in the pathogenesis of cancer, and that GH was reported to modulate EGFR expression and signaling, the objective of this study was to analyze the effects of increased GH levels on EGF-induced PI3K-Akt signaling.

EGF-induced signaling was evaluated in the liver of GH-overexpressing transgenic mice and in their normal siblings. While Akt expression was increased in GH-overexpressing mice, EGF-induced phosphorylation of Akt, relative to its protein content, was diminished at Ser473 and inhibited at Thr308; consequently, mTOR, which is a substrate of Akt, was not activated by EGF. However, the activation of PDK1, a kinase involved in Akt phosphorylation at Thr308, was not reduced in transgenic mice. Kinetics studies of EGF-induced Akt phosphorylation showed that it is rapidly and transiently induced in GH-overexpressing mice compared with normal siblings. Thus, the expression and activity of phosphatases involved in the termination of the PI3K-Akt signaling were studied. In transgenic mice, neither PTEN nor PP2A were hyperactivated; however, EGF induced the rapid and transient association of SHP-2 to Gab1, which mediates association to EGFR and activation of PI3K. Rapid recruitment of SHP2, which would accelerate the termination of the proliferative signal induced, could be therefore contributing to the diminished EGF-induced activity of Akt in GH-overexpressing mice.

1. Introduction

The phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway is activated by many types of cellular stimuli and regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival [1,2]. When PI3K is activated, it is able to convert phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) to PI(3,4,5)P3, whereas PTEN (phosphatase and tensin homologues) reverses this reaction. Akt translocates to the cell membrane and interacts with PI(3,4,5)P3 via its PH domain. The interaction of the PH domain of Akt with PI(3,4,5)P3 provokes conformational changes in Akt, resulting in the exposure of its two main phosphorylation sites, Thr308 in the kinase domain and Ser473 in the C-terminal regulatory domain. The PH domain of PDK1 also mediates its approach to PI(3,4,5)P3 and its interaction with AKt, this leads to PDK1-depent phosphorylation of AKt at Thr308, leading to stabilization of its active conformation. Although phosphorylation of Thr308 is a requirement for the activation of Akt, the concomitant phosphorylation at Ser473 leads to the full activation of this kinase [3–5]. Several studies have shown that phosphorylation at the two sites can occur independently [4,5]. Activated Akt modulates the function of numerous substrates involved in the regulation of cell proliferation, including glycogen synthase kinase-3 (GSK-3), cyclin-dependent kinase inhibitors P21/Waf1/Cip1 and P27/Kip2, and mammalian target of rapamycin (mTOR) [6,7]. The dysregulation of this pathway has been associated with the development of diseases such as cancer, diabetes mellitus, and autoimmunity [2,4]. The development and progression of cancer are the results of uncontrolled cell proliferation and survival and the PI3K-Akt signaling controls both of these events, therefore its dysregulation plays a major role in tumor growth. Akt has been described to be constitutively active in many types of human cancer due to amplification or mutations in components of the signaling pathway that activate this kinase. In different types of cancer the PI3KCA gene, which encodes the p110a catalytic subunit of PI3K, is amplified [4]. Cell surface receptors are commonly overexpressed or constitutively active in many types of cancers and downstream signaling pathways, including the PI3K-Akt pathway, are often activated as a result.

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Moreover, the dysregulation of negative regulators of the PI3K-Akt pathway is related to cancer progression, and thus absence of PTEN strongly correlates with activation of Akt in tumor cell lines [8].

The epidermal growth factor receptor (EGFR) is among the tyrosine kinase receptors that can activate the PI3K-Akt pathway. EGFR, also called ErbB-1, belongs to a family of receptors that comprises three additional proteins, ErbB-2, ErbB-3 and ErbB-4. ErbB receptors are activated by binding of growth factors of the EGF family which are produced in an autocrine or paracrine fashion. EGF binding to its receptor leads to the activation of the receptor tyrosine kinase domain and phosphorylation of multiple tyrosine residues within its intracellular domain [9,10]. This activation triggers intracellular signaling cascades such as the MAPKs, the PKC, the STAT and the PI3K-Akt pathways, involved in cell proliferation, survival and motility [11–13]. ErbB receptors have been shown to be involved in the pathogenesis and progression of different types of carcinoma [12,14,15]. EGFR undergoes different alterations including gene amplification, structural rearrangements, and somatic mutations in human carcinomas; moreover, some types of tumors produce an excess of EGF that leads to an increased activation of EGFR [13]. Besides anomalies in EGF receptor expression and ligand production, intracellular signaling pathways are often altered in cancer cells, including the PI3K-Akt pathway. All these alterations in EGF-mediated survival signaling pathways contribute to cancer progression and resistance to cancer therapies. In particular, amplifications or mutations of the efgr gene or overexpression of this receptor frequently occur in hepatocellular carcinoma (HCC) [16,17].

