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Site-Specific Control of Rat Preadipocyte Adipose Conversion by Ovarian Status

Possible Involvement of CCAAT/Enhancer-Binding Protein Transcription Factors

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The preadipocyte-adipocyte conversion process from two intraabdominal (parametrial and perirenal fat depots) is differently affected by ovarian status in the rat. We have tested the hypothesis that these site-specific alterations of adipogenesis might be related to changes in the expression of the transcription factors c-*myc* and CCAAT/enhancer binding proteins (C/EBPα, -β, and -ζ) that regulate proliferation and differentiation. The increased proliferation rates observed in parametrial and perirenal preadipocytes after ovariectomy were not linked to variations in c-*myc* mRNA levels. Expression of the early marker of adipogenesis, lipoprotein lipase (LPL), remained insensitive to the ovarian status in early differentiated parametrial and perirenal preadipocytes. By contrast, LPL expression increased in early differentiated sc preadipocytes from ovariectomized rats, an effect that was completely reversed by in vivo estradiol and progesterone treatment. Expression of C/EBPβ protein was unaffected by ovarian status whatever the anatomic origin of the preadipocytes. By contrast, the levels of p42 and p30 isoforms of C/EBPα were specifically decreased in parametrial preadipocytes, an alteration that was completely corrected by in vivo administration of estradiol and progesterone. C/EBPζ, a dominant inhibitor of C/EBPα and -β, exhibited a strong site-specific expression since C/EBPζ content was fivefold higher in sc preadipocytes than in deep intraabdominal cells whatever the ovarian status. Furthermore, ovariectomy selectively decreased C/EBPζ levels in sc cells. In conclusion, our study suggests that some of the site-specific effects of ovariectomy on adipogenesis could involve, at least in part, altered expressions of C/EBPα and -ζ, both of which are important transcriptional regulators of fat cell differentiation and metabolism.

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Introduction

It is now clear that ovarian steroid hormones play an important role in the regional specificities of fat metabolism and deposition *(1–4)*. Adipose tissue is an important site of estrogen biosynthesis and steroid hormone storage particularly in postmenopausal women *(5)*. Specific estrogen (estrogen receptor-α [ER-α]) and progesterone receptors are expressed in human and rodent precursors and mature fat cells *(3,6–10)*. In addition, ER numbers vary with the anatomic origin of the adipocytes *(11,12)*. Recently, another ER subtype (ER-β) was identified in human adipose tissue from various anatomic sites *(13)*. These findings suggest that estrogens could exert direct paracrine or intracrine effects on white adipose tissue.

In rats, ovariectomy is known to increase intraabdominal fat deposition *(14)*, which seems to result, in part at least, from altered preadipocyte-adipose conversion. In fact, we have recently reported that ovariectomy promotes the proliferation and differentiation as well as high mitogen-activated protein kinase (MAPK) activity of perirenal preadipocytes. Surprisingly, ovariectomy, which also increases the proliferation of parametrial preadipocytes, fails to alter the differentiation capacities of these cells. Moreover, in vivo treatment of ovariectomized (OVX) rats with estradiol and progesterone reversed the promoting effect of ovariectomy on proliferation and differentiation of perirenal preadipocytes. Finally, adipose conversion of sc preadipocytes was found insensitive to ovarian status *(15)*.

Cell proliferation and differentiation are often mutually exclusive processes and are controlled at the transcriptional level by ubiquitous or cell-specific factors. Among these transcriptional factors, the product of the protooncogene c-*myc* is a key regulator of cell proliferation *(16)*. The levels of c-*myc* mRNA and protein (c-Myc) are rapidly increased in response to growth factors. c-Myc forms a heterodimer with another transcriptional regulator, Max, and binds to DNA-specific sequences leading to transcriptional activation.

c-Myc thus regulates the expression of genes required for cell-cycle progression *(17)*. On the other hand, sex steroid hormones have been shown to regulate c-*myc* expression in several cell types and tissues *(18)*. For example, in vivo, estradiol increases c-*myc* expression in rat uterus *(19)*, anterior pituitary gland *(20)*, and ovary *(21)*. By contrast, progesterone was reported to exert a general downregulation of nuclear protooncogenes *(18)*, although in T-47D human breast cancer cells, c-*myc* expression is rapidly stimulated by progestins *(22)*.

The CCAAT/enhancer-binding proteins (C/EBPs) belong to a family of transcriptional factors, some members of which (C/EBPα, -β, -δ, and -ζ) play a critical role in preadipocyte differentiation *(23)*. Expressions of C/EBPβ and -δ are rapidly and transiently induced in response to hormonal stimulators *(24)*. In turn, these factors induce the expression of C/EBPα, which then stimulates its own expression and transactivates several important adipospecific genes such as the adipocyte fatty acid–binding protein aP2 *(25)* and leptin, the product of the *ob* gene *(26)*. Furthermore, C/EBPα is a potent inhibitor of cell proliferation mainly because it induces the expressions of the growth-arrested-associated gene *gadd45 (27)* and the cyclin-dependent kinase (CDK) inhibitor p21 *(28)*. C/EBPζ (CHOP or gadd153), another member of this family, is expressed later than C/EBPα during adipogenesis and also causes growth arrest when induced by stress *(23)*.

Regulation of these factors involves several hormonal signals. C/EBPβ expression is rapidly induced by glucocorticoids in rat liver *(29)* and intestinal cell line *(30)* but not in 3T3-L1 adipocytes *(31)*. C/EBPα expression was reported to be repressed by glucocorticoids in 3T3-L1 adipocytes *(31)*, but positively regulated following an in vivo estrogen treatment in rat ovarian follicles *(32)*.

A pivotal role has been assigned to c-*myc* and C/EBPα in the control of adipogenesis. In the 3T3-L1 preadipocyte cell line, overexpression of c-*myc* blocks the adipose conversion process and the induction of C/EBPα. Conversely, overexpression of C/EBPα overcomes the inhibitory effects of c-*myc* on adipogenesis *(33)*.

Thus, it is conceivable that the variations in adipogenesis found in deep intraabdominal preadipocytes following ovariectomy are linked to altered expressions of c-*myc* and C/EBPs. To test this hypothesis, we investigated the influence of ovariectomy on c-*myc* and C/EBP expression in rat preadipocytes isolated from two intraabdominal (parametrial and perirenal) and one sc (femoral) fat depots.

Results

Ovarian status differently affects adipose conversion of rat preadipocytes depending on their anatomic origins, as reported in (ref. *15*) and summarized in Table 1. To obtain information on these effects at the transcriptional level, we studied the expression of some transcription factors particularly involved in adipogenesis.

Table 1
Influence of Ovariectomy on Adipose Conversion of Rat Preadipocytes from Different Anatomic Origins

Preadipocytes from fat depots	Proliferation capacity (cell number)	Differentiation capacity (GPDH activity)
Femoral sc	Unchanged	Unchanged
Parametrial	Increased	Unchanged
Perirenal	Increased	Increased

*Ovarian Status and c-*myc *Expression*

First, the effect of ovariectomy on c-*myc* expression stimulated by serum was compared in proliferating preadipocytes from different fat depots. Preliminary experiments have shown that c-*myc* mRNA expression is maximally induced after 1 h of serum exposure and remains sustained up to 24 h. As indicated in Fig. 1, we observed that c-*myc* expression stimulated by 1 h of serum exposure was similar in sc and deep intraabdominal preadipocytes from sham rats. Under the same conditions, however, ovariectomy decreased the levels of c-*myc* mRNA in parametrial preadipocytes but not in sc and perirenal cells (Fig. 1).

In several cell types and tissues, estradiol and progesterone regulate c-*myc* expression *(18)*. In the present study, in vivo treatment with estradiol and progesterone completely reversed the reduced c-*myc* expression induced by ovariectomy in parametrial cells (Fig. 1). This finding led us to investigate in vitro the influence of estradiol and progesterone on c-*myc* expression in parametrial preadipocytes. Under our experimental conditions, however, none of these hormones did apparently influence c-*myc* mRNA expression in these cells no matter what the gonadal status of the rats (data not shown).

Ovarian Status and Early Events of Adipogenesis

The adipose conversion of rat deep intraabdominal preadipocytes is differently influenced by ovariectomy, as evidenced by glycerol-3-phosphate dehydrogenase (GPDH) activity, a late marker of differentiation *(15)* (see Table 1).

