Effects of Estrogen and Estrogen Receptors on Glucose Metabolism of Liver Cancer Cell

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Abstract

Epidemiology data show that men have a much higher incidence of hepatocellular carcinoma (HCC) than women, suggesting that estrogen and its receptors may inhibit HCC development and progression. To investigate the potential inhibitory mechanisms of estrogens on HCC development, human cancer cell line HepG2 was treated with different concentrations of estrogen and specific agonists for estrogen receptor (ER) ERα and ERβ to determine the roles of estrogen and its receptors in HepG2 cancer cell proliferation, apoptosis, gene expression, and metabolic pathways. Our findings indicated that estrogen and its receptors suppressed HepG2 cell growth by inhibiting cell proliferation and stimulating cell apoptosis via primary activation of ERβ, and by affecting HepG2 glucose metabolism via primary activation of ERα. Identifying roles of different estrogen receptors would provide comprehensive understanding of estrogenic mechanisms in HCC development and shed light on potential treatments for HCC patients.

Index Terms
HepG2, Estradiol, ERα, ERβ, Glycolysis, Oxidative phosphorylation

I. INTRODUCTION

Liver cells are highly specialized for their essential functions in detoxification and glucose metabolism. The most common primary liver cancer is hepatocellular carcinoma (HCC), one of the most malignant cancers with increasing incidence and mortality rate worldwide [1, 2]. The common risk factors of HCC include obesity, nonalcoholic fatty liver disease, chronic alcohol consumption, viral hepatitis infection, cirrhosis, and aflatoxin exposure, with obesity being a leading cause of HCC [3]. Data from clinical and epidemiological studies indicate that people with a body mass index (BMI) > 35 have an increased risk for developing HCC [4]. Thus metabolic defects in the liver could be a trigger for developing HCC.

Healthy cells produce energy via mitochondrial oxidative phosphorylation (OXPHOS) as their most common energy source. Cancer cells have characterized glucose metabolism with increased glucose uptake and increased aerobic and anaerobic glycolysis. Cancer cells grow more rapidly than blood vessels that nourish them, consequently obtain inadequate oxygen and experience hypoxia. In the absence of oxygen, cancer cells tend to use anaerobic glycolysis, *i.e.* lactic acid fermentation, as a primary source of ATP; along with reduction of pyruvate to lactic acid catalyzed by lactate dehydrogenase. Cancer cells also increase aerobic glycolysis even in the presence of oxygen, predominantly producing energy through glycolysis followed by lactic acid formation in the cytosol, rather than through mitochondrial OXPHOS as normal cells do, a phenomenon known as Warburg effect [5, 6]. It has been shown that high level of lactate, the end product of glycolysis, is associated with more aggressive cancer cells, such as drug-resistant and metastatic cancer cells [7]. Although glycolysis yields less ATP amount, its rate is faster than mitochondrial OXPHOS, providing sufficient energy to meet cancer cell demands [8].

Although Warburg effect is widely recognized, not all cancer types rely primarily on glycolysis for ATP production. Glycolysis contributes 1-64% ATP source in different types of cancers [9]. For example, among different leukemia cell lines, NB4 cells are more sensitive to treatment of 2-deoxy-D-glucose (2-DG), an inhibitor for glycolysis, than another leukemia cell line THP-1. NB4 cells are considered as "glycolytic" leukemia cells. In contrast, THP-1 cells are resistant to 2-DG treatment, but are sensitive to treatment of oligomycin, an inhibitor for OXPHOS; thus THP-1 cells are considered as "OXPHOS" leukemia cell line [10]. Therefore glucose metabolic pathways vary in different cancer cells. A specifically identified metabolic pathway could be used as a target for cancer therapy.

The unique change in HCC metabolism is the switch from glucose production to glucose usage. The activities of glucose-6-phosphatese, phosphoenolpyruvate carboxykinase, and fructose-diphosphatase involved in gluconeogenesis are decreased [11-13] leading to reduced gluconeogenesis in HCC. In addition, glycogenesis is decreased in liver cancer [14, 15]. On the country, the enzymes involved in glucose catabolism, including hexokinase-2, glucose-6-phosphate dehydrogenase, and pyruvate kinase-M2, have increased activities in liver cancer [16] to increase proliferation of cancer cells.

