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Metformin suppresses CYP1A1 and CYP1B1 expression in breast cancer cells by down-regulating aryl hydrocarbon receptor expression

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ABSTRACT

Induction of cytochrome P450 (CYP) 1A1 and CYP1B1 by environmental xenobiotic chemicals or endogenous ligands through the activation of the aryl hydrocarbon receptor (AhR) has been implicated in a variety of cellular processes related to cancer, such as transformation and tumorigenesis. Here, we investigated the effects of the anti-diabetes drug metformin on expression of CYP1A1 and CYP1B1 in breast cancer cells under constitutive and inducible conditions. Our results indicated that metformin down-regulated the expression of CYP1A1 and CYP1B1 in breast cancer cells under constitutive and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced conditions. Down-regulation of AhR expression was required for metformin-mediated decreases in CYP1A1 and CYP1B1 expression, and the metformin-mediated CYP1A1 and CYP1B1 reduction is irrelevant to estrogen receptor α (ERα) signaling. Furthermore, we found that metformin markedly down-regulated Sp1 protein levels in breast cancer cells. The use of genetic and pharmacological tools revealed that metformin-mediated down-regulation of AhR expression was mediated through the reduction of Sp1 protein. Metformin inhibited endogenous AhR ligand-induced CYP1A1 and CYP1B1 expression by suppressing tryptophan-2,3-dioxygenase (TDO) expression in MCF-7 cells. Finally, metformin inhibits TDO expression through a down-regulation of Sp1 and glucocorticoid receptor (GR) protein levels. Our findings demonstrate that metformin reduces CYP1A1 and CYP1B1 expression in breast cancer cells by down-regulating AhR signaling. Metformin would be able to act as a potential chemopreventive agent against CYP1A1 and CYP1B1-mediated carcinogenesis and development of cancer.

Introduction

The majority of breast cancer tumors constitutively express cytochrome P450 (CYP) 1A1 and CYP1B1 (Vinothini and Nagini, 2010). A recent study indicated that the expression of CYP1A1 regulates breast cancer cell proliferation and survival (Rodriguez and Potter, 2013). In human breast tissue, the catechol metabolite, 4-hydroxyestradiol (4-OHE_2), formed by CYP1B1, generates free radicals from reductive–oxidative cycling. Estrogenic quinones cause oxidative DNA damage as well as form mutagenic depurinating adenine and guanine adducts and lead to the development and evolution of breast cancer (Yager, 2012).

CYP1A1 and *CYP1B1* are regulated by the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor. The active transcriptional AhR/aryl hydrocarbon nuclear translocator (ARNT) heterodimer complex binds to xenobiotic responsive elements (XRE) in the *CYP1A1* and *CYP1B1* promoter, resulting in increased transcription. Emerging evidence demonstrates a tumor-promoting role of the AhR, CYP1A1 and CYP1B1 in breast, lung, and hepatocellular carcinoma (Androutsopoulos et al., 2009; Feng et al., 2013; Rodriguez and Potter, 2013). The activation of AhR may lead to deregulation of cell–cell contact, thereby inducing unbalanced proliferation, dedifferentiation and enhanced motility (Dietrich and Kaina, 2010). AhR activation also enhances the clonogenicity and invasiveness of cancer cells (Gramatzki et al., 2009). Transgenic mice with a constitutively active AhR spontaneously develop tumors (Moennikes et al., 2004). Knockdown of AhR reduced tumor growth and metastasis of human breast cancer cells (Goode et al., 2013), and the repressor of the AhR (AhRR) is a tumor suppressor in multiple human cancers (Zudaire et al., 2008).

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CYP, cytochrome P450; ER, estrogen receptor; GR, glucocorticoid receptor; Hsp90, heat shock protein 90; Sp1, specificity protein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TDO, tryptophan-2,3-dioxygenase.

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Tryptophan-2,3-dioxygenase (TDO), encoded by *TDO2*, degrades L-tryptophan to L-kynurenine to act as an endogenous ligand of AhR. L-kynurenine is produced during cancer progression and inflammation in the local microenvironment in amounts sufficient to activate AhR under constitutive conditions. TDO, together with indoleamine-2,3-dioxygenases 1 and 2 (IDO1/2), plays a crucial role in suppression of anti-tumor immune responses (Uyttenhove et al., 2003) and is associated with a poor prognosis in various malignancies (Lob et al., 2009). Interestingly, TDO expression correlates with the expression of AhR target genes such as CYP1A1 and CYP1B1 in glioma, as well as B-cell lymphoma, Ewing sarcoma, bladder carcinoma, cervix carcinoma, colorectal carcinoma, breast cancer, lung carcinoma and ovarian carcinoma (Opitz et al., 2011). The specific protein 1 (Sp1)/glucocorticoid receptor (GR) signaling pathway regulates *TDO2* in cancer cells (Kolla and Litwack, 1999; Soichot et al., 2013; Suehiro et al., 2004). There is increasing evidence suggesting that down-regulation of TDO expression or inhibition of TDO activity may have a therapeutic application in cancer treatment (Munn and Mellor, 2004; Opitz et al., 2011).

Sp1 regulates the expression of numerous genes related to cell proliferation, differentiation and apoptosis by binding to GC-rich promoter elements through three Cys_2His_2-type zinc fingers present at the C-terminal domain (Kadonaga et al., 1988). An increase in Sp1 transcriptional activity is associated with tumorigenesis through modulation of oncogenes and tumor suppressor genes (Castro-Rivera et al., 2001; Stoner et al., 2004). Sp1 plays an important role in regulation of the AhR transcriptional expression under constitutive conditions through a Sp1-site located in the AhR promoter (Racky et al., 2004).

Metformin belongs to the biguanide class of oral hypoglycaemic agents. Population studies indicate that metformin treatment is associated with a decreased incidence of breast (Bodmer et al., 2010), prostate (Wright and Stanford, 2009), pancreatic (Li et al., 2009) and liver cancer (Lai et al., 2012). Here, we show that metformin reduces constitutive and inducible CYP1A1 and CYP1B1 expression through mechanisms involving AhR down-regulation in breast cancer cells, which explains how metformin reduces breast cancer risk.

Materials and methods

Materials

Metformin, mithramycin A, L-kynurenine, L-tryptophan, dexamethasone, and mifepristone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 7-Ethoxyresorufin was obtained from Pierce Chemical Co. (Rockford, IL, USA). TCDD was purchased from Chemsyn Science Lab. (Lenexa, KS, USA). The plasmid pCMV-β-gal was obtained from Clontech (Palo Alto, CA, USA). Lipofectamine™ 2000 and nitrocellulose membranes were purchased from Invitrogen (Carlsbad, CA, USA). Oligonucleotide polymerase chain reaction (PCR) primers were custom-synthesized by Bioneer (Seoul, South Korea). A protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Antibodies against CYP1A1, CYP1B1, AhR, ARNT, GR, Hsp90, Sp1, Lamin B1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TDO antibody was purchased from Abnova (Walnut, CA, USA). Horseradish peroxidase (HRP)-linked anti-rabbit and anti-mouse IgG secondary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). All other chemicals and reagents were of analytical grade.

Cell culture and treatment

The human breast cancer MCF-7 and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Cells were cultured in a humidified 5% CO_2 incubator at 37 °C in complete medium supplemented with 10% heat-inactivated foetal bovine serum (FBS; Invitrogen) to 70–80% confluence for use in the assays. Metformin was dissolved in water. Mithramycin A, dexamethasone, mifepristone, and TCDD were dissolved in dimethyl sulphoxide (DMSO). L-kynurenine and L-tryptophan were initially dissolved in DMSO, followed dilution into fresh DMEM medium. The working concentrations were added directly to culture medium. Control cells were treated with vehicle only.

Ethoxyresorufin-O-deethylase (EROD) activity assay

The effects of metformin on CYP enzyme activity were determined in intact MCF-7 cells by measurement of EROD activity. The cells, in 24-well plates, were treated with metformin (1–5 mM) and/or 10 nM TCDD for 24 h. After incubation, the medium was removed, and the cells were washed twice with fresh medium. Then, 5 μM ethoxyresorufin was added in growth medium as a substrate. Additionally, the growth medium was supplemented with 1.5 mM salicylamide in order to inhibit conjugating enzymes. The fluorescence intensity was measured in a FL600 microplate reader (Biotek, Winooski, VT, USA), with excitation at 530 nm and emission at 590 nm.

Luciferase and β-galactosidase assays

Cells were transfected with 1 μg of human CYP1A1-Luc, CYP1B1-Luc vector, pXRE-Luc reporter plasmid and/or 0.2 μg of pCMV-β-gal per well using Lipofectamine™ 2000. Human CYP1B1-Luc vector (−1635 to +588) was a gift from Dr. Robert Barouki, and an XRE-driven luciferase reporter plasmid (−1306 to −824 of the murine CYP1A1) containing four XREs was used to examine specific activation of XREs. At 6 h after transfection, fresh medium was added. Cells were treated with metformin (1–5 mM) and/or 10 nM TCDD for 24 h and lysed. The lysed cell preparations were then centrifuged (12,000 rpm, 10 min), and the supernatant was assayed for both luciferase and β-galactosidase activity. Luciferase activity was normalized to β-galactosidase activity to determine relative luciferase activity.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated with RNAiso reagent (Takara, Osaka, Japan) and cDNA was synthesized with the ImProm-II™ Reverse Transcriptase system (Promega, Madison, WI, USA). Product formation was monitored continuously during PCR using Sequence Detection System software (ver. 1.7; Applied Biosystems, Foster City, CA). Accumulated PCR products were detected directly by monitoring an increase in SYBR® reporter dye fluorescence. The expression levels of CYP1A1, CYP1B1, AhR, Sp1, TDO, and GR mRNAs in metformin-treated cells were compared to those in control cells at each time point using the comparative cycle threshold (C_t)-method. The following primer sequences were used: human AhR forward, 5′-ACT CCA CTT CAG CCA CCA TC-3′; human AhR reverse, 5′-GTG CAC AGC TCT GCT TCA GT-3′; human CYP1A1 forward, 5′-CAA GAG GAG CTA GAC ACA GT-3′; human CYP1A1 reverse, 5′-AGC CTT TCA AAC TTG TGT CT-3′; human CYP1B1 forward, 5′-TTC GGC CAC TAC TCG GAG C-3′; human CYP1B1 reverse, 5′-AAG AAG TTG CGC ATC ATG CT-3′; human GR forward, 5′-GAA CTT CCC TGG TCG AAC AGT T-3′; human GR reverse, 5′-GAG CTG GAT GGA GGA GAG CTT-3′; human Sp1 forward, 5′-AAA CAT ATC AAG ACC CAC CA-3′; human Sp1 reverse, 5′-ATA TTG GTG GTA ATA AGG GC-3′; human TDO forward, 5′-GGG AAC TAC CTG CAT TTG GA-3′; human TDO reverse, 5′-GTG CAT CCG AGA AAC AAC CT-3′; human β-actin forward, 5′-TGG CAC CCA GCA CAA TGA A-3′; human β-actin reverse, 5′-CTA AGT CAT AGT CCG CCT AGA AGC A-3′. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of β-actin as a housekeeping gene.