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Gene deletion reveals roles for annexin A1 in the regulation of lipolysis and IL-6 release in epididymal adipose tissue

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Abstract

In this study, epididymal adipose tissue from male annexin 1 (ANXA1)-null and wild-type control mice were used to explore the potential role of ANXA1 in adipocyte biology. ANXA1 was detected by Western blot analysis in wild-type tissue and localized predominantly to the stromal-vascular compartment. Epididymal fat pad mass was reduced by ANXA1 gene deletion, but adipocyte size was unchanged, suggesting that ANXA1 is required for the maintenance of adipocyte and/or preadipocyte cell number. Epididymal tissue from wild-type mice responded *in vitro* to noradrenaline and isoprenaline with increased glycerol release, reduced IL-6 release, and increased cAMP accumulation. Qualitatively similar but significantly attenuated responses to the catecholamines were observed in tissue from ANXA1-null mice, an effect that was not associated with changes in β -adrenoceptor mRNA expression. Lipopolysaccharide (LPS) also stimulated lipolysis *in vitro*, but its effects were muted by ANXA1 gene deletion. By contrast, LPS failed to influence IL-6 release from wild-type tissue but stimulated the release of the cytokine from tissue from ANXA1-null mice. ANXA1 gene deletion did not affect glucocorticoid receptor expression or the ability of dexamethasone to suppress catecholamine-induced lipolysis. It did, however, augment IL-6 expression and modify the inhibitory effects of glucocorticoids on IL-6 release. Collectively, these studies suggest that ANXA1 supports aspects of adipose tissue mass and alters the sensitivity of epididymal adipose tissue to catecholamines, glucocorticoids, and LPS, thereby modulating lipolysis and IL-6 release.

Keywords

glucocorticoids; adipocytes; catecholamines

Dysregulation of adipose tissue function appears to contribute to the etiology of a number of pathophysiological conditions, including obesity, insulin resistance, and cardiovascular disease. Efficiency of energy mobilization by lipolysis, a process where the catecholamines play a crucial role, is an important consideration in the maintenance of body weight and in

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exercise-induced weight loss (20,47). Furthermore, adipose tissue is reported to contribute cytokines, notably interleukin-6 (IL-6), to a low-level chronic inflammatory state that has been linked to hepatic insulin resistance and endothelial dysfunction (25,51). Therefore, understanding the mechanisms by which lipolysis, notably catecholamine sensitivity, and IL-6 release are regulated is important to understanding the etiology of these significant health problems.

Annexin A1 (ANXA1; also known as lipocortin 1), a 37-kDa and Ca^{2+} - and phospholipid-binding protein, is widely but not ubiquitously distributed throughout the body (13). It has a diverse range of functions, including the suppression of inflammation (37) and hypothalamo-pituitary-adrenocortical activity (23) and modulation of the release and signaling of insulin (33,34). ANXA1, like other members of the annexin family, is found in adipose tissue (39), where it redistributes from the cytoplasm to plasma membrane after treatment with Ca^{2+} but not insulin (39). It is not yet known whether ANXA1 contributes to the processes regulating adipocyte differentiation, lipid metabolism, or the release of inflammatory mediators from adipose tissue. However, evidence that ANXA1 expression declines progressively with adipogenesis in differentiated 3T3-L1 adipocytes (49) raises the possibility that ANXA1 contributes to the process whereby preadipocytes in the stromal-vascular compartment mature into lipid-storing adipocytes (31).

ANXA1 has been intimately associated with glucocorticoid action ever since it was first identified in the medium of glucocorticoid-stimulated rat peritoneal macrophages (5) and is now known to be an important mediator of glucocorticoid action in the neuroendocrine (7) and host defense (37) systems. In accord with this premise, ANXA1-null mice show resistance to the anti-inflammatory actions of glucocorticoids in several experimental models of inflammation (18,50). Glucocorticoids also exert complex actions on the adipocyte. These include the promotion of adipogenesis, largely through induction of key transcription factors such as the peroxisome proliferator activated receptor- γ (PPAR γ) (31,46), lipogenesis (8), and inhibition of lipolysis (35). In addition, glucocorticoids modulate the endocrine function of adipose tissue by, for example, suppressing IL-6 release (15). However, it is unknown whether ANXA1 serves as a mediator of glucocorticoid action in adipose tissue.

In the present study, we used histological, molecular, and functional approaches to explore the effects of ANXA1 on adipocyte function, using the ANXA1 knockout (KO) mouse as an experimental model. Our results suggest that ANXA1 has a role in the regulation of epididymal adipose tissue mass. They also provide novel evidence to suggest that ANXA1 contributes to the signaling processes mediating the responses of adipose tissue to catecholamines and lipopolysaccharide (LPS) and that it thereby modulates both lipolysis and IL-6 release. The data do not, however, support a role for ANXA1 in mediating glucocorticoid actions on adipocytes and in adipose tissue and thus contrast with findings in other tissues.

MATERIALS AND METHODS

Animals and Treatments

The ANXA1 KO mouse was created as detailed in Hannon et al. (18). A colony of these mice was established at the Central Biomedical Services, Imperial College London, Hammersmith Campus by rederivation and bred alongside a colony of corresponding wild-type controls. The mice were genotyped by Southern blotting and maintained post-weaning according to sex in groups of five per cage in a room with controlled temperature (22°C) and humidity under a 12:12-h light-dark cycle. Food and water were normally available *ad libitum*. However, for measurements of fasting blood glucose and insulin, food, but not water, was withdrawn 24 h prior to autopsy. All experiments were performed under license on adult male mice (aged ~70 days) in accord with the UK Animals (Scientific Procedures) Act of 1986.