EGFR phosphorylation is induced not only by direct binding of specific ligands but also by growth factors such as growth hormone (GH) and prolactin (PRL). GH was reported to induce the transphosphorylation of the EGFR at tyrosines 845, 992, 1068 and 1173 [18–21]. Another level of interaction between GH and EGF signaling involves GH-induced EGFR phosphorylation at threonine residues. Such phosphorylation reduces EGF-induced EGFR degradation, thus modulating EGFR trafficking and signaling [21]. In addition, EGFR expression has been demonstrated to be regulated by GH [22–24]. Partially GH-deficient mutant mice and hypophysectomized mice showed reduced expression of the EGFR in liver. When GH was administered to these mice, EGFR expression was induced approaching EGFR levels found in normal mice [23]. We have recently demonstrated that EGFR expression is diminished in GH receptor knock-out mice while it is increased in transgenic mice over-expressing GH [24,25]. Moreover, crosstalk between GH and EGF signaling pathways comprising diverse signaling mediators has been described.

Growth hormone is a pituitary hormone involved in longitudinal growth promotion and metabolic processes. However, several studies have demonstrated that, in addition to its physiological effects, GH is involved in tumorigenesis and tumor progression. GH overexpression has been associated with cancer in animals as well as in humans; indeed, acromegalic patients show an increased incidence of this pathology [26–29]. On the contrary, a decreased incidence of cancer in the absence of GH or its receptor has been observed [30,31]. Transgenic mice overexpressing GH are more likely to develop cancer [32–34] and they have an increased tendency to develop liver tumors, including hepatocellular carcinoma, at advanced ages [34,35]; while GH resistance or deficiency are associated with reduced tendency to develop malignancies spontaneously [36,37] or in response to carcinogen administration [38,39]. The main GH-induced signaling pathway, JAK2-Stat5, is desensitized in transgenic mice overexpressing GH [40,41]. Nevertheless, these mice show increased expression and basal phosphorylation levels of EGFR and Akt [24,25]. Considering GH modulation of EGFR expression and activation, the relevance of the PI3K-Akt signaling in cell survival and in the pathogenesis of cancer, as well as the implication of GH and EGFR in the development of hepatocarcinoma, the objective of this study was to analyze the modulatory role of GH overexpression in EGF-induced PI3K-Akt signaling in mice liver.

2. Materials and methods

2.1. Reagents

Highly purified ovine growth homone (oGH) of pituitary origin was obtained through the National Hormone and Pituitary Program, NIDDK, NIH, USA. Recombinant human EGF, Trizma base, HEPES, Tween 20, Triton X-100, sodium dodecyl sulfate (SDS), glycine, ammonium persulphate, aprotinin, phenylmethylsulphonyl fluoride (PMSF), sodium orthovanadate, 2-mercaptoethanol, molecular weight markers and BSA-fraction V were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA.). PVDF membranes, high performance chemiluminescence film and enhanced chemiluminescence (ECL)-Plus are from Amersham Biosciences (GE Healthcare, Piscataway, NY, USA). Mini Protean apparatus for SDS-polyacrylamide electrophoresis, miniature transfer apparatus, acrylamide, bis-acrylamide and TEMED were obtained from Bio-Rad Laboratories (Hercules, California, USA). Secondary antibodies conjugated with HRP and antibody anti-Gab1 (H-198) were purchased from Santa Cruz Biotechnology Laboratories (Santa Cruz, CA, USA). Antibodies antiphospho-Gab1 Tyr627, anti-phospho-Akt Ser473, anti-phospho-Akt Thr308, anti-Akt, antiphospho-mTOR Ser2448, anti-mTOR, anti-PDK1, anti-PTEN, antiphospho-PDK1 Ser241, and anti-phospho-PTEN Ser380 were from Cell Signaling Technology Inc. (Beverly, MA, USA). Bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Scientific, Pierce Protein Research Products (Rockford, IL, USA). PP2A Immunoprecipitation Phosphatase Assay Kit was purchased from Millipore (Billerica, MA, USA).

2.2. Animals

Phosphoenolpyruvate carboxykinase (PEPCK)-bGH mice containing the bGH gene fused to control sequences of the rat pepck gene [42] were derived from animals kindly provided by Drs. T E Wagner and J S Yun (Ohio University, Athens, OH, USA). The hemizygous transgenic mice were derived from a founder male, and were produced by mating transgenic males with normal C57BL/6XC3H F1 hybrid females purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mating produced approximately equal proportions of transgenic and normal progeny. Normal siblings of transgenic mice were used as controls. Female and male adult animals (4–7 months old) were used. The mice were housed 3–5 per cage in a room with controlled light (12 h light/day) and temperature (22 ± 2 °C). The animals had free access to food (Lab Diet Formula 5001 containing a minimum of 23% protein, 4.5% fat and a maximum of 6% crude fiber, from Purina Mills Inc., St. Louis, MO, USA) and tap water. The appropriateness of the experimental procedure, the required number of animals used, and the method of acquisition were in compliance with federal and local laws, and with institutional regulations.

2.3. Animal treatments

PEPCK-bGH transgenic mice and their normal littermate controls were fasted for 6 h prior to i.p. injection with 2.5 mg oGH per kg of body weight (BW) or with recombinant human EGF 2 mg/kg BW in 0.9% w/v NaCl. Mice were killed 7.5 min after GH injection or 10 min after EGF administration [24,25], to study phosphorylation kinetics mice were killed 2.5, 5, 10 and 15 min after EGF stimulation. Additional mice were injected with saline to evaluate basal conditions. The livers were removed and stored frozen at −70 °C until homogenization.

2.4. Preparation of liver extracts

Liver samples were homogenized at the ratio 0.1 g/ml in buffer composed of 1% v/v Triton, 0.1 mol l^{-1} Hepes, 0.1 mol l^{-1} sodium