Subcellular distribution and glycosylation pattern of androgen receptor from sheep omental adipose tissue

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Abstract

Sex steroids are known to have an influence on the distribution, metabolism and accretion of adipose tissue. These steroids carry out their function via specific receptors. We have previously reported the presence of oestrogen and progesterone receptors in sheep adipose tissues. In this study, we have tested the subcellular distribution of androgen receptor (AR) in sheep omental adipose tissue. Subcellular fractions – microsomes, plasma membrane and nuclei-cell debris – were isolated by differential and sucrose gradient centrifugation and confirmed by electron microscopy. The AR was determined in each fraction by Western blot analyses. As anticipated, the receptor was found in the cytosolic fraction, but a high concentration was also present in the microsomal fraction, a lesser amount in the plasma membrane fraction, and only a small amount was left in the nuclei-cell debris fraction. Two minor immunostaining bands with approximate molecular weights of 250 and 140 kDa and a major band

at 110 kDa were detected in the cytosolic fraction, but only the 110 kDa band was detected in the membrane fractions. A 104 kDa band was observed on occasion and believed to be a degradation product.

The cytosolic isoforms were tested for sensitivity to glycosidases. This treatment resulted in a decrease in the amount of the 250 and 140 kDa bands. To substantiate that the 250 and 140 kDa isoforms were glycoproteins, the cytosolic fraction was chromatographed on Concanavalin A-Sepharose. The 110 kDa band was eluted in the 0.4 M KCl salt wash while the 250 and 140 kDa bands were eluted with α -methylmannoside. Treatment of the glycoprotein (α -methylmannoside) peak with glycosidases converted the 250 and 140 kDa bands to the 110 kDa band. These data confirm the presence of AR in subcellular fractions of adipose tissue and suggest that it exists in various glycosylated isoforms.

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Introduction

Obesity is a risk factor for non-insulin-dependent diabetes, cardiovascular disease, some types of cancer, and certain reproductive and metabolic disorders (Bray 1996). This risk generally relates more to the central (abdominal, omental) male distribution pattern of fat than to the amount of fat. Men have a more central accumulation of fat, whereas women have a more gluteal/femoral accumulation (Bjorntorp 1990). Men also have a higher incidence of cardiovascular disease than women, and menopause in women increases the incidence of cardiovascular disease and the central distribution of adipose tissue (Tchernof & Poehlman 1998). This would suggest a role for sex steroid hormones in the regulation of adipose tissue distribution and in risk factors for certain disorders. It is generally held that androgens may be a member of key hormones that regulate fat accumulation in central depots

because androgenization increases central stores of fat in men (Bjorntorp 1990), in women with polycystic ovary syndrome (Rebuffe-Scrive *et al.* 1989) and in animal models (York 1996). It is unclear whether the role of androgens in adipose tissue accretion is direct or indirect. If it is a direct effect, androgen receptor (AR) should be present in adipose tissue. In support of this Pedersen *et al.* (1996) reported the presence of AR in human adipose tissue.

We have been studying obesity in a sheep model (McCann *et al.* 1992). Oestrogen receptor (ER) was present in adipose tissues from these animals (Watson *et al.* 1993) and the ER appeared to be functional as oestrogen treatment of ovariectomized ewes resulted in the up-regulation of progesterone receptor (PR) in adipose tissues (Mayes *et al.* 1996). This study is our initial investigation into the presence of AR in omental adipose tissue from female sheep.

Figure 1 Diagram for isolation of subcellular fractions from omental adipose tissue.

Materials and Methods

Animals and preparation of tissues

All procedures were approved by the Institution Animal Use and Care Committee, Oklahoma State University. Four adult Dorset ewes were ovariectomized and maintained a minimum of 30 days to decrease the endogenous steroids before experiments were begun. Animals were then treated for 8–12 days with subcutaneous oestradiol-17 β implants to maintain physiological concentrations of oestrogen. At necropsy, animals were anaesthetized deeply with pentobarbital and exsanguinated. Tissues were removed expediently, frozen in liquid nitrogen and stored at -80°C .

Isolation of subcellular fractions

Omental adipose tissue (1 g/ml) was homogenized at 4°C with Medium I (0.25 M sucrose, 1 mM EDTA, 10 mM

Tris-HCl, pH 7.4) which contained protease inhibitors (aminoethyl-benzenesulfonyl fluoride, leupeptin, bacitracin, aprotinin and pepstatin; Watson *et al.* 1993). Unless otherwise stated, all chemicals and reagents were obtained from Sigma (St Louis, MO, USA). The aqueous phase was removed with a Pasteur pipette and the subcellular fractions – microsomes, plasma membrane and nuclei-cell debris – isolated by differential and sucrose gradient centrifugation as shown in Fig. 1. Microsomes were isolated by differential centrifugation (St John *et al.* 1991). Plasma membrane and nuclei-cell debris fractions were isolated by sucrose gradient centrifugation (Lewis *et al.* 1981). Electron micrographs of the microsomal and plasma membrane pellets were very similar in appearance to those reported by Steinsapir *et al.* (1990) and electron micrographs of the nuclei-cell debris pellet showed mainly debris with very few intact nuclei. The

electron micrographs of these pellets did not show any major contamination by mitochondria or peroxisomes-lysosomes. Uterine cytosol was prepared from each animal to serve as a control.

Western blot analyses

Briefly, denatured proteins were separated by SDS-PAGE using 7.5% gels in the Mini-protein II system (Bio-Rad, Hercules, CA, USA). Prestained protein markers (Bio-Rad) were used as standards. Proteins were transferred from the polyacrylamide gels to nitrocellulose membranes by electro-blotting in a Trans Blot apparatus (Bio-Rad) at a 100 V setting for 1 h. After blocking with 3% gelatin, the membranes were then immunostained with rabbit polyclonal anti-AR (PG-21) as the first antibody. This was followed with goat anti-rabbit IgG-alkaline phosphatase conjugate as the second antibody. Alkaline phosphatase colour development solution was added and incubated at room temperature to visualize the AR specific bands. Image analyses were performed on a GS 710 calibrated imaging densitometer (Bio-Rad).

The PG-21 antibody and the 21 amino acid peptide immunogen were gifts from Dr G Greene, University of Chicago, Chicago, IL, USA (Prins *et al.* 1991). This antibody was produced by immunizing rabbits with a peptide corresponding to the N-terminal 21 amino acids of rat and human AR. It is specific for AR and will not cross-react with ER, PR or glucocorticoid receptor. By Western blot analysis, the antibody reacts with a 110 kDa band which is the molecular weight of AR. Competition experiments with the 21 amino acid peptide immunogen inhibits the immunostaining for AR. Using the above-mentioned criteria, we feel that the immunostaining which we observed in extracts of omental adipose tissue was specific for AR. In beginning experiments, we also used two other AR antibodies, U407 and R489, which were gifts from Dr M McPhaul, University of Texas Southwestern Medical Center, Dallas, TX, USA (Husmann *et al.* 1990). The R489 antibody did cross-react with the three cytosolic AR isozymes from sheep omental adipose tissue, while the U407 antibody did not cross-react. The PG-21 antibody was employed in all experiments reported in this article.

Concanavalin A chromatography

For binding to Concanavalin A (Con A), omental adipose tissue was extracted with 0.1 M acetate buffer, pH 6.0, containing 1 mM MgCl₂, 1 mM MnCl₂ and 1 mM CaCl₂ with protease inhibitors. The aqueous phase was removed with a Pasteur pipette and centrifuged at 253 000 g for 45 min. Supernatant (cytosolic fraction) was added to a 4 ml Con A-Sepharose 4B column. After washing with 0.4 M KCl in the acetate buffer containing the divalent metals and protease inhibitors, the glycoproteins were

eluted with 0.5 M α -methylmannoside in the acetate buffer with protease inhibitors, but without divalent metals. Fractions were pooled, concentrated with Centrprep-30 and analyzed for AR by Western blot.

Treatment with endoglycosidase F/N-glycosidase F

The cytosolic fraction or the glycoprotein peak from the Con A column was incubated overnight (18 h) with 0.4 units endoglycosidase F/N-glycosidase F (Sigma) at 37 °C. An equal volume of the denaturing buffer for gel electrophoresis was added and Western blot analysis for AR was performed.

