Methylenecin acid is a novel suppressor of aromatase expression

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Abstract

Elevated circulating estrogen levels, as a result of increased peripheral aromatization of androgens by aromatase, have been indicated to underlie the association between obesity and a higher risk of breast cancer in postmenopausal women. Although aromatase inhibitors have been used as a first-line therapy for estrogen receptor-positive breast cancer in postmenopausal women, their potential as breast cancer chemopreventive agents has been limited due to toxicities and high costs. It is therefore imperative to develop new aromatase-inhibiting/suppressing agents with lower toxicities and lower costs for breast cancer chemoprevention, especially in obese postmenopausal women. The expression of the aromatase gene, CYP19, is controlled in a tissue-specific manner by the alternate use of different promoters. In obese postmenopausal women, increased peripheral aromatase is primarily attributed to the activity of the glucocorticoid-stimulated promoter, PI.4, and the cAMP-stimulated promoter, PII. In the present study, we show that methylenecin acid (MSA), a second-generation selenium compound, can effectively suppress aromatase activation by dexamethasone, a synthetic glucocorticoid, and forskolin, a specific activator of adenylate cyclase. Unlike the action of aromatase inhibitors, MSA suppression of aromatase activation is not mediated via direct inhibition of aromatase enzymatic activity. Rather, it is attributable to a marked downregulation of promoters PI.4- and PII-specific aromatase mRNA expression, and thereby a reduction of aromatase protein. Considering the low-cost and low-toxicity nature of MSA, our findings provide a strong rationale for the further development of MSA as a breast cancer chemopreventive agent for obese postmenopausal women.

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Introduction

Cytochrome P450 aromatase (CYP19) is the key enzyme for estrogen biosynthesis, and is responsible for converting androgens to estrogens (Simpson et al. 1994). In premenopausal women, aromatase is predominantly expressed in ovarian granulosa cells or placental syncytiotrophoblasts during pregnancy (Simpson et al. 1994). In postmenopausal women, after the ovaries have ceased to produce estrogens, adipose tissue becomes the principal site of estrogen biosynthesis (Simpson et al. 1994). Estrogen is known to play a critical role in breast cancer development and progression (Evans 1988), making aromatase an important target for breast cancer prevention and therapy.

The human aromatase gene has several promoters. Although the aromatase-coding region and protein are identical in all tissues that express aromatase, the promoter usage for transcription is somewhat tissue-specific (Bulun et al. 2005). The distal promoter I.4, which is located ~70 kb upstream of the translation start site, is the major promoter used in skin and adipose stromal cells for aromatase expression. Promoter I.4 can be activated by glucocorticoid (Mahendroo et al. 1993). The proximal promoter PII, just upstream of the translation start site, is used in premenopausal ovarian granulosa cells, breast cancer cells, and cancer adjacent breast adipose stromal cells (Agarwal et al. 1996). PII can be activated by FSH and LH in ovarian tissue, and cytokines such as prostaglandin E2 in breast tumors, through the cAMP–protein kinase A signaling pathway (Simpson et al. 1994, Zhao et al. 1996a). It has also been reported that altered adipokine milieu associated with obesity, e.g. elevated leptin level, can activate strong PII-induced aromatase expression in the peripheral
adipose tissue of obese women, resulting in elevated peripheral aromatization of androgens and increased circulating estrogen levels (Geiser et al. 2007, Maccio et al. 2009, Hursting 2011). This may underlie the association between obesity and increased risk of breast cancer in postmenopausal women (Brown et al. 2009, Brown & Simpson 2010).

Aromatase inhibitors are a first-line therapy for estrogen receptor-positive breast cancer in postmenopausal women. However, due to systemic suppression of estrogen biosynthesis, treatment with aromatase inhibitors often leads to side effects associated with estrogen depletion, including arthralgia, bone loss, and bone fracture, as well as possible cardiovascular and neurocognitive defects (Thurlimann et al. 2005, Buzdar et al. 2006, Coombes et al. 2007). As a result of these adverse effects and also the high costs of aromatase inhibitors, 23–30% of patients could not complete aromatase inhibitor therapy (Hershman et al. 2010, Sedjo & Devine 2011). With this high rate of nonadherence in patients with life-threatening cancer, it is reasonable to believe that patient acceptance of aromatase inhibitors as a breast cancer chemopreventive option could be limited. Therefore, it is imperative to develop new agents with lower toxicities and lower costs for breast cancer prevention.


