GENERAL GYNECOLOGY

Chemopreventive effects of metformin on obesity-associated endometrial proliferation

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OBJECTIVE: Obesity is a significant contributing factor to endometrial cancer risk. We previously demonstrated that estrogen-induced endometrial proliferation is enhanced in the context of hyperinsulinemia and insulin resistance. In this study, we investigate whether pharmacologic agents that modulate insulin sensitivity or normalize insulin levels will diminish the proliferative response to estrogen.

STUDY DESIGN: Zucker fa/fa obese rats and lean controls were used as models of hyperinsulinemia and insulin resistance. Insulin levels were depleted in ovariectomized rats following treatment with streptozotocin, or modulated by metformin treatment. The number of BrdU-incorporated cells, estrogen-dependent proliferative and antiproliferative gene expression, and activation of mTOR and ERK1/2 MAPK signaling were studied. A rat normal endometrial cell line RENE1 was used to evaluate the direct effects of metformin on endometrial cell proliferation and gene expression in vitro.

RESULTS: Streptozotocin lowered circulating insulin levels in obese rats and decreased the number of BrdU-labeled endometrial cells even in the presence of exogenous estrogen. Treatment with the insulin-sensitizing drug metformin attenuated estrogen-dependent proliferative expression of c-myc and c-fos in the obese rat endometrium compared to untreated controls and was accompanied by inhibition of phosphorylation of the insulin and IGF1 receptors (IRβ/IGF1R) and ERK1/2. In vitro studies indicated metformin inhibited RENE1 proliferation in a dose-dependent manner.

CONCLUSION: These findings suggest that drugs that modulate insulin sensitivity, such as metformin, hinder estrogen-mediated endometrial proliferation. Therefore, these drugs may be clinically useful for the prevention of endometrial cancer in obese women.

Key words: estrogen, insulin resistance, obesity

Cite this article as: Zhang Q, Celestino J, Schmandt R, et al. Chemopreventive effects of metformin on obesity-associated endometrial proliferation. Am J Obstet Gynecol 2013;209:24.e1-12.

Overweight and obesity not only increase the risk of a variety of chronic illnesses, including cardiovascular disease and type 2 diabetes, but also are known risk factors for a variety of cancer types.[1-3] Among all cancers, increasing body mass index is most strongly associated with endometrial cancer risk, with >50% of all endometrial cancers attributable to obesity.[4] While hyperestrogenism associated with obesity is a significant contributor to the development of endometrial cancer, other factors, including hyperinsulinemia, contribute to its pathogenesis and progression.

We previously evaluated the effect of obesity-associated insulin resistance and hyperinsulinemia on estrogen-associated endometrial proliferation in a rat model. Specifically, we showed that the expression of the proproliferative genes was increased while the expression of antiproliferative genes was inhibited in the endometrium of estrogen-treated obese, insulin-resistant rats as compared to lean controls.[5] These data suggested that insulin potentiates estrogen-regulated endometrial proliferation in the context of obesity.

To address the effects of insulin modulation as a chemopreventive strategy for endometrial cancer, circulating insulin levels and insulin sensitivity were manipulated in obese female Zucker rats using streptozotocin (STZ) and metformin, respectively, in the presence and absence of estrogen. Like obese human beings, the Zucker rat model develops insulin resistance, hyperinsulinemia, and ultimately, noninsulin-dependent diabetes.[6,7]

STZ, a glucosamine-nitrosourea compound, has been used to treat cancer of the pancreatic islets of Langerhans in human beings. It is extremely toxic to the beta cells of the pancreas, inhibiting insulin production, and therefore has

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Received Dec. 3, 2012; revised Jan. 30, 2013; accepted March 7, 2013.

This project was supported in part by grant number P50CA098258 from the National Cancer Institute and in part by the National Institutes of Health through MD Anderson Cancer Center Support Grant CA016672.

The authors report no conflict of interest.

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0002-9378/$36.00 • • http://dx.doi.org/10.1016/j.ajog.2013.03.008

limited clinical utility. However, this drug can be used to permanently reduce circulating insulin levels in laboratory animals. Metformin, a biguanide drug commonly used to treat type 2 diabetes, has recently been demonstrated to exert chemopreventive and antiproliferative effects for a variety of cancers.[8-10] Metformin inhibits cell growth both by insulin- and noninsulin-dependent mechanisms. Metformin increases insulin receptor sensitivity, increases insulin uptake, thereby reducing systemic insulin levels. Metformin also inhibits cell proliferation by activating the growth-inhibitory 5″ adenosine monophosphate activated protein kinase (AMPK), which counteracts signaling through both the PI3K/AKT and mitogen-activated protein kinase (MAPK) pathways downstream of the insulin and insulin growth factor 1 (IGF1) receptors.