Results

A representative Western blot analysis of AR from sub-cellular fractions of sheep omental adipose tissue is shown in Fig. 2. The cytosolic AR showed isoforms with molecular weights of approximately 250, 140 and 110 kDa (Fig. 2A). An immunostaining band of approximately 104 kDa was sometimes observed below the 110 kDa band. The 110 kDa immunostaining band for AR was also present in the microsomal fraction and to a lesser extent in the plasma membrane fraction while very little remained in the nuclei-cell debris fraction (Fig. 2B). When the cytosolic fraction was treated with endoglycosidase F/N-glycosidase F, the 250, 140 and 110 kDa bands decreased in intensity, while the 104 kDa band increased (Fig. 3). Image analyses showed the quantity (intensity $\times$ mm) of the 250 kDa band decreased from 0.313 to 0.070, the 140 kDa band decreased from 0.288 to 0.112 and the 110 kDa band decreased from 0.448 to 0.316, while the 104 kDa band increased from 0.182 to 0.315. This suggested that these bands were glycoproteins. To confirm this possibility, a cytosolic fraction was chromatographed on Con A-Sepharose (Fig. 4A). The 110 kDa band was eluted in the salt wash while the 250 and 140 kDa bands were eluted with α -methylmannoside (Fig. 4B). Subsequent treatment of the α -methylmannoside (glycoprotein) fraction with endoglycosidase F/N-glycosidase F resulted in the conversion of the 250 and 140 kDa bands to the 110 kDa band (Fig. 5). Image analyses showed the quantity (intensity $\times$ mm) of the 250 kDa band decreased from 0.604 to 0.095 and the 140 kDa band decreased from 0.409 to 0.286, while the 110 kDa band increased from 0.133 to 0.404.

Discussion

Androgens affect the metabolism, deposition and distribution of fat (Vermeulen *et al.* 1999). However, it is not known whether these effects on adipose depots are direct

Figure 2 (A) Western blot analysis of AR in the cytosolic fraction from sheep omental adipose tissue. Lane 1: prestained protein standards (low range) with molecular weight on left. Lane 2: cytosolic fraction (21·8 µg protein) from uterine tissue. Lane 3: cytosolic fraction (800 µg protein) from omental adipose tissue. (B) Western blot analysis of AR in subcellular fractions from sheep omental adipose tissue. Lane 1: prestained protein standards (low range) with molecular weight on left. Lane 2: cytosolic fraction (21·8 µg protein) from uterine tissue. Lane 3: cytosolic fraction (800 µg protein) from omental adipose tissue. Lane 4: nuclear fraction from 6 g omental adipose tissue. Lane 5: plasma membrane fraction from 6 g omental adipose tissue. Lane 6: microsomal fraction from 6 g omental adipose tissue.

Figure 3 Treatment of cytosolic fraction with endoglycosidase F/N-glycosidase F followed by Western blot analysis for androgen receptor. Lane 1: prestained protein standards (broad range) with molecular weight on left. Lane 2: cytosolic fraction (800 µg protein) from omental adipose tissue incubated without glycosidases. Lane 3: cytosolic fraction (800 µg protein) from omental adipose tissue incubated with glycosidases.

or indirect. If they are direct, an AR should be present in the tissues. Sjogren *et al.* (1995) used ligand-binding assays to show androgen binding to isolated nuclei from adipose depots of male rats. Pedersen *et al.* (1996) employed ligand binding and RT-PCR to demonstrate the presence of AR in adipocytes from subcutaneous abdominal fat from patients undergoing intra-abdominal surgery. Our initial Western blot results showed the presence of AR protein in the cytosolic fraction of sheep gluteal, omental and perirenal adipose tissues. As abdominal obesity appears to be associated with androgen action, we have concentrated on the subcellular distribution and apparent glycosylation pattern of AR in omental adipose tissue. In omental adipose tissue the receptor was found mainly in the cytosolic and microsomal fractions with a smaller amount in the plasma membrane fraction, while very little remained in the nuclei-cell debris fraction. It is generally accepted that, with homogenization of tissues in hypotonic buffers, many steroid receptors are released from the

Figure 4 (A) Con A chromatography of cytosolic fraction from omental adipose tissue. Tubes 3–11=peak 1 (unbound fraction). Tubes 12–21=0.4 M KCl salt wash. Tubes 22–28=peak 2 (elution with α -methylmannoside). (B) Western blot analysis of AR in fractions from Con A chromatography. Lane 1: prestained protein standards (low range) with molecular weight on left. Lane 2: cytosolic fraction (21.8 μ g protein) from uterine tissue. Lane 3: cytosolic fraction (800 μ g protein) from omental adipose tissue. Lane 4: peak 1 (unbound fraction) from Con A column. Lane 5: salt wash from Con A column. Lane 6: peak 2 (α -methylmannoside fraction) from Con A column.

