Adipocyte-derived Factor as a Modulator of Oxidative Estrogen Metabolism: Implications for Obesity and Estrogen-dependent Breast Cancer

H. LEON BRADLOW*, DANIEL W. SEPKOVIC*, NITIN TELANG** and RAJ TIWARI***

Strang Cancer Research Laboratory, New York, NY, USA

Abstract. The role of body fat as a risk factor for breast cancer has been well established. A decrease in the urinary 2/16α-hydroxyestrone ratio has also been shown to be a risk marker for breast cancer. These two observations are connected by the fact that obese women have decreased levels of 2-hydroxyestrone. To test the hypothesis that fat depots secrete factors that inhibit 2-hydroxylation, the effect of substances released into the media from adipocytes incubated in Krebs-Ringer buffer, on estrogen metabolism by MCF-7 cells in minimum essential medium eagle (MEM) plus adipocyte-conditioned media (ACM) was studied. The 1:1 ACM-MEM culture system resulted in a substantial and highly significant decrease in 2-hydroxylation of estradiol. This inhibition was partially reversed by the addition of indole-3-carbinol, a potent inducer of 2-hydroxylation of estradiol. Centrifugal sizing showed that the active 2-hydroxylation inhibitor in the medium had a molecular weight of about 30 kDa. These results suggest a mechanism for the decrease in 2-hydroxylation of estradiol that is observed in obese women and the increase in 2-hydroxylation observed in women with depleted fat depots.

The role of obesity as a risk factor for breast cancer has been well established (1-5), but a convincing mechanism for this observation has not been proposed. Decreased formation of 2-hydroxyestrone in obese persons was shown in our laboratory (6), while women with decreased fat depots resulting from vigorous exercise or anorexia have greatly elevated levels of 2-hydroxylation (7-13). These latter observations are pertinent because we and others have shown that a decrease in the urinary 2/16α-hydroxyestrone ratio is associated with increased risk for breast cancer (14-18). Recently, Im et al. have reported the converse: that an increased 2/16α hydroxylation ratio is associated with reduced risk for breast cancer (19). 2-Hydroxyestrone itself has been shown to be non-carcinogenic, unlike its 4-hydroxy isomer, which is a known carcinogen (20). The aim of the present study was to test the hypothesis that reduced 2-hydroxylation of estradiol in obese persons is due to the release of a factor from adipocytes that inhibits 2-hydroxylation. To test this hypothesis, the effect of incubating MCF-7 breast cancer cells in a 1:1 mixture of minimum essential medium and adipocyte-conditioned medium on the metabolism of added radioactively labeled estradiol was investigated.

The pattern of estradiol metabolism is exclusively oxidative. The various pathways of estradiol metabolism are carried out by specific enzymes: CYP4501A1/2 for 2-hydroxylation, CYP4501B1 for 4-hydroxylation, CYP4503A4 for 16α-hydroxylation, and 17β-steroid dehydrogenase for 17-oxidation.

Materials and Methods

MCF-7 breast cancer cells were initially obtained from the Michigan Cancer Foundation (Detroit, Michigan) and maintained in our laboratory. Radioactivity counting was carried out in a Packard Tri-Carb liquid scintillation counter. Statistics were carried out using the t-test. Conditioned media human body fat was obtained from either buttok or abdominal fat of volunteer subjects using a trocar needle (21). The tissue was incubated with collagenase in Krebs-Ringer buffer for 4 h at 37˚C. The medium was then cleared of cellular debris by centrifugation. The preparation of the conditioned media was carried out in the laboratory of Dr. Rudy Leibel at the Rockefeller University (New York, NY, USA) and was a generous gift from him. The supernatant media designated as (ACM) was initially tested directly by 1:1 addition to MEM for MCF-7 cell culture studies.

Key Words: Obesity, 2-hydroxylation, estradiol metabolism, breast cancer.
Substrates. 2-3H-estradiol was purchased from New England Nuclear Corp (Boston, MA, USA). 16α-3H-estradiol was prepared in this laboratory (22).

Experimental procedure. MCF-7 cells were cultured in MEM media containing 5 mg/ml of insulin, 2 mM L-glutamine, 50 IU/ml of penicillin, 50 μg/ml of streptomycin, plus 10% fetal calf serum (23). Prior to the experiments the cells were plated in 24-well plates (10⁴ cells in 1 ml of MEM). After the cells reached 80% confluence the medium was replaced with 0.5 ml of ACM plus 0.5 ml of MEM, 100,000 dpm of C-2 or C-16α-3H estradiol in 10 μl of ethanol was added and the incubation was continued for an additional 48 h. Two 0.1 ml aliquots were taken for counting to determine the total dose and a single 0.5 ml aliquot was diluted with 3 ml of distilled water and lyophilized, and the water trapped in a receiver cooled in acetone-dry ice. Two aliquots of the sublimed water (1.0 ml) were counted to determine the extent of the reaction as described previously (23). The cells remaining in the wells were analyzed colorimetrically for protein using the Pierce reagent as described previously (25). The results are reported as the percentage reaction per 100 mg of protein. Each measurement is the mean of 3 wells for each ACM sample assayed.