The overall goal of these studies is to provide preclinical data to determine the ability of insulin-sensitizing drugs to attenuate estrogen-induced endometrial proliferation and serve as chemopreventive agents for endometrial cancer in obese individuals.

Materials and Methods

Cell lines

RENE1, a Sprague-Dawley rat normal endometrial cell line, was purchased from Sigma-Aldrich (St. Louis, MO).

Cell proliferation assay

RENE1 cells were treated with metformin or vehicle for 72 hours and cell proliferation was evaluated using 3-*(4,5-dimethylthiazol-2-Yl)*-2,5-diphenyltetrazolium bromide assay as previously described.[11]

Western blot

The effect of metformin on cell-signaling pathways was evaluated by Western blot analysis. RENE1 cells were plated in 6-well plates at 2×10^5/well. Following 24 hours, cells were treated by metformin (5 mmol/L in culture medium) for 72 hours. Cells were lysed in protein extraction reagent (ThermoScientific, Rockford, IL). Equal amounts of protein for each treatment group were resolved by sodium dodecyl sulfate poly acrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes, and probed for phospho-AMPK (T172), phospho-ERK1/2 (Thr202/Tyr204), phospho-S6 protein (Cell Signaling Technology Inc, Danvers, MA), or β-actin (Sigma-Aldrich) followed by horse radish peroxide-conjugated secondary antibody, per manufacturer's instructions. When necessary, polyvinylidene fluoride membranes were stripped (62.5 mmol/L Tris-HCl, PH 6.8, 2% SDS, and 100 mmol/L 2-mercaptoethanol) at 50°C for 30 minutes), washed twice in tris buffered saline/tween 20, then reprobed.

Animals care and use

All animal experiments were conducted in compliance with federal guidelines and approved by the institutional animal care and use committee. Mature (5 weeks old) female Zucker fa/fa rats and their lean littermates were purchased from Harlan Laboratories (Indianapolis, IN). After 1 week of acclimation, animals were ovariectomized, and held for 5 days to clear endogenous ovarian hormones.

STZ treatment

Obese and lean animals were randomized to 4 treatment groups (n = 6 per group): vehicle, estradiol (Innovative Research of America, Sarasota, FL), STZ (Sigma-Aldrich), and estradiol plus STZ. Estradiol (10 μg/kg body weight/d, for 2 weeks) was administrated by subcutaneously implanting the timed-releasing estradiol pellets 2 weeks before the end of the experiment, while in control group animals were implanted with the placebo pellets. Seven days after estradiol administration, animals were injected intraperitoneally with either vehicle (citrate buffer) or STZ (45 mg/kg). Seven days after STZ administration, animals were sacrificed for tissue collection.

Metformin treatment

Obese and lean rats were randomized to 3 treatment groups (17-26 animals per group): vehicle alone, estradiol, and estradiol plus metformin. Metformin (300 mg/kg body weight/d in 1% methyl-cellulose solution) was administrated by daily oral gavage for 3 weeks. Control animals received vehicle alone. Estradiol (40 μg/kg body weight/d, for 3 days) was administrated intraperitoneally for the last 3 days of the experiment. Control animals received saline alone. Animals were sacrificed and uteri were collected for histochemical evaluation and RNA isolation.

Plasma glucose level and insulin level detection

Three to 5 rats from each treatment group were fasted overnight, and were subjected to an oral glucose tolerance test.[5] Plasma glucose concentrations were tested with a blood glucose monitoring system (Ascensia Contour; Bayer Health Care, New York, NY). Insulin levels were by enzyme-linked immunosorbent assay (insulin ultrasensitive EIA kit; ALPCO Diagnostics, Salem, NH).

Immunohistochemistry

All rats were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU) at a dose of 100 mg/kg body weight 90 minutes before sacrifice. Fresh uterine tissues were collected and fixed in 10% neutral-buffered formalin, and processed for paraffin embedding. BrdU immunostaining was performed using BrdU in situ detection kit (BD Biosciences, San Diego, CA). The slides were counterstained with Mayer hematoxylin for 1 minute. The total number of BrdU-stained nuclei per 200 endometrial cells was counted in 10 randomly selected fields (200×).

Immunohistochemical analysis of rat uterine tissue was performed using Ki67 (BD Biosciences), phospho-IGF1R (Tyr1131)/insulin receptor β (Tyr1146), phospho-S6 ribosomal protein (Ser235/236), phospho-ERK1/2 (Thr202/Tyr204), phospho-acetyl-CoA carboxylase (Ser79) (ACC), and cleaved caspase-3 (Asp175) (Cell Signaling Technology Inc) per manufacturers' instructions. The sections were counterstained with Mayer hematoxylin. The average number of positively Ki67 or caspase-3 stained cells in 5-10 high-power microscopic fields were counted per slide, and calculated as: 200 × (numbers of stained endometrial cell/total endometrial cells). For all other markers, staining was scored based