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Nandrolone and stanozolol upregulate aromatase expression and further increase IGF-I-dependent effects on MCF-7 breast cancer cell proliferation

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ABSTRACT

Several doping agents, such as anabolic androgenic steroids (AAS) and peptide hormones like insulin-like growth factor-I (IGF-I), are employed without considering the potential deleterious effects that they can cause. In addition, androgens are used in postmenopausal women as replacement therapy. However, there are no clear guidelines regarding the optimal therapeutic doses of androgens or long-term safety data. In this study we aimed to determine if two commonly used AAS, nandrolone and stanozolol, alone or in combination with IGF-I, could activate signaling involved in breast cancer cell proliferation. Using a human breast cancer cell line, MCF-7, as an experimental model we found that both nandrolone and stanozolol caused a dose-dependent induction of aromatase expression and, consequently, estradiol production. Moreover, when nandrolone and stanozolol were combined with IGF-I, higher induction in aromatase expression was observed. This increase involved phosphatidylinositol 3-kinase (PI3K)/AKT and phospholipase C (PLC)/protein kinase C (PKC), which are part of IGF-I transductional pathways. Specifically, both AAS were able to activate membrane rapid signaling involving IGF-I receptor, extracellular regulated protein kinases 1/2 (ERK1/2) and AKT, after binding to estrogen receptor (ER), as confirmed by the ability of the ER antagonist ICI182, 780 to block such activation. The estrogenic activity of nandrolone and stanozolol was further confirmed by their capacity to induce the expression of the ER-regulated gene, CCND1 encoding for the cell cycle regulator cyclin D1, which represents a key protein for the control of breast cancer cell proliferation. In fact, when nandrolone and stanozolol were combined with IGF-I, they increased cell proliferation to levels higher than those elicited by the single factors. Taken together these data clearly indicate that the use of high doses of AAS, as occurs in doping practice, may increase the risk of breast cancer. This potential risk is higher when AAS are used in association with IGF-I. To our knowledge this is the first report directly associating AAS with this type of cancer.

1. Introduction

Androgenic anabolic steroids (AAS) are widely prescribed for the treatment of male hypogonadism. However, they may play a significant role in the treatment of other conditions as well, such as cachexia associated with human immunodeficiency virus infection, cancer, burns, renal and hepatic failure, and anemia associated with leukemia or kidney failure (Basaria et al., 2001). Unfortunately, AAS are also used for illicit, self-administration to enhance athletic performance or body image (for review Kanayama et al., 2008; Trenton and Currier, 2005). Another alarming factor is that exposure to AAS starts at an earlier age since a significant increase of their use among adolescent athletes and non-athletes has been observed (Calfee and Fadale, 2006; Dawson, 2001; Fitness, 1997). Clinical reports highlight a link between AAS abuse and different kinds of cancer, such as hepatocellular adenomas and adenocarcinomas (Socas et al., 2005). In addition, we have recently reported that AAS, through the activation of estrogen-dependent pathways, are involved in the progression of a type of testicular cancer (Chimento et al., 2012). To date no data regarding the relationship between AAS abuse and breast cancer risk are available.

Effects of androgen administration on breast cancer is a very current topic. Indeed, recently, androgens have been indicated for hormone replacement therapy (HRT) in postmenopausal women, or in young women who had both ovaries removed, as an alternative to estrogen/progestin administration in order to prevent cardiovascular and metabolic alterations. Several investigations examined the effects of androgens in postmenopausal women (Braunstein, 2007). A potential risk associated with androgen treatment in postmenopausal women is due to the potential conversion of C19 steroids into estrogens, allowing the same negative

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effects observed in women undergoing HRT with estrogen/progestin. Despite this potential risk, postmenopausal androgen replacement is becoming increasingly widespread. However, there are no clear guidelines regarding the optimal therapeutic doses or long-term safety. Therefore it appears extremely urgent to clarify all the aspects associated with the mechanism of action of androgens in breast cancer.

The role of androgens in normal breast tissue and breast cancer is still controversial. Emerging evidence indicate that androgens mainly exert inhibitory effects on the growth of normal mammary epithelial cells and play a protective role in the pathogenesis of breast cancer (Labrie et al., 2003; Somboonporn and Davis, 2004; Tworoger et al., 2005; Yeh et al., 2003). Nonetheless, there are also some epidemiologic reports supporting the concept that androgens, in certain settings, can contribute to breast cancer growth (Missmer et al., 2004; Tong et al., 2002). These latter effects elicited by androgens can be attributed to their ability to directly activate estrogen receptor (ER)-dependent pathways (Aspinall et al., 2004; Rochefort and Garcia, 1976), as well as to their ability to be converted into estrogens by the aromatase enzyme encoded by the CYP19 gene (Simpson et al., 2000; Sonne-Hansen and Lykkesfeldt, 2005). Aromatase expression in breast cancer tissues is higher than in the normal breast tissues (Harada, 1997; Miller et al., 1997). In breast cancer cells aromatase has been shown to promote tumor growth in both an autocrine and a paracrine manner (Catalano et al., 2009; Sun et al., 1997; Yue et al., 1994), leading to an increase of 17β-estradiol (E2) production that, in turn, activates ER and stimulates tumor growth. This is confirmed by the observation that overexpression of aromatase in mammary gland of transgenic mice causes premalignant lesions, such as atypical ductal hyperplasia (Gill et al., 2001; Tekmal et al., 1996).