To determine whether the early steps of differentiation are also affected by ovariectomy, the expression of lipoprotein lipase (LPL), an early marker of adipogenesis, was compared in early differentiated preadipocytes (EDPs) from the three different fat depots. Moreover, ovariectomy with or without supplemental estradiol and progesterone treatment failed to significantly modify LPL expression in parametrial and perirenal EDPs. However, LPL expression was markedly enhanced (+110%) by ovariectomy in sc EDPs,

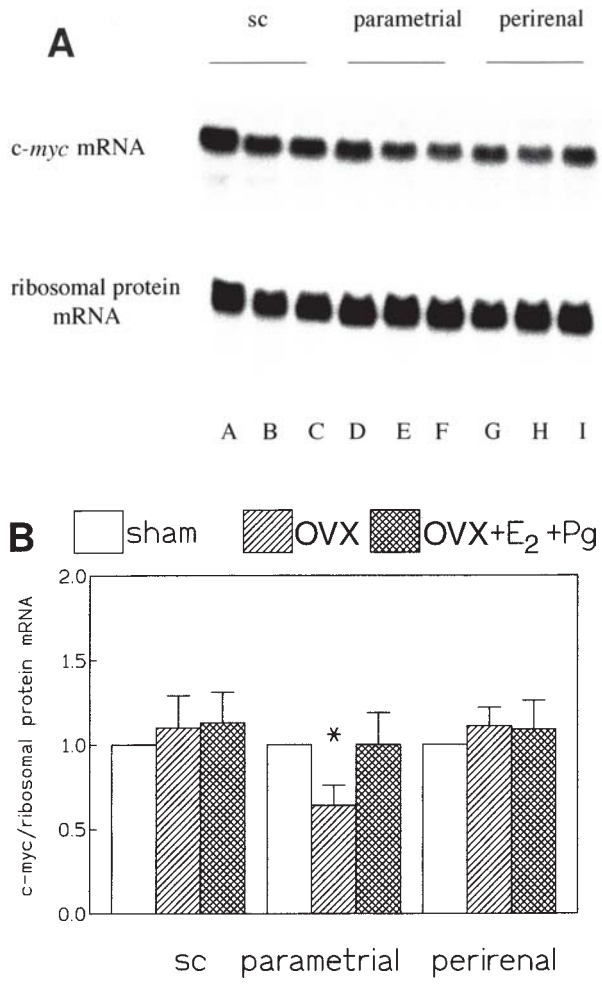

Fig. 1. Influence of ovarian status on c-*myc* mRNA expression in rat proliferating preadipocytes from femoral sc, parametrial, and perirenal fat depots. Total RNAs of proliferating preadipocytes from femoral sc (lanes A, B, and C), parametrial (lanes D, E, and F), and perirenal (lanes G, H, and I) fat depots from sham (lanes A, D, and G), OVX (lanes B, E, and H), and OVX+E_2+Pg (lanes C, F, and I) rats were hybridized with rat c-*myc* probe. (**A**) Representative Northern blot of c-myc mRNA. (**B**) Densitometric analysis of c-*myc* Northern blots. The data are means ± SEM of six separate experiments. Variations in c-*myc*/ribosomal protein mRNA percentage ratios as shown. One hundred percent was assigned to each type of preadipocyte from sham rats for c-*myc* mRNA and ribosomal protein mRNA. Then, for each experimental group, the respective c-*myc* mRNA and ribosomal protein mRNA were expressed relative to the corresponding controls. Finally, c-*myc* mRNA/ribosomal protein mRNA percentage ratios were calculated. *$p < 0.05$, OVX vs sham and OVX+E_2+Pg rats.