nucleus and move into the cytoplasm. The reason for the presence of receptor in the microsomes is not clear. Earlier work by Watson & Muldoon (1985) showed the presence of a sex steroid receptor (ER) in rat uterine microsomes and Steinsapir *et al.* (1990) showed androgen-binding sites in endoplasmic reticulum from rat ventral prostate. Mora & Mahesh (1999) recently demonstrated the accumulation of AR mRNA in rat ventral prostate polyribosomes. It is possible that AR mRNA also accumulates in microsomes of adipose tissues and leads to the sequestering of AR that we observed in microsomes from omental adipose tissue. Alternatively non-classical subcellular distribution of

Figure 5 Treatment of glycoprotein fraction with endoglycosidase F/N-glycosidase F followed by Western blot analysis for AR. Lane 1: prestained protein standards (broad range) with molecular weight on left. Lane 2: glycoprotein fraction (410 μ g protein) from Con A column incubated without glycosidases. Lane 3: glycoprotein fraction (410 μ g protein) from Con A column incubated with glycosidases.

steroid receptors has been associated with non-genomic actions of these hormones (Brann *et al.* 1995, Wehling 1997).

We noticed that the AR from the cytosolic fraction showed three isoforms with approximate molecular weights of 250, 140 and 110 kDa. When the cytosolic fraction or glycoprotein fraction from the Con A column was treated with endoglycosidase F/N-glycosidase F, the 250 and 140 kDa immunostaining bands decreased with an increase in the 110 and/or 104 kDa bands. This was a strong indication that the 250 and 140 kDa bands were glycosylated. To our knowledge, this is the first report of glycosylation of AR. However, the glycosylation of ER has been studied in some detail. Karthikeyan & Thampan (1995) reported that non-activated ER bound to Con A and could be eluted with α -methylglucoside. When the

non-activated ER was treated with N-glycopeptidase F, it was deglycosylated to the nuclear ER R-II. They were also able to show that the purified non-activated ER stained for glycoprotein in Western blot analyses. The molecular weight of the glycosylated form was about the same as the deglycosylated form. Glycosylated ER, but not deglycosylated ER, dimerized with ER activation factor. The plasma membrane appears to be the primary site of localization of the glycosylated non-activated ER (Karthikeyan & Thampan 1996). Jiang & Hart (1997) reported a subpopulation of murine, bovine and human ERs that contained O-linked N-acetylglucosamine and the O-glycosylation alternates with O-phosphorylation (Cheng *et al.* 2000). The glycosylation of AR that we observed in omental adipose tissue appears to be different from that reported for ER. Binding of AR to Con A and decrease in molecular weight when treated with endoglycosidase F/N-glycosidase F would suggest large N-linked high mannose complex oligosaccharides. The sex steroid receptors may be synthesized as large glycoproteins, then the oligosaccharides are processed to form smaller units. Glycosylation of proteins is important in recognition markers, targeting, stability, storage or protein interactions and alterations of normal glycosylation patterns have been linked to several disease states (Dwek 1995). The function of glycosylation of AR and its role in obesity are unknown, and will have to be delineated by future experiments.

A fourth band with an approximate molecular weight of 104 kDa was sometimes observed in the cytosolic fraction. This band was particularly present after long incubations of the crude cytosolic fraction with endoglycosidase F/N-glycosidase F (Fig. 3). However, the 104 kDa band was not seen when the glycoprotein fraction was treated with endoglycosidase F/N-glycosidase F (Fig. 5). These results would suggest that the 104 kDa band could be a proteolytic degradation product. The increase in the 104 kDa band as compared with control after treatment of the crude cytosolic fraction with endoglycosidase F/N-glycosidase F (Fig. 3) may be the result of the deglycosylated receptor being more readily degraded by proteasomes as has been reported for oligosaccharide-modified glycoprotein (Liu *et al.* 1999). Proteasome-mediated proteolysis of ER has been reported (Alarid *et al.* 1999, El Khissiin & Leclercq 1999, Nawaz *et al.* 1999) and recently Shefin *et al.* (2000) reported that inhibiting proteasomes increased the amount of AR in HepG2 and LNCAP cells. Some diffuse bands with molecular weights below 104 kDa were often seen in the Western blots. Bands in this region are routinely seen in blots and often represent non-specific staining. When proteolysis is occurring, it would also be expected that fragments would show up in these areas which may cross-react with the antibody. However, quantification and tracking of fragments in this lower region is generally complicated due to lower antibody avidity and higher background due to broad non-specific staining.

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