Dexamethasone Treatment

Where appropriate, ANXA1-null and wild-type mice ($n = 5/\text{group}$) were treated with dexamethasone sodium phosphate ($1 \mu\text{g}/\text{ml}$ in the drinking water; David Bull Laboratories, Warwick, UK) for 16 h prior to tissue collection (38). Controls ($n = 5/\text{group}$) were given normal drinking water.

Collection of blood and tissues

Animals were killed by decapitation between 9 and 11 h to avoid any changes associated with the circadian rhythm. Blood was collected from the trunk into chilled heparinized tubes; after centrifugation, the plasma was removed and stored at -80°C for subsequent analysis of IL-6, insulin, and glucose. Epididymal fat tissue was collected, weighed, and processed for histology, Western blot analysis, or in vitro studies.

For measurements of β -adrenoceptor mRNA and tissue macrophage density, the mice ($n = 4\text{--}6/\text{group}$) were killed by cervical dislocation and perfused transcardially with 5–10 ml of sterile physiological saline (0.9% NaCl solution; Hammersmith Hospital Pharmacy) for 10–15 min until the perfusate was clear. The epididymal and retroperitoneal fat pads and liver were excised. The liver was cut into pieces ($n = 8/\text{group}$, 10–50 mg) and placed in RNeasy lysis buffer (Qiagen; Crawley, West Sussex, UK) in a 1.5-ml RNase-free Eppendorf tube, maintained for 24 h at 4°C , and then stored at -80°C . The fat pads were either frozen in liquid N_2 and stored at -80°C in plastic tubes for histological analysis ($n = 4/\text{group}$) or cut into pieces ($n = 8/\text{group}$, 50–100 mg), frozen in liquid N_2 in round-bottomed RNase- and DNase-free tubes, and stored at -80°C .

Histology

Measurement of adipocyte size—Pieces of adipose tissue ($n = 5/\text{group}$) were fixed in 4% paraformaldehyde for 15 h at 4°C , washed in 0.1 M phosphate buffer, dehydrated through a series of increasing ethanol concentrations, and cleared in xylene at room temperature. The tissue was impregnated with paraffin overnight at 60°C and cooled to room temperature. Sections ($12 \mu\text{m}$) were cut from the paraffin blocks, mounted onto gelatin-coated slides, and stained with Ehrlich's hematoxylin and eosin. Images of the sections were captured using a MicroImager attached to an Eclipse E800 microscope (Nikon), and cell area was analyzed at a magnification of $\times 10$ using Image Pro Plus. For each tissue block, 20 adipocytes per section (selected randomly) and six sections were analyzed.

Measurements of macrophage density in adipose tissue—Pieces of epididymal and retroperitoneal adipose tissue ($n = 4/\text{group}$) were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (15 h, 4°C), dehydrated, cleared, and embedded in paraffin. Serial sections ($5 \mu\text{m}$) were taken and processed for immunohistochemistry as described previously, using F4/80 as a macrophage marker (9). Briefly, dewaxed $5\text{-}\mu\text{m}$ sections were treated with 3% hydrogen peroxide to inactivate endogenous peroxidases and with normal goat serum to reduce nonspecific staining. The sections were incubated overnight with anti-mouse F4/80 (1:100; Serotec, Kidlington, UK), washed in PBS, and then incubated for 2 h in secondary antibody, goat anti-rat biotinylated IgG (1:100; Vector Laboratories, Burlingame, CA). Negative controls in which the primary antibody was omitted were run in parallel. Histochemical reactions were performed using the Vectastain ABC kit (Vector Laboratories) and Sigma Fast 3,3'-diaminobenzidine as substrate (Sigma-Aldrich, Poole, UK). Sections were counterstained with hematoxylin and photographed using an Olympus BH microscope fitted with an Axiovision digital camera (Zeiss, Hemel Hempstead, UK). Immunoidentified macrophages were counted under standardized conditions and expressed as macrophage

number per total cell number per high power field. A total of six sections at different block depths were analyzed for each animal ($n = 4$).

Separation of Adipocyte and Stromal-Vascular Cell Fractions

Epididymal fat pads were minced, digested with collagenase for 30 min, filtered, washed three times, and spun (10 min, 300 g) as described above for the isolation of adipocytes. The floating adipocyte layer was then removed and spun twice again. The pellets formed from each spin were resuspended in 10 mM EDTA with 1% Triton X (vol/vol) and pooled, representing the stromal-vascular fraction. The floating cell layer from the final spin, which constitutes the adipocyte fraction, was resuspended in 10 mM EDTA with 1% Triton X (vol/vol). The samples were then processed for ANXA1 analysis by SDS-PAGE and Western blot analysis.

SDS-PAGE and Western Blot Analysis

Adipose tissue ANXA1, IL-6, PPAR γ , and glucocorticoid receptor (GR) protein content ($n = 4$ /group) were determined by SDS-PAGE and Western blot analysis in accordance with a published protocol (44). Samples (tissue pieces or separated adipocyte and stromal-vascular fractions) for ANXA1, IL-6, and PPAR γ analysis were extracted in 10 mM EDTA with 1% Triton X (vol/vol), whereas those for GR were extracted in 50 mM Tris, 40 mM EDTA, 0.5% wt/vol deoxycholic acid, and 0.5% vol/vol Nonidet P-40. After total protein estimation (6), the proteins (30 μ g protein/channel) were separated electrophoretically and transferred to nitrocellulose paper. Using polyclonal antiserum, the blots were then probed for IL-6 (anti-IL-6, diluted 1:1,000; R&D Systems, Abingdon, UK), ANXA1 (anti-ANXA1, diluted 1:10,000; Cambridge Bioscience, Cambridge, UK), PPAR γ (anti-PPAR γ , diluted 1:1,000; Autogen Bioclear, Wiltshire, UK), and GR (anti-GR, diluted 1:1,000; Autogen Bioclear, Caine, Wiltshire, UK), and the resultant immunoreactive bands were visualized by enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles Media Cybernetics, Wokingham, UK) (18). Molecular weights of the immunoreactive bands were determined by comparison with the migration of molecular mass standards (Precision Protein Standards; Bio-Rad Laboratories, Richmond, CA). Densitometry was performed using Image Pro Plus (Media Cybernetics, Berkshire, UK). Data were normalized to percentage of the wild-type (vs. KO) or adipocyte (vs. stromal-vascular fraction).

Quantitative PCR

Isolation of total RNA and reverse transcription—After the addition of QIAzol reagent (750 μ l; Qiagen, Crawley, UK), liver and adipose tissues were disrupted with a TissueLyser (Qiagen; 4 min, 20 Hz) and suspended in chloroform (150 μ l). The tubes were shaken vigorously by hand (15 s), allowed to stand for 3 min, and centrifuged (12,000 rpm, 15 min, 4°C). Total RNA in 300 μ l of the upper aqueous phase was extracted using an EZ1 RNA kit (Qiagen) and the BioRobot EZ1. All RNA samples were measured at 260 nm (NanoDrop Technologies). The absorbance ratio (260/280 nm) was >1.8 for all samples. Each RNA sample (1 μ g) was treated with the gDNA Wipeout buffer (42°C, 2 min), and cDNA was synthesized using the Quantitect RT kit (Qiagen).

Real-time PCR—Real-time quantitative PCR was performed using a LightCycler rapid thermal cycler system (Roche Applied Science, Lewes, UK). Reactions were done in a 10- μ l volume using 0.5- μ M sense and antisense primers, dNTPs, *Taq* DNA polymerase, and reaction buffer provided in the QuantiTect SYBR Green kit (Qiagen).

The following murine primer sequences were designed and used to generate short products: β_1 -adrenoceptor (NM_007419): sense 5'-CATCATGGGTGTGTTACAG-3', antisense 5'-GGAAAGCCTTCACCACGTT-3' (product 65 bp); β_2 -adrenoceptor (NM_007420): sense 5'-

CGAGCTGAGTGTGCAGGAC-3', antisense 5' GACTCCTGGAAGCTTCATTCA-3' (product 68 bp); β_3 -adrenoceptor (NM_013462): sense 5'-CCTTCCGTCGTCTTCTGTGT-3', antisense 5'-TTCCCGAAGAAGGGAAGTGT-3' (product 125 bp).

Three reference (housekeeping) genes were measured to normalize β -adrenoceptor gene expression data using geometric averaging of multiple internal control genes. The reference murine gene primers were 28S rRNA (X00525): sense 5'-TTAAAATCCGGGGGAGAG-3', antisense 5'-ACATTGTTCCAACATGCCAG-3' (product 100 bp); cytoplasmic β -actin (NM_007393): sense 5'-TGACAGGATGCAGAAGGAGA-3', antisense 5'-CGCTCAGGAGGAGCAATG-3' (product size 75bp); TATA box-binding protein (BC012685): sense 5'-GGGGAGCTGTGATGTGAAGT-3', antisense 5'-CCAGGAAATAATTCTGGCTCA-3' (product size 93 bp).

All real-time PCR assays included an initial step (15 min, 95°C) to activate *Taq* polymerase followed by 40 cycles of denaturation (95°C, 15 s), annealing (57°C, 20 s), and extension (72°C, 10 s). The fluorescence of the accumulating product was acquired during each cycle after an additional 3-s step to 3°C below the product melting temperature (T_M). PCR products were separated by agarose gel electrophoresis (2.5% wt/vol) with ethidium bromide (0.5 µg/ml) staining, purified, and prepared for use as standards in quantitative PCR as described previously (1). All assays were sensitive and linear (all $r^2 = -1.00$) over a very wide concentration range (10^1 to 10^7 copies). Amplification efficiencies were β_1 -adrenoceptor 99%, β_2 -adrenoceptor 97%, β_3 -adrenoceptor 100%, β -actin 93%, 28S rRNA 91%, TATA box-binding protein 100%. Each assay included a no-template control and tissue RNA sample that had not been reverse transcribed. No product was detected in the absence of reverse transcription in any assay for any of the products analyzed, indicating that there was no genomic contamination. A single peak was observed for each of the products through melting curve analysis, performed routinely for all samples after amplification. Product T_M values were β_1 -adrenoceptor 80°C, β_2 -adrenoceptor 83°C, β_3 -adrenoceptor 86°C, β -actin 77.6°C, 28S rRNA 82.1°C, TATA box-binding protein 80.2°C.

In Vitro Experiments

Preparation and incubation of adipose tissue explants—Epididymal fat pads were removed postmortem and placed in Krebs buffer (120 mM NaCl, 4 mM KH_2PO_4 , 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75 mM CaCl_2 , 10 mM NaHCO_3 , 30 mM HEPES, pH 7.4, 2.5 mM D-glucose) supplemented with 5% free fatty acid (FFA)-free BSA (Krebs-FFA-free BSA buffer). Each fat pad was cut into pieces (explants) weighing 10–20 mg and distributed into 48-well plates containing 0.5 ml Krebs-5% FFA-free BSA buffer, with an explant from each animal ($n = 5$ /genotype/experiment) being allocated to each in vitro treatment group. The explants were preincubated for 2 h at 37°C in a humidified atmosphere saturated with 95% O_2 -5% CO_2 , with medium replacement after 1 and 1.5 h. The explants were transferred into fresh medium containing 1 µM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and incubated for a further 2 or 4 h in the presence or absence of isoprenaline (0.1–10 µM; Sigma-Aldrich) or LPS (1 µg/ml, *E. coli*; Sigma-Aldrich). The medium was then collected and stored at –20°C. The tissue was weighed on a torsion balance, and the data were expressed per milligram wet weight.

Preparation and incubation of isolated adipocytes—The method used is described in detail elsewhere (16). Briefly, epididymal fat pads from mice ($n = 5$ /premortem treatment group/experiment) were removed postmortem and transferred immediately to Krebs-FFA-free BSA buffer. Each pad was minced into small pieces, transferred to Krebs-FFA-free BSA buffer containing 8 mg/ml type II collagenase (Sigma-Aldrich), and incubated at 37°C for 30 min with gentle shaking. The resultant cell suspensions were filtered through a 400-µm nylon mesh,

washed three times, and centrifuged (10 min, 300 g). The floating adipocyte layers were then removed, resuspended in fresh Krebs-FFA-free BSA buffer, and left shaking gently for 2 h at 37°C in a humidified atmosphere saturated with 95% O₂-5% CO₂. The cells from each animal were adjusted to a concentration of 30,000 cells/ml, plated in a 48-well plate (15,000 cells/well), and incubated in the presence or absence of norepinephrine (0.1 and 10 µM), isoprenaline (0.1 and 10 µM), or LPS (1 µg/ml) for 2 or 4 h. The medium was collected by spinning the contents of each well (770 g, 10 min, 4°C) with sesame seed oil (50 µl; Sigma-Aldrich) to capture the cells in the oil. The medium below the oil layer was aspirated and stored immediately at -20°C for subsequent analysis.

Assay of Glycerol, IL-6, cAMP, Glucose, and Insulin

All samples were assayed in duplicate, and samples from individual experiments were always determined in single assay runs to avoid problems of interassay variance.

Glycerol was determined according to a protocol adapted from a published method (28) for use in a 96-well plate. Twenty microliters of reagent solution [2 mM adenosine triphosphate, 3 mM nicotinamide adenine dinucleotide, 20 mM L-cysteine-hydrochloride (made up in 0.4 M NaOH), 0.7 M hydrazine-HCl, pH 9.4, 1.05 mM MgCl₂, 1 µg/ml glycerokinase (all Sigma-Aldrich), 50 µg/ml glycerophosphate dehydrogenase (Roche Molecular Biochemicals)] were added to 40 µl of sample. The tubes were then sealed and left at room temperature for 2 h. Four hundred microliters of diluent (0.01 M NaOH and 1 mM EDTA) were added to each tube, and 200 µl were then dispensed into each well of a 96-well plate (Greiner, Dursley, UK). Each well was then subjected to a 40-ms, 360-µm incident ray, and the emission at 460 µm from the sample was measured using a Fluoroskan Ascent FL (Thermo Labsystems, Vantaa, Finland) and Ascent Software. The values were deduced from the standard curve of glycerol (500–1 µg/ml; Sigma), prepared in parallel.

IL-6 was determined in duplicate by a specific sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems) in accordance with manufacturer's guidelines. The sensitivity of the assay was 50 pg/ml; inter- and intra-assay coefficients of variation were 4.2 and 7.1% respectively.

3'5' cAMP was measured by radioimmunoassay (22). The sensitivity of the assay was 1 fmol/ml, with 95% confidence limits, and the intra-assay coefficient of variation was 4%.

Plasma glucose was measured by the glucose oxidase method (YSI 2300 glucose analyzer; YSI Bioanalytical Products, Yellow Springs, OH). Plasma insulin was measured in duplicate by an in-house radioimmunoassay (3,26), using an antibody raised in guinea pigs against porcine insulin conjugated to bovine serum albumin by glutaraldehyde (diluted 1:400,000) and ¹²⁵I-labeled insulin as a probe (specific activity 48 Bq/fmol). The sensitivity of the assay was 5 pmol/l plasma, with 95% confidence limit. The inter- and intra-assay coefficients of variation were 6.2 and 9.5% respectively.

Statistics

The data are presented as means (n = no. of animals used/treatment group) $\pm$ SE. All statistical analyses were performed using SigmaStat (Jandal Scientific, San Rafael, CA). As basal glycerol and IL-6 release varied between experiments, statistical comparisons were made within experiments only. The data shown are typical of two experiments. Data that showed a normal distribution were analyzed by two- or three-way ANOVA followed by Tukey's multiple comparisons test or Student's t -test as appropriate. Western blot data were analyzed using the

Mann-Whitney *U*-test (nonparametric). A *P* value of ≤ 0.05 was considered to be statistically significant.

RESULTS

Body Weight and Characterization of Epididymal Adipose Tissue

There were no significant between-strain differences in body weight in age-matched animals (ANXA1 KO = 34.6 ± 1.8 g, wild type = 31.9 ± 1.9 g, $P > 0.05$, $n = 5$). The epididymal fat pad weight was, however, lower in the ANXA1 KO mouse ($P < 0.05$ vs. wild type; Fig. 1, A and B), although histological analysis showed no differences in adipocyte size between the two mouse strains (Fig. 1C). Macrophage number in the epididymal fat pad was unaffected by ANXA1 gene deletion (Table 1); similarly, there was no change in macrophage number in the retroperitoneal fat pad (Table 1).

Full length ANXA1 (37 kDa) was readily detectable by SDS-PAGE and Western blot analysis in the adipocyte and stromal-vascular fractions of epididymal adipose tissue from wild-type mice (Fig. 1D), as was the 34-kDa NH₂-terminally clipped metabolite (23). However, the degree of expression of both species of the protein was significantly greater in the stromal-vascular compartment cells ($P < 0.05$ vs. adipocytes). By contrast, ANXA1 was not detectable in epididymal adipose tissue from ANXA1-null mice (Fig. 1E).

IL-6, GR, and PPAR γ were readily detectable by Western blot analysis in the epididymal fat pad of both ANXA1 KO and wild-type control mice. IL-6 expression (Fig. 2A) was more pronounced in the ANXA1 KO ($P < 0.05$ vs. wild type). However, the levels of GR (Fig. 2B) and PPAR γ (Fig. 2C) protein were unaffected by the ANXA1 gene deletion.

Table 2 shows the expression of mRNAs for β_1 -, β_2 -, and β_3 -adrenoceptors in epididymal and retroperitoneal adipose tissue and liver control tissue from ANXA1-null and wild-type control mice, as determined by quantitative PCR. Expression of mRNAs for β_1 -, β_2 -, and β_3 -adrenoceptors was readily detected in all three tissues studied. There were no significant effects of ANXA1 gene deletion on the amounts of mRNA expression for any of the three adrenoceptors in either of the fat pads tested or in the liver (control).

Plasma Variables

The basal plasma IL-6 concentration was unaffected by ANXA1 gene deletion (ANXA1 KO = 11.4 ± 0.99 pg/ml, wild type = 10.1 ± 2.45 pg/ml, $P > 0.05$). Similarly, there are no between-strain differences in plasma triglycerides or glycerol levels (48). Fasting blood glucose concentrations were also unaffected by ANXA1 gene deletion (ANXA1 KO = 7.23 ± 0.24 mmol/l, wild type = 9.07 ± 0.85 mmol/l, $n = 9$, $P > 0.05$). However, the fasting blood insulin level was raised significantly ($P < 0.05$) in the ANXA1-null mice vs. wild-type controls (ANXA1 KO = 55.22 ± 5.39 pmol/l, wild type 41.32 ± 3.23 pmol/l, $n = 9$).

Catecholamine-Induced Lipolysis and cAMP Accumulation

Figure 3 illustrates the effects of isoprenaline (2 h contact) on glycerol release and cAMP accumulation within adipose tissue explants from ANXA1 KO and wild-type mice. There were no between-strain differences in basal glycerol release from the tissue explants (Fig. 3A); however, resting cAMP levels were significantly lower in the ANXA1 KO tissue ($P < 0.05$ vs. wild type; Fig. 3B). Explants from both mouse strains responded to isoprenaline (0.1 and 1 μ M) with increases ($P < 0.01$) in glycerol release and cAMP accumulation. The magnitude of the glycerol response to isoprenaline (1 μ M) was, however, attenuated ($P < 0.05$) by ANXA1 gene deletion (Fig. 3A). In addition, tissue from ANXA1-null mice showed a reduced cAMP

response to the isoprenaline (0.1 μM). Furthermore, although the reduction in cAMP accumulation observed with a higher (1 μM) concentration of isoprenaline did not reach significance ($P > 0.05$), there was an overall shift of the isoprenaline-cAMP concentration-response curve to the left in tissue from the KO animals and, hence, a reduction in the area under the curve. Intracellular cAMP levels are regulated by many factors. The finding that basal cAMP levels are reduced in the ANXA1 KO suggests that the protein has an important role in controlling the total cellular pool of this nucleotide as well as modifying the pool regulated by β -adrenoceptor stimulation.

The effects of isoprenaline and norepinephrine (0.1 and 10 μM , 2 h contact) on the release of glycerol from isolated adipocytes from ANXA1 KO and wild-type control mice are shown in Fig. 4. As with the tissue explants, there were no significant between-strain differences in basal glycerol release. Isoprenaline (Fig. 4A) and norepinephrine (Fig. 4B) caused significant ($P < 0.001$) increases in glycerol release from cells from both mouse strains. However, as with the tissue explants, the isolated adipocytes from the ANXA1-null mice showed attenuated lipolytic responses to the higher (10 μM), but not the lower (0.1 μM), concentration of isoprenaline and norepinephrine tested ($P < 0.001$ vs. wild-type; Fig. 4, A and B).

Pretreatment of the rats with dexamethasone (1 $\mu\text{g}/\text{ml}$ in the drinking water) reduced the basal release of glycerol in vitro from adipocytes from the wild-type control and ANXA1-null mice. The steroid treatment also virtually abolished the lipolytic responses to isoprenaline (0.1 and 10 μM , $P < 0.001$; Fig. 4A) and norepinephrine (10 μM , $P < 0.001$; Fig. 4B) in adipocytes from both mouse strains.

Catecholamine-Induced Changes in IL-6 Release

Figures 5 and 6 illustrate the effects of catecholamines on IL-6 release from adipose tissue explants (Fig. 5A) and isolated adipocytes (Figs. 5B and 6) from ANXA1-null and wild-type mice. Basal IL-6 release from the tissue explants over a 4-h period was significantly reduced by ANXA1 gene deletion ($P < 0.001$ vs. wild type; Fig. 5A). Isoprenaline (10 μM , 4 h) caused a marked reduction in IL-6 release from explants from wild-type mice ($P < 0.001$ vs. untreated control). However, explants from the ANXA1 KO mouse were relatively unresponsive to the catecholamine, and hence, only a small ($\sim 30\%$) reduction in IL-6 release was observed ($P < 0.05$ vs. untreated control). When a shorter contact time was used (2 h), no between-strain differences in basal IL-6 release from the tissue explants were observed and isoprenaline (10 μM) was without effect (data not shown). In parallel studies on isolated adipocytes, basal IL-6 release was also lower in cells from ANXA1 KO vs. wild-type control. This difference was pronounced after a 4-h incubation ($P < 0.01$; Fig. 5B) but already apparent after a 2-h incubation ($P < 0.05$; Fig. 6, A and B). However, in contrast to the data on tissue explants, isoprenaline (10 μM , 2 or 4 h) had no effect on IL-6 release from isolated adipocytes of either strain (Figs. 5B and 6A). Similarly, norepinephrine (10 μM , 2 h) was without effect (Fig. 6B).

Dexamethasone (1 $\mu\text{g}/\text{ml}$ in the drinking water for 24 h prior to autopsy) reduced basal IL-6 release from isolated adipocytes from wild-type mice ($P < 0.05$ vs. untreated control; Fig. 6, A and B). In contrast, the steroid treatment increased the basal release of the cytokine from cells from the ANXA1 KO mouse ($P < 0.05$; Fig. 6, A and B). The effects of dexamethasone in the wild-type, but not the ANXA1 KO, mice were partially attenuated by isoprenaline ($P < 0.05$; Fig. 6A) but not by norepinephrine (Fig. 6B).

Responses to LPS

Figure 7 demonstrates the effects of LPS (1 $\mu\text{g}/\text{ml}$, 4 h) on the in vitro release of glycerol (Fig. 7A) and IL-6 (Fig. 7B) from isolated adipocytes derived from ANXA1-null and wild-type

mice. In contrast to observations made over a 2-h incubation (Figs. 3 and 4), basal glycerol release was significantly lower ($P < 0.05$) in cells from ANXA1-null mice compared with the wild-type controls. LPS increased glycerol release from adipocytes from both the wild-type and ANXA1-null mice ($P < 0.001$). In terms of absolute changes in glycerol release, its effects were less pronounced in the ANXA1-null cells ($P < 0.01$; Fig. 7A), although in both cases there was an $\sim 50\%$ increase above basal. In both strains, the lipolytic responses to LPS were comparable with those induced by isoprenaline (10 μM), included as a positive control for cell viability (data not shown).

Basal IL-6 release was again significantly lower in the ANXA1 KO group ($P < 0.01$ vs. wild-type controls; Fig. 7B). LPS had no effect on IL-6 release from cells derived from the wild-type control but caused a significant increase in IL-6 release from adipocytes from the ANXA1-null mice ($P < 0.01$ vs. basal).

DISCUSSION

In the present study, we used adipose tissue from adult male ANXA1-null and wild-type control mice to explore the role of ANXA1 in a number of key metabolic processes. Our data support the premise that ANXA1 plays a role in tissue mass, possibly affecting adipogenesis (49), and provide new evidence to suggest that the protein also modulates the sensitivity of epididymal adipose tissue to catecholamines, glucocorticoids, and LPS and, hence, the processes of lipolysis and IL-6 release. In addition, the data reveal for the first time that fasting plasma insulin, but not glucose, is raised by ANXA1 gene deletion, a finding that supports the premise that ANXA1 has a role in regulating the release of and/or the tissue sensitivity to insulin (33, 34).

ANXA1 Expression and Adipose Tissue Mass

The adult male ANXA1-null mouse showed a somewhat leaner phenotype, as indicated by the significant reduction in epididymal fat pad weight, compared with wild-type controls. As the mean area of the adipocytes was unaffected by ANXA1 gene deletion, it seems likely that this difference reflects a reduction in preadipocyte and/or adipocyte cell number. The latter could suggest a perturbation of adipogenesis, the process by which preadipocytes in the stromal-vascular compartment mature into lipid-storing adipocytes (31). Although ANXA1 gene deletion did not affect the expression of PPAR γ , a transcription factor concerned with adipogenesis (31), several other lines of evidence support a role for the protein in this complex process. First, ANXA1 expression declines with progressing adipogenesis in 3T3-L1 cells in vitro (49). Second, our Western blot analysis showed that ANXA1 is localized predominantly in the stromal-vascular compartment where the preadipocytes reside. Third, ANXA1 is strongly implicated in the processes regulating cell growth and differentiation in other tissues (41,43). Additionally, in related studies we (19) have shown that ANXA1 expression in epididymal adipose tissue is reduced by a diet-induced increase in adipose tissue mass, a process that involves both tissue hypertrophy and hyperplasia and, hence, reduction in preadipocyte and increase in adipocyte number. This observation further supports the notion that ANXA1 is important in regulating epididymal adipose tissue mass. Additional studies are now required to delineate the role of ANXA1 in early adipogenesis.

Catecholamine Sensitivity

Previous studies (25) have shown that β -adrenoceptor stimulation promotes lipolysis, and hence, glycerol release, in adipose tissue via a mechanism involving cAMP generation and protein kinase A-dependent phosphorylation of hormone-sensitive lipase. In accord with these findings, we observed an increase in glycerol release in isolated adipocytes and explants of

adipose tissue treated for 2 or 4 h with isoprenaline or norepinephrine and an associated increase in cAMP. Although basal glycerol release in vitro was unaffected by ANXA1 gene deletion, as is also the case in vivo (44), the lipolytic responses to isoprenaline and/or noradrenaline were attenuated from cells and/or tissues from ANXA1-null mice compared with wild-type controls. These findings suggest that ANXA1 may play a role in the regulation of lipolysis by modulating the sensitivity of adipose tissue to β -adrenoceptor stimulation. Further evidence for a role for ANXA1 in adrenoceptor signaling emerged from our observation that, over a 4-h period, isoprenaline reduces IL-6 release from adipose tissue explants from the wild-type but not the ANXA1-null mouse. Interestingly, no such effect was evident in isolated adipocytes from mice of either strain, perhaps because the stromal-vascular cells contribute significantly to the total IL-6 released from tissue explants (15) or because the stromal tissue plays an essential role in mediating the responses to catecholamines. Interestingly, and in contrast to our findings in explants, over a longer time frame (24 h) isoprenaline has been shown to increase IL-6 release from isolated adipocytes (36,45).

Our measurements of β -adrenoceptor mRNA expression show that the loss of sensitivity of epididymal adipose tissue to catecholamines in the ANXA1-null mouse cannot be explained merely by a reduction in β -adrenoceptor expression. Interactions between ANXA1 and catecholamine-signaling cascades have been implicated in other systems. For example, in vitro ANXA1_{Ac2-26} reduces the ability of LPS and interferon- γ to attenuate isoprenaline-induced contractions in rat papillary muscle (40). Furthermore, ANXA1 augments the responsivity of the pig epidermis to β -adrenoceptor stimulation in vitro apparently by inhibiting cytosolic phospholipase A₂ (cPLA₂), which ordinarily depresses the β -adrenoceptor-stimulated adenylyl cyclase (21). Because ANXA1 inhibits both the expression and the activity of cPLA₂ in many systems (10,18,42), it is possible that adipocyte adenylyl cyclase is protected from the inhibitory influence of cPLA₂ by ANXA1. Such a protective action would explain the reduction in cAMP levels observed before and after isoprenaline stimulation in adipose tissue from the ANXA1-null mice and thus could offer a potential mechanism whereby ANXA1 serves to sensitize tissues to β -adrenoceptor stimulation.

Effects of Glucocorticoids

Since ANXA1 is a mediator of glucocorticoid action in the neuroendocrine and immune systems (7,37), and the ANXA1-null mouse is resistant to some facets of glucocorticoid action (18,50), we examined the effects of ANXA1 gene deletion on the responses of adipose tissue to glucocorticoids. Our results showed that GR expression is unaffected by ANXA1 gene deletion. They also confirmed reports (27,35) that dexamethasone readily suppresses catecholamine-stimulated glycerol release in vitro. Because adipocytes from both mouse strains appeared to be equally sensitive to the steroid, it seems likely that this particular facet of steroid action is independent of ANXA1.

Our data on IL-6 release are more difficult to interpret. Complex interactions between IL-6, glucocorticoids, and ANXA1 have been described in other systems (12,30). Our study shows that the IL-6 content of epididymal adipose tissue is increased markedly by ANXA1 gene deletion, as it is in several other tissues (50), although basal release of the cytokine in vitro is reduced. Our histological findings show that the increased IL-6 content cannot be explained by an increase in the local macrophage number and must therefore reflect increased synthesis and/or storage of the cytokine. In line with previous reports (15,45), we noted that glucocorticoids suppress basal IL-6 release from wild-type adipocytes. However, paradoxically, our data revealed a stimulatory effect of the steroids on IL-6 release from adipocytes from ANXA1-null mice. Thus the impairment in basal IL-6 release caused by ANXA1 gene deletion is reversed by dexamethasone treatment. The mechanism responsible

for this apparent “switch” in steroid action is unknown, but it is conceivable that it reflects other glucocorticoid-induced modifications that are ordinarily masked by the effects of ANXA1, thus raising the possibility that, contrary to the dogma, ANXA1 may oppose some facets of glucocorticoid action.

Responses to LPS

LPS acts via the toll-like receptor 4 (17,29) on adipose tissue to induce cytokine release (2, 14) and alterations in lipid mobilization (32) due in part to its ability to increase hormone-sensitive lipase and decrease lipoprotein lipase (24). Since LPS modulates the expression and cellular disposition of ANXA1 (12) and the ANXA1 KO mouse is hypersensitive to LPS-induced sepsis (11), we hypothesized that ANXA1 gene deletion may modify the sensitivity of adipose tissue to LPS. Our results show for the first time that LPS exerts a direct stimulatory effect on glycerol release from adipocytes *in vitro*; they also demonstrate that this action is attenuated by ANXA1 gene deletion and thus raises the possibility that ANXA1 plays a significant role in mediating the lipolytic action of the toxin.

Although LPS readily stimulates the release of IL-6 from a number of cells and/or tissues, controversy surrounds its ability to promote the release of the cytokines from adipocytes. Data on subcutaneous adipocytes from the male pig suggest that they exert a positive effect, possibly via ERK1/2 (2), whereas adipocytes derived from human males are unresponsive to the toxin (14). Here, we show that LPS stimulates IL-6 release from murine epididymal adipocytes from ANXA1 KO mice but not from corresponding wild-type cells. Thus ANXA1 appears to blunt the IL-6 response to LPS in these cells, an effect that could be achieved by the ability of the protein to suppress ERK activity (4).

It is noteworthy that, although the basal release of IL-6 from epididymal adipose tissue is reduced by ANXA1 gene deletion, both glucocorticoids and LPS facilitate the release of the cytokine from ANXA1 KO but not wild-type adipose tissue. The reduction in basal IL-6 release from the ANXA1-null tissue does not appear to be reflected *in vivo*, as there were no significant between-strain differences in basal plasma IL-6 release. However, if the glucocorticoid and/or LPS-induced release of IL-6 is translated to the *in vivo* situation, it could have important implications in the pathogenesis of diseases associated with stress and/or chronic low inflammatory states, e.g., atherosclerosis. In addition, since the plasma IL-6 response to LPS is exaggerated by ANXA1 gene deletion (8), it is conceivable that adipose tissue provides a major source of the cytokine.

In conclusion, this study indicates that ANXA1 plays an important role in epididymal adipose tissue biology by 1) participating in the mechanisms regulating adipocyte number and 2) modulating the sensitivity of the tissue to catecholamines, LPS, and glucocorticoids and, hence, contributing to the processes regulating lipolysis and IL-6 release. Whether these changes reflect direct actions of ANXA1 on the adipose tissue or whether they are secondary to other changes induced by ANXA1 gene deletion remains to be determined. Interestingly, our data do not support the premise that ANXA1 is a mediator of glucocorticoid action in epididymal adipose tissue, as it is in other tissues (reviewed in Ref. 23). To the contrary, they raise the intriguing novel possibility that ANXA1 may oppose some facets of steroid action within this tissue. Taken together, the findings raise the possibility that disturbances in ANXA1 expression and/or function might, therefore, contribute to the etiology of metabolic and cardiovascular/inflammatory disease associated with adipose dysfunction.

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Fig 1.

Effects of annexin 1 (ANXA1) gene deletion on the epididymal fat pad. *A*: wet weight as % body weight. *B*: absolute wet weight. *C*: adipocyte size in adult male wild-type (WT; white bars) and ANXA1 knockout (KO; black bars) mice ($n = 5 \pm \text{SE}$). *D* and *E*: Western blot analysis showing ANXA1 protein expression in the adipocyte and stromal-vascular fractions of the epididymal fat pad from the adult male WT mouse ($n = 4 \pm \text{SE}$; *D*) and expression of ANXA1 expression in epididymal fat tissue from WT but not ANXA1-null (ANXA1 KO) mice (*E*).

Fig 2.

Western blot analysis showing expression of interleukin-6 (IL-6; *A*), glucocorticoid receptor (GR; *B*), and peroxisome proliferator activated receptor- γ (PPAR γ ; *C*) protein in the epididymal fat pad of adult male WT (white bars) and ANXA1 KO (black bars) mice; $n = 4 \pm$ SE. # $P < 0.05$ (Mann-Whitney *U*-test).

Fig 3.

Effects of treatment of adipose tissue explants from male WT (white bars) and ANXA1 KO (black bars) mice with isoprenaline (2 h) in vitro on glycerol release (A) and cAMP accumulation (B); $n = 5 \pm \text{SE}$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. basal control; # $P < 0.05$ as indicated. NS, not significant. Two-way ANOVA plus Tukey's multiple comparisons test.

Fig 4.

Effects of dexamethasone (Dex; 2.5 μg/ml in the drinking water for 16 h prior to autopsy) on the ability of isolated adipocytes from the epididymal fat pad of adult male WT and ANXA1 KO mice to release glycerol in vitro in response to a 2-h challenge with isoprenaline (A) or norepinephrine (B). White bars, basal; hatched bars, isoprenaline or norepinephrine (0.1 μM); black bars, isoprenaline or norepinephrine (10 μM); $n = 5 \pm \text{SE}$. * $P < 0.05$; *** $P < 0.001$ vs. drug-free control; ### $P < 0.001$ vs. corresponding WT control; ++ $P < 0.01$; +++ $P < 0.001$ vs. respective Dex-free group. Three-way ANOVA plus Tukey's multiple comparison test.

Fig 5.

Effects of isoprenaline (10 μ M, 4 h) on the release of IL-6 from adipose tissue explants (A) and isolated adipocytes (B) from the epididymal fat pad of WT (white bars) and ANXA1 KO (black bars) mice; $n = 5 \pm$ SE. * $P < 0.05$; *** $P < 0.001$ vs. basal; ## $P < 0.01$ WT vs. ANXA1 KO. Two-way ANOVA plus Tukey's multiple comparison test.

Fig 6.

Effects of Dex (2.5 μ g/ml in the drinking water for 16 h prior to autopsy) and isoprenaline (10 μ M, 2 h; **A**) and norepinephrine (10 μ M, 2 h; **B**) on IL-6 release from isolated adipocytes from adult male WT and ANXA1 KO mice. White bars, basal; black bars, isoprenaline or norepinephrine; $n = 5 \pm$ SE. [#] $P < 0.05$ vs. WT; ^{*} $P < 0.05$ vs. basal; ⁺ $P < 0.05$ vs. respective steroid-free group. Three-way ANOVA plus Tukey's multiple comparison test.

Fig 7. Effects of lipopolysaccharide (LPS; 1 $\mu\text{g}/\text{ml}$, 4 h) on the release in vitro of glycerol (A) and IL-6 (B) from isolated adipocytes derived from epididymal adipose tissue from WT (white bars) and ANXA1 KO (black bars) mice; $n = 5 \pm \text{SE}$. ** $P < 0.01$; *** $P < 0.001$ vs. basal; # $P < 0.05$; ## $P < 0.01$ vs. corresponding WT control. Two-way ANOVA plus Tukey's multiple comparisons test.

Table 1

Epididymal and retroperitoneal fat pad macrophage number, as determined by F4/80 expression, in tissue from WT and ANXA1 KO mice

	Epididymal Fat Pad, %F4/80 Positive Cells	Retroperitoneal Fat Pad, %F4/80 Positive Cells
WT	7.2±0.5	5.1±0.5
ANXA1 KO	6.9±0.7	5.5±0.4

Values are means ± SE. WT, wild type; ANXA1 KO, annexin 1 knockout. Fraction of F4/80-expressing cells for each sample was calculated as the number of nuclei of F4/80-expressing cells divided by the total number of nuclei and expressed as a percentage; $n = 4 \pm$ SE. No significant differences between groups were observed ($P > 0.05$).

Table 2

Expression of mouse β_1 -, β_2 -, and β_3 -AR mRNAs in the epididymal and retroperitoneal fat pads and liver of WT ANXA1 KO mice

	Strain	Epididymal Fat Pad, %WT	Retroperitoneal Fat Pad, %WT	Liver, %WT
β_1 -AR	WT	100±13.1	100±63.9	100±21.9
	ANXA1 KO	156.5±21.9	172.2±31.1	164.9±27.7
β_2 -AR	WT	100±22.4	100±41.8	100±17.5
	ANXA1 KO	107.0±47.0	175.8±59.0	160.9±35.8
β_3 -AR	WT	100±21.2	100±28.1	100±9.5
	ANXA1 KO	76.1±14.2	44.0±21.6	145.6±40.0

Values are means $\pm$ SE. AR, adrenoreceptor. Data were normalized using the geometric average of 3 reference genes and are expressed as %expression in WT tissue; $n = 4-6 \pm$ SE. No significant differences were observed between the WT and ANXA1 KO mice were observed in any of the tissues studied ($P > 0.05$, ANOVA and Student's *t*-test).