In the present study, we characterized the effect of MSA on estrogen biosynthesis that has never been investigated before. We focused on the effect on promoters PI.4- and PI.1-driven aromatase expression because of the important role of these two promoters in regulating estrogen level in obese postmenopausal women. The long-term objective of the present study is to develop MSA as a low-cost, low-toxicity breast cancer chemopreventive agent for obese postmenopausal women.

Materials and Methods

Cell culture and reagents

The KGN human ovarian granulosa tumor cell line was established from a postmenopausal patient with invasive ovarian granulosa cell carcinoma (Nishi et al. 2001). KGN cells are undifferentiated, and maintain physiological characteristics of ovarian cells, including the expression of functional FSH receptor, relatively high aromatase activity, and the expression of estrogen receptor-β as the predominant isoform of estrogen receptor (Nishi et al. 2001, Chu et al. 2004). The cells were regularly cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The human preadipocyte cell strain, SGBS, was derived from an adipose depot of an infant with Simpson–Golabi–Behmel syndrome (Wabitsch et al. 2001). SGBS cells were routinely cultured in growth medium comprising DMEM/F12 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 33 μM biotin, and 17 μM pantethenolic acid. All the SGBS cells used in this study were in passage 30 to passage 35. The cells were switched to hormone-deprived medium (phenol red-free medium containing charcoal-stripped-FBS) for analysis of aromatase activity, protein, and mRNA. MSA was obtained from PharmaSe (Lubbock, TX, USA), 3H-Androst-4-ene-3,17-dione was from PerkinElmer (Waltham, MA, USA). Dexamethasone (Dex), forskolin (FSK), and other reagents were purchased from Sigma–Aldrich.

Establishment of aromatase-overexpressing MCF-7arom cells

The aromatase cDNA with the 129 bp 5’-UTR sequence was PCR amplified from the CYP19A1-coding plasmid (SC107980, OriGene, Rockville, MS, USA) and subcloned into pcDNA3.1/Zeo (+) between the HindIII and XbaI sites. The resulting pcDNA3.1-aromatase construct was transfected to MCF-7 cells and selected with 100 μg/ml Zeocin for 3 months to generate stable aromatase-overexpressing MCF-7 cells, MCF-7arom. MCF-7arom cells were regularly cultured in DMEM medium containing 5% FBS, 1% penicillin/streptomycin, and 50 μg/ml Zeocin.

Aromatase activity assay

Aromatase activity was determined by using the tritiated water release assay that measures the amount of 3H2O formed during the conversion of 3H-androstenedione to estrone by aromatase (Silva et al. 1989). SGBS cells were seeded to six-well plates (2 × 10⁴ cells/well) in hormone-deprived medium and allowed to attach overnight. The cells were treated with 250 nM Dex in the presence or absence of MSA for 6 or 16 h, and then washed twice with PBS before being incubated in fresh hormone-deprived medium containing 6 nM 3H-androst-4-ene-3,17-dione for an additional 4 h. Following incubation, the medium was removed and extracted with two volumes of chloroform. The samples were then centrifuged at 2000 g for 10 min, and the aqueous upper layer was mixed with 2% charcoal followed by an additional centrifugation at 12 000 g for 10 min to remove any trace amount of unreacted substrate. A 500 μl aliquot of the supernatant for each sample was subsequently subjected to liquid scintillation counting.

For KGN cells, the aromatase activity assay was performed the same way as for SGBS cells except that 25 μM FSK were
used to replace Dex to induce aromatase activity. For determining the direct effect of MSA on aromatase enzymatic activity in SGBS and KGN cells, MSA was not added to the Dex- or FSK-containing medium, but was present during the last 4 h of incubation together with 3H-androst-4-ene-3,17-dione.

Western blot analysis

KGN cells were seeded to hormone-deprived medium and cultured overnight. The cells were treated with FSK in the presence or absence of MSA for 6 or 16 h. Following treatment, the cells were washed twice with ice-cold PBS, and scraped in lysis buffer (Cell Signaling, Danvers, MA, USA). SDS-PAGE and western blotting procedures were done as described before (Liu et al. 2010). The mouse aromatase monoclonal antibody, 677/H7, was developed as described (Sasano et al. 2005), and the GAPDH antibody was obtained from Millipore (Billerica, MA, USA).