Fractionation of ACM. To further characterize the biologically active component of the ACM, the conditioned medium was fractionated by centrifugal filtration through 10 kDa, 30 kDa, and 50 kDa Filtron membranes (Pall Filtron Corp, Port Washington, NY), which act as molecular weight sieves (26). The filtrate and the supernatant were separately diluted 1:1 with MEM and tested for inhibitory activity. The 50 kDa filtrate was also re-fractionated using a 30 kDa membrane and the upper and lower layers were again separately assayed.

Assay of potential inhibitors. Because leptin had been previously reported to be an inhibitor of 2-hydroxylation, ACM was assayed for leptin by Linco Diagnostics (St Louis, MO) and none was found. The ACM was also analyzed in the laboratory of Dr. Michael Lazar of the University of Pennsylvania (Philadelphia, PA) for resistin, which has approximately the same molecular weight, and none was found.

Results

Dilution of MEM 1:1 with ACM resulted in a highly significant decrease of 63% in 2-hydroxylation (p=0.001) and a minimal, non-significant increase in 16α-hydroxylation (p=0.11) (Figure 1). In some experiments, 50 μM indole-3-carbinol (I3C), a known stimulator of 2-hydroxylation of estradiol (24), was added to the incubation mixture. A partial increase of 2-hydroxylation towards the control level was demonstrated. This increase was not quite significant (p=0.11) (Figure 2). In contrast, the addition of I3C alone to the undiluted MEM resulted in a highly significant (p=0.001) increase of 2-hydroxylation, as reported by Niwa et al. (24), When the 2-/16α-hydroxylation ratio was compared with and without I3C, the increase in the 2/16α ratio in the presence of I3C MEM-ACM medium was far below the increase in that observed with MEM + I3C (Table I).

In order to further characterize the active component of ACM, MCF-7 cells were incubated in fractionated subsamples diluted 1:1 with MEM. The fraction passing the 10 kDa membrane had no activity; all of the active material filtered through the 50 kDa membrane, but was mostly retained by a 30 kDa membrane, suggesting a molecular weight between 10 and 30 kDa. In a reverse experiment using a cell system containing 50 μM of I3C, the individual
molecular-weight fractions were used to dilute the MEM (Figure 3). Only fraction E (10-30 kDa) was able to inhibit the estradiol 2-hydroxylation-promoting effect of I3C. The inhibition cannot be attributed to the Krebs-Ringer buffer in the medium, since the addition of untreated Krebs-Ringer buffer produced no inhibitory effect (data not shown). In addition, neither leptin nor resistin were found in the ACM.

**Discussion**

These results present a possible mechanism for the decreased 2-hydroxylation of estradiol that has been reported in obese women (6) and the increased 2-hydroxylation that has been reported in anorectic girls (7) and highly athletic women engaged in 8-oar-crew rowing with depleted fat depots (12).

The decrease in 2-hydroxylation in ACM was roughly the same, whether the adipocytes were derived from buttock fat or abdominal fat (data not shown). There was a large, consistent, highly significant decrease in 2-hydroxylation and a small, non-significant increase in 16α-hydroxylation, suggesting that the effect of ACM on 2-hydroxylation was specific, whereas the effect on 16α-hydroxylation was most likely secondary and indirect, resulting from the increased availability of estradiol substrate for 16α-hydroxylation when 2-hydroxylation is inhibited. When I3C was added to the MCM+ACM, a partial, but not quite statistically significant restoration of 2-hydroxylation to that of the control level was demonstrated. When I3C was added alone to the control medium, Niwa et al. found a 6-fold increase in 2-hydroxylation (24).

The modest increase in 2-hydroxylation following the administration of I3C to obese women reported by Michnovicz (27) relative to the greater increase observed in normal subjects by Michnovicz and Bradlow (28) is consistent with the results in these cell culture studies.

Following molecular-weight fractionation of the ACM, only the fraction around 30 kDa showed significant inhibitory activity. The absence of any inhibitory activity for the <10 kDa fraction established that the inhibitor is not a simple small molecule, but did not exclude the possibility that it is a small molecule firmly bound to a larger one. Further studies to identify the active principle in ACM are under-way. The fact that I3C partially blocked the inhibitory effect of ACM suggested that I3C and the inhibitory ACM subfraction may be acting on some common mechanism, although not necessarily at the same locus. Further studies to identify the active principle are underway.

In light of the protective effect of 2-hydroxyestrone against breast cancer, the inhibitory effect of ACM on 2-hydroxylation of estradiol provides a possible mechanism for the connection between obesity and the increased risk of breast cancer observed in obese women. Conversely, the decreased levels of ACM in women who exercise, with depleted fat depots, results in higher levels of 2-hydroxyestrone. Higher levels of 2-hydroxyestrone are correlated with decreased risk of breast cancer.

**Acknowledgements**

The financial support of the Murray and Isabella Rayburn Foundation, the Tiger Fund and the Irving Weinstein Foundation for this work is acknowledged. We would also wish to thank Dr. Rose Frisch (Harvard University, Boston, MA, USA) for stimulating discussions about ACM.

**References**

26 Filtration, Separation, Detection, and Analysis in the Lab. Pall Filtron Corp Brochure.