Epidemiological data indicate that the risk for men to develop HCC is 3-5 times greater than women. Sex hormones may contribute to the gender disparity in HCC development [17]. Whether estrogens play a protective or destructive role in HCC is still under debate. Evidence has shown that estrogens suppress progression of tumor growth, fibrosis, and carcinogenesis in HCC [18, 19]. Estrogens act on classic nuclear estrogen receptors (ERs), ERα and ERβ, presented in all types of liver cancer tissues [20]. ERα is usually considered

as a proliferation activator in many reproductive cancer cells, including breast, ovarian, and endometrial cancers in females [21, 22]. ERβ is less abundant in liver cells comparing with ERα [23]. Decreases in levels of gene and protein of ERβ have been found in many cancers, such as breast, prostate, and ovarian cancers [24-26]. The biological functions and significance of different subtypes of ERs in HCC development remain unclear. We tested the hypothesis that estradiol, the predominant and most biologically active estrogens in non-pregnant subjects, acted on ERs to inhibit liver cancer cell growth via changing glucose metabolism, using HepG2 cell line, the most commonly used liver cancer cell line in metabolic studies.

II. METHODS AND MATERIALS

Cell line and reagents

The human hepatocellular cancer-derived cell line HepG2 (American Type Culture Collection, Manassas, VA) was maintained in phenol red-free DMEM supplemented with 10% (v/v) heat-inactivated and charcoal-stripped FBS, 1% antibiotics of 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen, Grand Island, NY) at 37 ℃. Culture medium was starved in low serum (0.1% v/v FBS) for 16 h prior to experiments.

To examine major metabolic pathways utilized by HepG2 cells, an inhibitor for glycolysis 2-deoxy-D-glucose (2-DG, 1 - 10 mM; Santa Cruz, Dallas, TX), an inhibitor for lactate dehydrogenase sodium oxamate (OX, 5 – 50 mM; Santa Cruz, Dallas, TX), and an inhibitor for mitochondrial ATP synthase oligomycin (OM, 0.1 - 1µg/ml; Santa Cruz, Dallas, TX) were used. 2-DG and OX inhibit glycolysis, whereas OM inhibits mitochondrial OXPHOS. To examine the roles of estradiol and different ERs involved in growth of HepG2 cells, cells were treated with vehicle (1 µM dimethyl sulphoxide [DMSO]), a serial concentrations of water soluble 17β-estradiol (E2, 0 - 1000 nM; Sigma-Aldrich, St. Louis, MO), ERα selective agonist 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT, 1 µM; Fisher, Waltham, MA), or ERβ selective agonist 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN, 1 µM; Fisher, Waltham, MA). The dose of 1 µM for selective ER agonists is commonly used in liver cancer cell research [27], as well as other non-reproductive cancer cells with lower expression of ERs than reproductive cancer cells [23], such as adrenal carcinoma cells [28, 29], medulloblastoma cells [30], thyroid carcinoma cells [31], and colon cancer cells [32].

Cell counting and viability assay

HepG2 cell growth was evaluated using light microscopy. Cell number and cell viability were measured using a TC10TM automated cell counter (Bio-Rad, Hercules, CA) that counted cells within a 6-50 µm cell diameter range.

Cell proliferation assay

Bromodeoxyuridine (BrdU) incorporation analysis was performed using an enzyme-linked immunosorbent assay (ELISA) kit (Millipore Corporation, Billerica, MA). Approximately 5×10^4 HepG2 cells were seeded for 24 h before BrdU was added to the cultures. Four hours later HepG2 cells were fixed and DNA denatured. Prediluted BrdU detection antibody conjugated with peroxidase at a 2000X concentrate binds to the newly BrdU incorporated cellular DNA. The resultant immune complexes were quantified using a spectrophotometer microplate reader set at 450/550 nm double wavelength. Relative light units / second is proportional to amount of DNA synthesis and number of proliferating cells.

Western blotting

Protein was extracted by homogenization using lysis buffer with sodium orthovanadate, phenylmethylsulf inhibitor (Santa Cruz Biotechnology, Santa Cruz, CA), and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein lysates were resolved in 4%-15% tris-glycine gels and transferred to a nitrocellulose membrane (Bio-Rad). Cleaved-caspase 3 and total caspase 3 (1:1000; Cell Signaling, Danvers, MA) were detected via chemiluminescence (Amersham™ ECL™ Prime, GE Healthcare). Protein band density was visualized and quantified using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Quantitative densitometric values of Cleaved-caspase 3 were normalized to total caspase 3.

Quantitative real-time PCR

Total RNA was isolated from HepG2 cells using an RNeasy Plus kit (Qiagen, Foster City, CA), and was reverse transcribed using a cDNA synthesis kit (Bio-Rad, Hercules, CA). Interested genes included cytochrome c oxidase subunit 6B (*Cox6b*), glycogen synthase 2 (*Gys2*); phosphoenol-pyruvate carboxykinase (*Pepck*), ATP-dependent 6-Phosphofructokinase (*6Pfk*), pyruvate kinase (*Pk*), and glycogen phosphorylase L (*Pygl*). The primers were synthesized by Integrated DNA Technologies (San Jose, CA; Table 1). Quantitative real-time PCR was carried out using SYBR green master mixes and an iCycler (Bio-Rad, Hercules, CA). Amplified products were confirmed via gel electrophoresis and melt curve analysis. Results were generated from triplicate experiments, calculated by a $2^{-\Delta\Delta Ct}$ method, and normalized using the housekeeping gene β-actin.

TABLE I.

Table 1	Quantitative PCR primers	
Genes	*Forward primers 5'-3'*	*Reverse primers 5'-3'*
β-actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG
Cox6b	CTCAACGTGTTCCTCAAGTC	ATGGAGGACAGAGGAAAGG
Gys2	GCCAGACACCTGACATTAAG	CTCCACTTCATCTTCCACATC
Pepck	CCAGGCAGTGAGGGAGTTTCT	ACTGTGTCTCTTTGCTCTTGG
6Pfk	CGAGGACCCTTTCAACATC	CGAGGACCCTTTCAACATC
Pk	TCGTCTTTGCCTCCTTTG	CTCACCTCCAGGATTTCATC
Pygl	CCTGTGATGAGGCCATTTAC	GTATCCATAGGCTGCAAGTC

Statistical analysis

Data were presented as Mean ± SEM. Prism5 GraphPad Software (La Jolla, CA) was used to conduct statistical analyses. Two-way ANOVA comparing treatments with leptin and E2 or ER agonists followed by Bonferroni posttest was used to analyze cell numbers, proliferation and apoptosis, and intracellular signaling protein levels. One-way ANOVA followed by Tukey posttest was used to compare gene expressions. Statistically significance was set at $p < 0.05$.

III. RESULTS

Effects of OX, OM, or 2-DG on HepG2 cell number

2-DG at all doses tested, and OX at the high doses (10, 20, and 50 mM), but not the low dose of OX (5 mM) tested, decreased numbers of HepG2 cells comparing with control treatment (Fig 1). Treatment with up to 1 µg/ml of OM did not reduce HepG2 cells significantly (Fig 1). Thus, HepG2 cells were resistant to OM, but sensitive to OX and 2-DG, indicating that HepG2 cell line was mainly dependent on glycolysis, instead of mitochondrial OXPHOS, for ATP production.
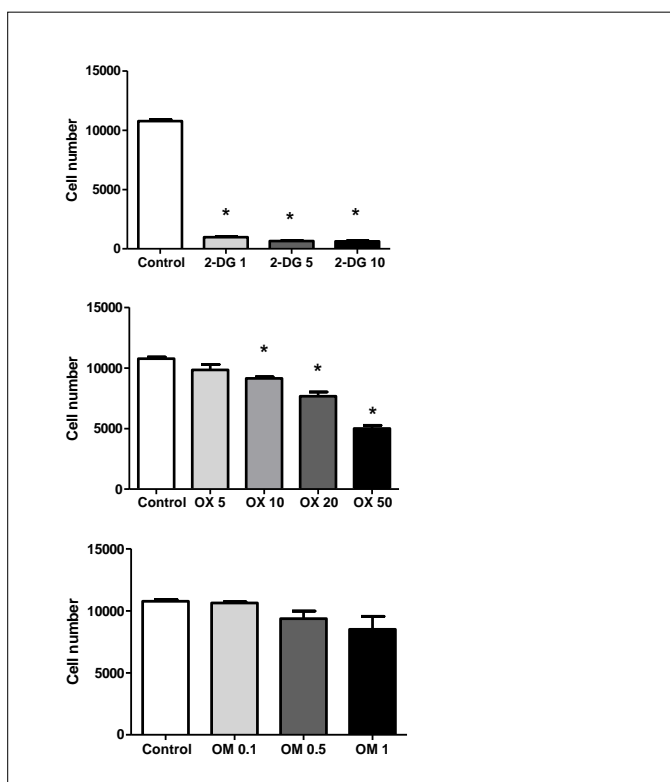

Fig. 1. Effects of 2-deoxy-D-glucose (2-DG), sodium oxamate (OX), and oligomycin (OM) on HepG2 cell number.

Effects of E2 and ER agonists on HepG2 cell number

The highest dose of E2 tested in this experiment (1000 nM), but not lower doses (1 nM, 10 nM, or 100 nM) decreased the numbers of HepG2 cells comparing with control (Fig 2). Additionally cell numbers were decreased by PPT and DPN comparing with control, with DPN having a greater effect (Fig 2).
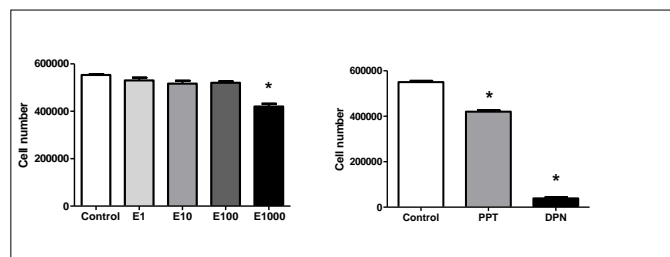

Fig. 2. Effects of estradiol (E2), ERα agonist PPT, and ERβ agonist DPN on HepG2 cell number.

Effects of E2 and ER agonists on HepG2 cell proliferation

Cell proliferation affects cell number and thus was assessed. E2 at 1000 nM, but not at 1 nM, reduced cell proliferation, indicated by lower BrdU incorporation following 1000 nM E2 treatment than control treatment. Additionally, proliferation was inhibited by ERα agonist PPT and ERβ agonist DPN. Thus, treatment of either E2 or each of ER agonists suppressed cell proliferation (Fig 3).
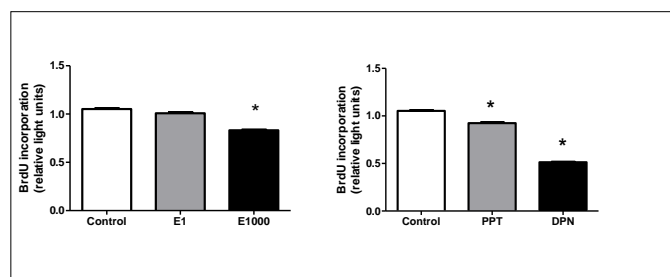

Fig. 3. Effects of estradiol (E2), ERα agonist PPT, and ERβ agonist DPN on HepG2 cell proliferation.

Effects of E2 and ER agonists on HepG2 cell apoptosis

Both cell proliferation and apoptosis determine cell number. Thus apoptosis was assessed subsequently. Protein level of cleaved-caspase 3 is a critical executioner in apoptotic cells responsible for proteolytic cleavage of many key proteins [33]. Caspase 3 cleavage, indicated using Western blotting, was increased following E2 treatment at 1 nM or 1000 nM, with a greater increase induced by 1000 nM E2 than 1 nM E2. Furthermore, cleaved-caspase 3 level was increased by treatment of PPT or DPN (Fig 4). Thus, E2 and its receptor agonists promoted caspase 3-dependent apoptosis in HepG2 cells.
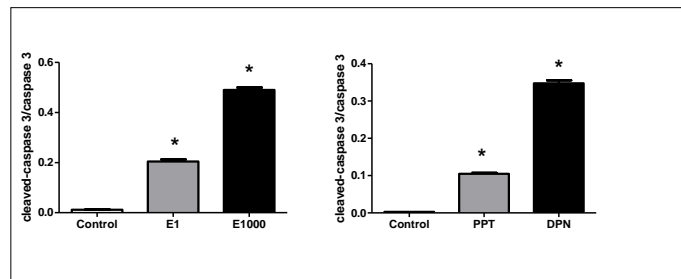

Fig. 4. Effects of estradiol (E2), ERα agonist PPT, and ERβ agonist DPN on HepG2 cell apoptosis.

Effects of E2 and ER agonists on gene expression involved in energy metabolism

HepG2 cells display enhanced rates of glucose usage. E2 and ERα agonist suppress HepG2 cell growth (Fig 2). Inhibition of glycolysis, but not inhibition of OXPHOS, suppressed HepG2 cell growth (Fig 1). We then tested the hypothesis that E2 and ER agonists could suppress glycolysis and thus glucose usage to achieve anti-cancer effects.

Gene expressions of *6Pfk* and *Pk*, which codes for two rate-limiting enzymes involved in glycolysis, ATP-dependent 6-phosphofructokinase and pyruvate kinase, were measured. E2, PPT, and DPN treatments all significantly lowered *6Pfk* expression comparing with the control treatment group, with E2 having the most prominent effects (Table 2). E2 treatment and DPN treatment, but not PPT treatment, significantly reduced *Pk* expression (Table 2). Expression of *Cox6b*, an essential gene involved in OXPHOS, and *Gys*, a critical gene for the formation of glycogen, however, were not significantly changed by any E2 or ER agonist treatment (Data not shown). The mRNA levels of *Pygl*, coding the enzyme glycogen phosphorylase L that breaks down glycogen in the liver, was significantly increased by E2 treatment (Table 2). Additionally, PPT induced *Pepck*, a gene that codes phosphoenol-pyruvate carboxykinase an enzyme regulating gluconeogenesis, comparing with E2 and DPN groups (Table 2).

Table 2	Gene Expression Measured Using Quantitative PCR		
Genes	**Control**	**E2**	**PPT**	**DPN**
Pepck	100.00 ± 3.87	93.42 ± 6.81	146.50 ± 13.68 *	97.73 ± 10.74
6Pfk	100.00 ± 5.85	31.41 ± 2.06 *	75.37 ± 0.70 *	67.45 ± 7.61 *
Pk	100.00 ± 3.89	32.44 ± 1.61 *	81.88 ± 10.49	44.35 ± 3.04 *
Pygl	100.00 ± 5.39	160.60 ± 4.95 *	89.13 ± 6.29	96.10 ± 2.73

IV. DISCUSSION

Women have a much lower rate of liver cancer than men [17]. Although this lower incidence of HCC in women may be attributed to female sex hormone estrogens, the protective role of estrogens in HCC development remains unclear. Reprogrammed energy metabolism is critical for the survival of tumor cells. The Warburg effect, a well-known metabolic change in cancer cells, allows cancer cells being more dependent on glycolysis to produce ATP instead of using OXPHOS for ATP production, even in the presence of oxygen. Inhibition of hexokinase 2, pyruvate kinase isozymes muscle type 2, or lactate dehydrogenase profoundly suppresses tumor progression in HCC, suggesting the critical role of the Warburg effect in cancer physiology [34-36]. In this study, we showed that HepG2 cell growth was suppressed by a glycolysis inhibitor 2-DG and a lactate dehydrogenase inhibitor sodium oxamate, but not by a mitochondrial ATP synthase inhibitor

oligomycin (Fig 1), which suggested that HepG2 cells more dominantly relied on glycolysis other than OXPHOS for ATP production. In the present study, we investigated the effects of E2 and selective agonists of ER subtypes on HCC development and glucose metabolism. Our findings indicated that E2 and its agonists reduced HepG2 cell numbers (Fig 2) and suppressed cell proliferation (Fig 3). Thus it is possible that E2 and its agonists inhibit glycolysis to reduce cancer cell growth.

Cell number is a net outcome between cell proliferation and cell apoptosis. It has been reported that estrogen and estrogen receptor agonists can stimulate cell apoptosis in a number of cancers, including ovarian cancer. The apoptotic effect of estrogen or its receptor agonists in hepatocellular carcinoma was tested. E2 increased cell apoptosis indicated by increased caspase 3 cleavage and decreased cell proliferation indicated by reduced BrdU incorporation (Fig 4). It is noteworthy that, although E2 at 1 μM has been used previously in vitro studies of liver culture cells that display specific binding of E2 and with similar properties of ERs as in reproductive tissues [37] but at a much lower expression level [23], caution should be taken into account that E2 greater than in vivo physiological concentration would potentially impact cell growth and intracellular molecules of HepG2 cells.

Selective activation of different subtypes of ERs elicited distinct effects. The decrease in HepG2 cell number was more evident by ERβ selective agonist DPN than ERα selective agonist PPT (Fig 2). E2 binding affinity for ERα is higher than for ERβ [38]. Thus, E2 acts mainly via ERα and provokes similar effects as ER-α selective agonist PPT. In contrast, DPN is an ERβ selective agonist and has a 70-fold higher binding affinity for ERβ than for ERα. DPN had far greater effects in regulating cell number than E2 (Fig 2), suggesting that ERβ, instead of ERα, plays major roles in regulating cell viability, including suppressing cell proliferation (Fig 3) and inducing cell apoptosis (Fig 4).

We also examined whether E2 affected regulatory genes for glucose metabolism of HepG2 cells and which ER subtype was more relevant to metabolism. The results from the qPCR experiment showed that E2, PPT, and DPN reduced *6Pfk* and *Pk* mRNA levels (Table 2), but not mRNA level of *Cox6b*, an OXPHOS enzyme, which indicated that E2 could suppress glycolysis via either ER-α or ER-β. Glycogen breakdown (indicated by *Gypl* expression) was enhanced by E2 treatment, and gluconeogenesis (indicated by *Pepck* expression) was enhanced by PPT treatment, which indicated that increased glucose production by E2 via ERα activation could be achieved by alternative pathways, such as pentose phosphorylation pathway, besides by glycolysis and OXPHOS [13].

E2 is known to either induce cell proliferation or induce cell apoptosis, by stimulating either oncogenes or tumor suppressor genes, depending on whether cell types are estrogen sensitive or insensitive [39-43]. The mechanisms underlying these opposite E2 effects could be partially explained by genomic and

non-genomic estrogenic action via different ER isoforms, which then either modulates estrogen target gene transcription or rapidly activates intracellular signaling pathways, respectively [44, 45]. Therefore investigating the effects of ERα and ERβ in HepG2 cell growth to understand genomic and non-genomic estrogenic actions in liver cancer development is of great interest and should be investigated in future.

To summarize, findings from the present study supported that estrogens attenuated HepG2 cell growth by facilitating apoptosis and suppressing proliferation. More importantly, we found that in HepG2 cells, neither ERα nor ERβ was pro-proliferative, as they are in breast, endometrial and ovarian cancer cells; instead, both selective agonists of ER-α and ER-β stimulated cell apoptosis. This study provides better understanding of estrogenic protective role in HCC development and indicates that ER-β agonists may have implications in potential HCC treatment. In future, the effects of ERs can be further confirmed using knock-down of respective receptor. Taken together, our data provided a better understanding of the protective role of estrogen in HCC development, and suggested an attractive target of estrogen receptor in the prevention and/or treatment of leptin-induced HCC. Glucose metabolism, including glycolysis and OXPHOS, plays a fundamental role in cancer cell survival. Certain metabolic genes identified in this study could be next generation of tumor treatment targets. Further research is required to recognize the ultimate functions of metabolic pathways as unexpected drivers of progression of human normal cells toward malignancy.

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