Aromatase total mRNA quantification

SGBS and KGN cells were seeded to hormone-deprived medium and cultured overnight before treatment. The cells were treated with MSA for 3 h. RNA extraction and real-time RT-PCR procedures were done as previously described (Dong et al. 2004). The primer–probe sets for aromatase (Hs00903409-m1) and β-actin (Hs99999903-m1) were from Applied Biosystems (Carlsbad, CA, USA).

Promoter-specific aromatase mRNA quantification

Promoter-specific aromatase PCR amplifications were performed with the use of the Sybr Green Supermix (Bio-Rad). The primer sequences specific to PI.4 (sense, GTGACC-ACTGGAGCCTG; antisense, CAGGAATCTGCGGAGA) and PII (sense, GCAACAGGAGCTATAGAT; antisense, CAGGAATCTGCGGAGA) were as previously described (McInnes et al. 2008). The data were normalized to β-actin levels.

Statistical analysis

Mean activities were calculated from at least three independent experiments done in triplicate. The Student's two-tailed t-test was used to determine the significant differences between two groups. *P<0.05 is considered statistically significant.

Results

MSA inhibits aromatase activation

As described in the Introduction, promoters PI.4 and PII can be activated by glucocorticoid and cAMP–protein kinase A signaling respectively. We first assessed the effect of MSA on aromatase activity induced by glucocorticoid and cAMP–protein kinase A. SGBS and KGN cells were chosen as the cell models for our study because their aromatase expression is driven mainly by promoter PI.4 or PII respectively (Ghosh et al. 2008, McInnes et al. 2008). Consistent with previous reports (McInnes et al. 2008, Ohno et al. 2009), the basal activity of aromatase was almost undetectable in both cell models, and the activity was induced respectively by Dex, a synthetic glucocorticoid, or FSK, a highly specific activator of adenyate cyclase (Fig. 1). Treatment of SGBS cells with MSA led to a dose-dependent inhibition of Dex-induced aromatase activation (Fig. 1A). The inhibitory effect of MSA on FSK induction of aromatase appears to be even more robust (Fig. 1B). A more than 60% inhibition was already evident with 0.6 µM MSA (Fig. 1B).

MSA inhibition of aromatase activation is not mediated at the enzymatic level

Aromatase inhibitors suppress aromatase activity through disrupting the binding of the substrates to aromatase (Chen et al. 2007). In order to determine whether the same mechanism underlies MSA inhibition of aromatase activation, we assessed the direct effect of MSA on the enzymatic activity of aromatase. The experiment was done by adding MSA to the culture at the same time as 3H-androstenedione so that MSA was not present when aromatase expression was induced.

Figure 1 Aromatase activity in (A) SGBS and (B) KGN cells in response to MSA treatment. Cells were treated with or without inducers in the presence or absence of MSA in phenol red-free medium for 6 or 16 h. The inducers and MSA were then removed, and cells were incubated with 3H-androstenedione for an additional 4 h. *P<0.05 compared to inducer-treated sample.
by Dex or FSK. We used the aromatase inhibitor letrozole as the positive control. The data, as presented in Fig. 2A and B, showed that, in both SGBS and KGN cells, while letrozole almost completely abolished the activity of aromatase, no significant change of aromatase activity was detected after MSA treatment. We also determined the response of an aromatase-overexpressing stable transfectant, MCF-7arom, to MSA treatment. The expression of aromatase in MCF-7arom cells is driven by a constitutive promoter. Consistently, we did not observe modulation of aromatase activity by MSA in these cells (Fig. 2C). The data were apparently different from that obtained when MSA was added to the culture together with the aromatase expression inducers (Fig. 1), indicating that the effect of MSA on aromatase is not mediated through affecting aromatase enzymatic activity.

To unravel the mechanism by which MSA inhibits aromatase activation, we assessed MSA modulation of aromatase protein. As shown in Fig. 3A, 0.6 μM MSA inhibited FSK–induced aromatase protein by more than 50%, and 2.5 μM MSA totally blocked the induction. We also examined the effect of MSA on Dex–induced aromatase protein expression in SGBS cells. However, the low level of aromatase expression in SGBS cells, even after Dex induction, was under the detection limit of aromatase western blot analysis. We next characterized the effect of MSA on the level of total aromatase mRNA by real-time RT-PCR using primers recognizing all aromatase transcripts. As shown in Fig. 3B and C, MSA significantly suppressed Dex and FSK induction of aromatase mRNA, and the effect on FSK–induced expression was even more pronounced. In order to confirm that the decrease in total aromatase mRNA was due to suppression of transcription from promoter PI.4 or PII, we repeated the real-time RT-PCR analysis using primers specific to PI.4 or PII. The results, as shown in Fig. 4, are in great concordance with that presented in Fig. 3B and C. Taken together, the data indicated that MSA suppressed aromatase activation through downregulating promoter PI.4- and PII-driven aromatase mRNA expression.