The ability of estrogens, via ER activation, to stimulate proliferation and differentiation in normal and cancerous epithelium has been widely discussed (Katzenellenbogen et al., 1987; Osborne et al., 1985). More than 60% of breast cancers express ER (Clark et al., 1994; Keen and Davidson, 2003) which is considered a marker for breast cancer diagnosis and prognosis (Sisci et al., 2007) and represents a target for antiestrogen therapy.

Normal mammary development depends on E2 surge, which, however, does not act alone. It relies on pituitary growth hormone (GH) to induce insulin-like growth factor I (IGF-I) production in the mammary stromal compartment. In turn, IGF-I permits E2 action (Kleinberg and Barcellos-Hoff, 2011). In breast cancer tissue, local IGF-I levels are mainly contributed by breast cancer cells and surrounding stromal cells in adjacent normal breast tissue (Chong et al., 2006a). IGF-I is a potent mitogen for both normal and transformed breast epithelial cells (Cullen et al., 1990), and serum IGF-I level is associated with the development of mammary gland hyperplasia and cancer in a primate model (Ng et al., 1997). The actions of IGF-I are mediated primarily by the type I insulin-like growth factor receptor (IGF1R). The activation of the IGF1R tyrosine kinase results in the stimulation of intracellular pathways such as Ras/Raf mitogen activated protein kinase (MAPK), PI3K/AKT and PLC/PKC (Rubin and Baserga, 1995). IGF1R expression is up-regulated by estrogens (Pandini et al., 2007), which also enhance IGF1R-dependent signaling (Panno et al., 2006). In ERα-positive breast tumors and cancer cell lines, ERα and IGF1R are often co-expressed and E2 acts in synergy with IGF-I to stimulate the maximal cell proliferation (Jerome et al., 2004; Surmacz, 2000). It has been reported that athletes, in addition to the use of androgens, (Evans, 1997) also abuse IGF-I (Evans, 1997), which could work in synergy with androgens aggravating any potential proliferative effect exerted on breast cancer cell growth. In addition, during hormone replacement therapy with androgens in postmenopausal women, locally produces IGF-I could further increase androgen-induced proliferative effects.

Based on these observations, using MCF-7 breast cancer cells as a model, in the present study we have investigated the effects of commonly used AAS, differentially metabolized by aromatase, nandrolone (aromatizable) (Reznik et al., 2001; Sundaram et al., 1995) and stanozolol (non-aromatizable) on aromatase expression, ER activation and breast cancer cell proliferation. We also evaluated the effects of combined treatment of cells with AAS and IGF-I.

2. Materials and methods

2.1. Cell cultures

Wild-type human breast cancer (MCF-7) cells were a gift from Dr. E. Surmacz (Temple University, Philadelphia, PA, USA). Human uterine cervix adenocarcinoma (HeLa) cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). MCF-7 cells were cultured in DMEM/F-12 containing 5% calf serum and antibiotics (Sigma Aldrich, Saint Louis, MO, USA) (complete medium). HeLa cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and antibiotics (Sigma Aldrich) (complete medium). Cell monolayers were subcultured onto 30 mm dishes for protein or RNA extraction (1×10^6 cells/plate), 12 well culture dishes for steroid measurement (2.5×10^5 cells/well), 24 well culture dishes for transfection and proliferation assay (1×10^5 cells/well). Twenty-four hours later complete medium was replaced with DMEM/F-12 containing 1% Pen/Strep and 1% glutamine (serum-free medium). Cells were treated 24 h later in DMEM/F-12 medium containing 2.5% dextran charcoal coated (DCC) FBS with nandrolone, stanozolol, IGF-I, AG1024, ICI182,780 (Sigma Aldrich), PD98059, LY294002 and/or GF109203X (Calbiochem, San Diego, CA, USA). The reagents used to treat cells were resuspended in DMSO except for IGF-I, which was resuspended in water. Basal, untreated cells were added with vehicle only (DMSO) that never exceeded 0.002% (V/V).

2.2. Radioimmunoassay

Cells were treated for 48 h with nandrolone and stanozolol in DMEM F-12 serum-free medium. Estradiol, released in the medium, was extracted by solvent partition using diethyl ether (Sigma Aldrich). After evaporation of the organic phase, the extracts were redissolved in 200 μl of kit standard n.1 (0 pg/ml of estradiol). Estradiol concentration was determined against standards using a radioimmunoassay kit (Diagnostic System Laboratories, Webster, TX, USA). Results were normalized to the cellular protein content per dish.

2.3. Western blot analysis

Cells were lysed in ice cold lysis buffer (50 M HEPES (pH 7.5), 150 nM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, protease inhibitors (Sigma Aldrich)). Equal amounts of total protein were resolved on 11% SDS-polyacrilamide gels and transferred onto a nitrocellulose membrane. Blots were incubated overnight at 4 °C and probed with the following antibodies: (a) anti-human cytochrome P450aromatase (Serotec, Oxford, UK) (1:50), (b) anti-cyclin D1 (M-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000), (c) anti-pIGF1R (Cell Signaling Technology, Beverly, MA, USA) (1:500), (d) anti-pERK (Cell Signaling Technology) (1:500), (e) anti-pAKT (Santa Cruz Biotechnology) (1:500), (f) anti-IGF1R (Santa Cruz Biotechnology) (1:500), (g) anti-ERK (Cell Signaling Technology) (1:1000), (h) anti-AKT (Santa Cruz Biotechnology) (1:1000), (i) anti-ERα (D12) (Santa Cruz Biotechnology) (1:1000). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia,