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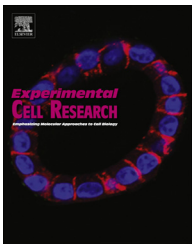

Research Article

Selective insulin resistance in hepatocyte senescence

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ABSTRACT

Insulin resistance has been described in association with chronic liver disease for decades. Hepatocyte senescence has been demonstrated in chronic liver disease and as many as 80% of hepatocytes show a senescent phenotype in advanced liver disease. The aim of this study was to understand the role of hepatocyte senescence in the development of insulin resistance. Senescence was induced in HepG2 cells *via* oxidative stress. The insulin metabolic pathway was studied in control and senescent cells following insulin stimulation. GLUT2 and GLUT4 expressions were studied in HepG2 cells and human liver tissue. Further, GLUT2 and GLUT4 expressions were studied in three independent chronic liver disease cohorts. Signalling impairment distal to Akt in phosphorylation of AS160 and FoxO1 was evident in senescent HepG2 cells. Persistent nuclear localisation of FoxO1 was demonstrated in senescent cells despite insulin stimulation. Increased GLUT4 and decreased GLUT2 expressions were evident in senescent cells, human cirrhotic liver tissue and publically available liver disease datasets. Changes in GLUT expressions were associated with a poor clinical prognosis. In conclusion, selective insulin resistance is evident in senescent HepG2 cells and changes in GLUT expressions can be used as surrogate markers of hepatocyte senescence.

Introduction

The liver and hepatocytes in particular, play a crucial role in glucose metabolism and metabolic homeostasis by maintaining plasma glucose concentrations. Exposure of hepatocytes to insulin leads to uptake, conversion and storage of glucose into glycogen or lipid nd inhibits both glycogenolysis and gluconeogenesis. In hepatocytes and other cells, the effects of insulin are mediated through two main cellular pathways, namely the phosphatidylinositol 3-kinase (PI3K)-Akt and Ras-MAP kinase (MAPK) pathways. PI3K-Akt signalling mediates the metabolic actions of insulin, while the Ras-MAPK pathway, in conjunction with PI3K-Akt pathway, regulates cellular growth, proliferation and differentiation [1]. Under normal circumstances, activation of the PI3K-Akt pathway by insulin regulates four crucial downstream pathways resulting in activation of mammalian target of rapamycin complex 1 (mTORC1) and its downstream substrate S6 Kinase, inactivation of both glycogen synthase kinase-3 (GSK3) and AS160, as well as nuclear exclusion of the Forkhead box protein FoxO1 [1,2].

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Since the first description of "hepatogenous diabetes" over 100 years ago [3], a number of studies have shown an association between advanced chronic liver disease and the development of insulin resistance and as many as 70% of patients with cirrhosis have impaired glucose tolerance [4–12]. Impairment of the response to insulin at both receptor and post-receptor levels in adipocytes was considered one potential mechanism behind the development of impaired glucose tolerance in patients with liver cirrhosis [4]. Impairment of insulin binding to its receptor in monocytes or erythrocytes of patients with liver cirrhosis was also demonstrated [4,9,13]. Perhaps surprisingly, the role of the liver and more specifically, that of hepatocytes, in the development of insulin resistance in chronic liver disease have received much less attention.

Hepatocyte senescence has been demonstrated in a number of diverse chronic parenchymal liver disorders [14–19]; in similar fashion, cholangiocyte senescence has been demonstrated in chronic biliary liver disease [20,21]. Wherever sought, a close association has only been demonstrated between the proportion of senescent hepatocytes and insulin resistance in patients with chronic parenchymal liver disease [15–17]. There are no comparable studies investigating chronic biliary liver disease and insulin resistance. These findings suggest there may be a direct role for hepatocyte senescence in the development of insulin resistance in chronic parenchymal liver disease. This putative relationship was investigated by more detailed examination of insulin mediated PI3K-Akt signalling following induction of hepatocyte senescence *in vitro*.

Materials and methods

Induction of senescence in HepG2 cells

As described previously, HepG2 cells (ATCC HB-8065), grown in Dulbecco's Modified Eagle Medium containing 10% foetal calf serum and antibiotics (100U/ml Penicillin, 100μg/ml Streptomycin), were seeded in 6-well plates at a density of $5x10^5$ per well and allowed to adhere overnight. Cells were incubated with 0.5mM H_2O2 in culture media for 60 minutes to induce senescence, whilst control HepG2 cells were incubated in culture media alone. Cells were then washed with PBS and incubated at 37°C, 5% CO_2 for 5 days. Senescence in H_2O_2-treated HepG2 cells was confirmed using changes in cell morphology, SA-β-GAL activity, expression of both cell cycle phase markers and cell cycle inhibitors, the presence of senescence-associated heterochromatic foci and the senescence-associated secretary phenotype as described [43].

Insulin stimulation of senescent and control HepG2 cells

Both senescent and control HepG2 cells were then incubated in serum-free Dulbecco's Modified Eagle Medium for 4 h followed by stimulation with 100 nM insulin (Sigma-Aldrich) in serum-free Dulbecco's Modified Eagle Medium for 10 min.

The (metabolic) insulin pathway in control and senescent HepG2 cells

Protein was extracted from control and senescent HepG2 cells with and without insulin stimulation, using CelLytic™ M cell lysis and protein solubilisation and extraction reagent (Sigma-Aldrich) according to the manufacturer's recommendations. Protease Inhibitor Cocktail (Sigma-Aldrich) was added to the CelLytic M reagent to prevent protein degradation. Halt Phosphatase Inhibitor Cocktail (Thermo Scientific) was added to the protein-containing supernatant to preserve protein phosphorylation.

Proteins were resolved on 4–20% mini-PROTEAN TGX Gels (Bio-Rad Laboratories) and transferred to polyvinylidene difluoride membranes using Trans-Blot® Turbo™ Mini PVDF Transfer Packs and Trans-blot turbo transfer system (Bio-Rad Laboratories).

The cellular location of FoxO1 in control and senescent HepG2 cells following insulin stimulation

Control and senescent HepG2 cells (grown on coverslips) with and without insulin stimulation were fixed with 4% PFA for 15 min at room temperature and permeabilised with 0.2% Triton X-100 for 5 min followed by incubation with unconjugated rabbit anti-FoxO1 (Cell Signaling, catalogue number 2880; concentration 1:100) for 45 min at room temperature. FITC-conjugated donkey anti-rabbit secondary antibody was used to identify FoxO1. DAPI served as a nuclear stain. Coverslips were mounted on glass slides using fluorescence mounting medium (Dako).

Expression of glucose transporters (GLUT2 and GLUT4) in control and senescent HepG2 cells, normal and cirrhotic human liver and public datasets

RNA was extracted from control and senescent HepG2 cells using Qiagen RNeasy Plus Mini Kit according to the manufacturer's recommendations. Real time one step RT-PCR reactions were carried out on the Rotor-Gene 6000 (Qiagen) using the TaqMan® Fast Virus 1-Step Master Mix system (Life Technologies) in a final volume of 20 μl, using primer and 5′FAM™ labelled TaqMan® MGB probe sets (Life Technologies; catalogue number 4331182) following the manufacturer's recommendations. A total of 5 ng RNA was amplified using the reaction conditions 50 °C for 5 min, denaturation at 95 °C for 20 s followed by 50 cycles of 95 °C for 3 s and 60 °C extension step for 30 s. Expression of all genes was normalised to 18s levels.

Protein was extracted from control and senescent HeG2 cells and western blots were probed with anti-GLUT2 (Abcam; catalogue number ab95256; concentration 1:1000) or anti-GLUT4 (Cell Signaling; catalogue number 2213; concentration 1:1000).

Formalin-fixed paraffin-embedded liver needle biopsy specimens were obtained in accordance with and approval of Cambridge University Hospital local research ethics committee. Five patients with liver cirrhosis and increased hepatocyte p21 expression (a marker of cellular senescence) were selected to the study expression of GLUT2 and GLUT4. Background liver from three patients with colorectal cancer metastases served as normal control tissue. Sections were stained with unconjugated mouse monoclonal anti-GLUT2 (R&D Systems; concentration 1:600) or unconjugated mouse monoclonal anti-GLUT4 (Abcam; concentration 1:800) using an automated immunohistochemical analysis Bond™ machine (Leica Microsystems).

Publically available microarray data were then used to study the changes in expression of GLUT2 and GLUT4 and their clinical significance in patients with chronic liver disease. Datasets (GSE33814 and GSE28619) containing diseased liver tissue expression profiles from patients with steatohepatitis ($n=12$) and alcohol-related hepatitis ($n=15$), respectively were compared

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