**Discussion**

Elevated circulating estrogen levels, as a result of increased peripheral aromatization of androgens, have been indicated to underlie the association between obesity and a higher risk of breast cancer in postmenopausal women (Brown et al., 2009, 2012).
Brown & Simpson 2010). In the present study, we showed that both PI.4- and PII-driven aromatase transcription can be efficiently suppressed by MSA, leading to a marked down-regulation of aromatase mRNA, protein, and thereby activity. Considering the low-cost and low-toxicity nature of MSA, the data provide a strong rationale for the further development of MSA as a breast cancer chemopreventive agent for obese postmenopausal women.

Our data on MSA suppression of PII- and PI.4-driven aromatase expression also suggest a role of MSA in reducing breast intratumoral estrogen level. In postmenopausal breast cancer patients, tumor concentration of estrogens has been reported to be \(\sim 10\) times the concentration in plasma (van Landeghem et al. 1985, Bulun et al. 1993, Agarwal et al. 1996, Miller et al. 1997). This is attributable to the heterotypic interaction between breast tumor cells and tumor adjacent adipose stromal cells (Bulun et al. 2005). In response to estrogen stimulation, breast tumor cells secrete large amounts of cytokines, such as tumor necrosis factor \(\alpha\) and interleukin 11, to inhibit adipogenic differentiation of adjacent stromal cells (Crichton et al. 1996, Meng et al. 2001). This leads to an upregulated PII promoter activity (Irahara et al. 2006) and thereby an elevated local aromatase expression and estrogen level (Meng et al. 2001), thus creating a localized, growth-stimulatory environment for tumor cells. In addition, aromatase expression has also been detected in breast tumor cells, although at a much lower level compared to the adipose stromal cells (Miki et al. 2007). Promoter PII is also a main promoter driving the expression of aromatase in breast tumor cells (Agarwal et al. 1996). Therefore, reducing intratumoral production of estrogen may represent an additional novel mechanism of MSA anticancer action.

In fact, we have sought to study the effect of MSA on aromatase expression in breast cancer cell lines. However, none of the breast cancer cell lines that we have tested, including MCF-7, T47D, and MDA-MB-468, have detectable aromatase expression even after inducer treatment. Whether cultured breast cancer cell lines express a detectable amount of aromatase is still debatable. While some groups were able to detect aromatase expression in cell lines such as MCF-7, T47D, and MDA-MB-468 (Kijima et al. 2006, Miki et al. 2007, Ciolino et al. 2011), others could not (Sanderson et al. 2001, Heneweer et al. 2005). Nevertheless, the data that we obtained from KGN cells should be applicable to breast tumor cells as PII-mediated expression is regulated mainly by the cAMP–protein kinase A pathway in both cell types (Zhao et al. 1996a, Ghosh et al. 2008).

A number of transcription factors have been implicated in PII regulation, including LRH-1, CREB, CRT2, ATF2, SF-1, C/EBPs, Jun, and several orphan nuclear receptors (Zhou et al. 2001, Clyne et al. 2002, Yang et al. 2002, Sofi et al. 2003, Ghosh et al. 2008, Kijima et al. 2008, Brown et al. 2009). PI.4 is a TATA-less promoter that contains a glucocorticoid response element, Sp1-binding site, and an interferon-\(\gamma\) activation site element (Zhao et al. 1996b). The JAK/STAT signaling pathway has been reported to be involved in PI.4 regulation (Zhao et al. 1995). MSA has been shown to alter the expression levels of a number of proteins in stromal cells, including cAMP-responsive element–binding protein 6 (CREB6) (Jiang et al. 1999, Tsavachidou et al. 2009, Zhang et al. 2010). We are currently investigating the effect of MSA on signal transduction from the cAMP–protein kinase A and glucocorticoid receptor/JAK/STAT pathways to elucidate the mechanisms by which MSA inhibits aromatase expression.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y D, R G, L Z, B G R, and Q Y designed the research; R G and X L conducted the study; R G, B G R, Q Y, and Y D wrote the paper; M W supplied SGBS cells; D P E supplied the aromatase antibody; Y N and T Y supplied KGN cells; Y D and Y Q had primary responsibility for the final content.

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