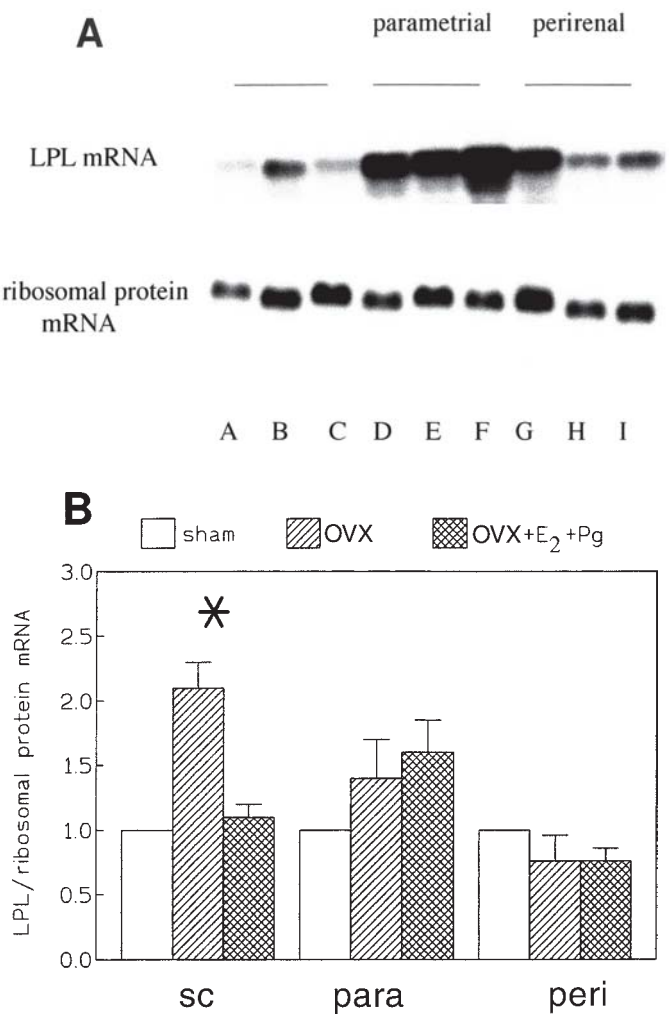

Fig. 2. Influence of ovarian status on LPL mRNA expression in rat EDPs from femoral sc, parametrial, and perirenal fat depots. Total RNAs of EDPs from femoral sc (lanes A, B, and C), parametrial (lanes D, E, and F), and perirenal (lanes G, H, and I) fat depots from sham (lanes A, D, and G), OVX (lanes B, E, and H), and OVX+E_2+Pg (lanes C, F, and I) rats were hybridized with rat LPL probe. (**A**) Representative Northern blot of LPL mRNA. (**B**) Densitometric analysis of LPL Northern blots. The data are means ± SEM of six separate experiments. Variations in LPL/ribosomal protein mRNA percentage ratios are shown. One hundred percent was assigned to each type of preadipocyte from sham rats for LPL mRNA and ribosomal protein mRNA. Then, for each experimental group, the respective LPL mRNA and ribosomal protein mRNA were expressed relative to the corresponding controls. Finally, LPL mRNA/ribosomal protein mRNA percentage ratios were calculated. *$p < 0.05$, OVX vs sham and OVX+E_2+Pg rats.

an effect that was completely prevented by in vivo estradiol and progesterone replacement (Fig. 2). A similar pattern of LPL expression was observed in late differentiated preadipocytes (LDPs) from sc depots.

These experiments indicate that the early marker of adipogenesis LPL is site specifically regulated by ovariectomy because its expression is either increased in sc or unchanged in deep intraabdominal EDP.

Ovarian Status and C/EBP Transcription Factors

To gain more information about the molecular basis underlying the effects of ovariectomy on adipogenesis, the expression of C/EBP (C/EBPα, -β, and -ζ) transcription factors was studied next.

C/EBPβ, which is induced early during adipogenesis, has an mRNA that can be translated to give rise to the transcriptional activator liver-enriched activator protein (LAP) and

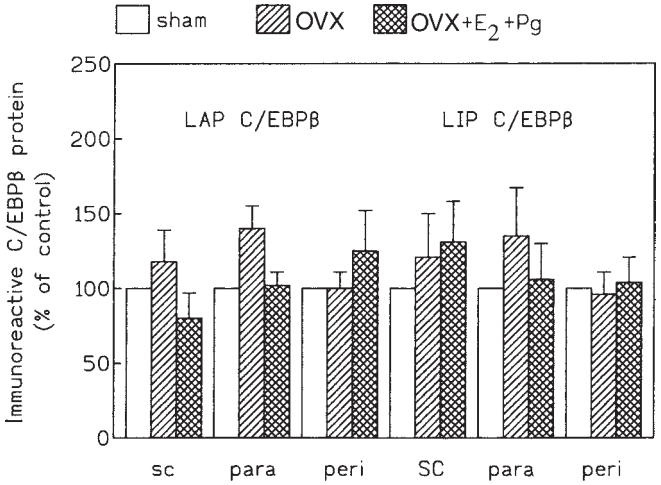

Fig. 3. Influence of ovarian status on C/EBPβ protein expression in rat EDPs from femoral sc, parametrial, and perirenal fat depots. Cellular extracts of EDPs from femoral sc, parametrial, and perirenal fat depots from sham, OVX, and OVX+E_2+Pg rats were probed with C/EBPβ polyclonal antiserum. For the densitometric analysis of C/EBPβ Western blots, the data are means ± SEM of six separate experiments and are expressed as percentage-of-control values (one hundred percent is assigned to each type of preadipocyte from sham rats).

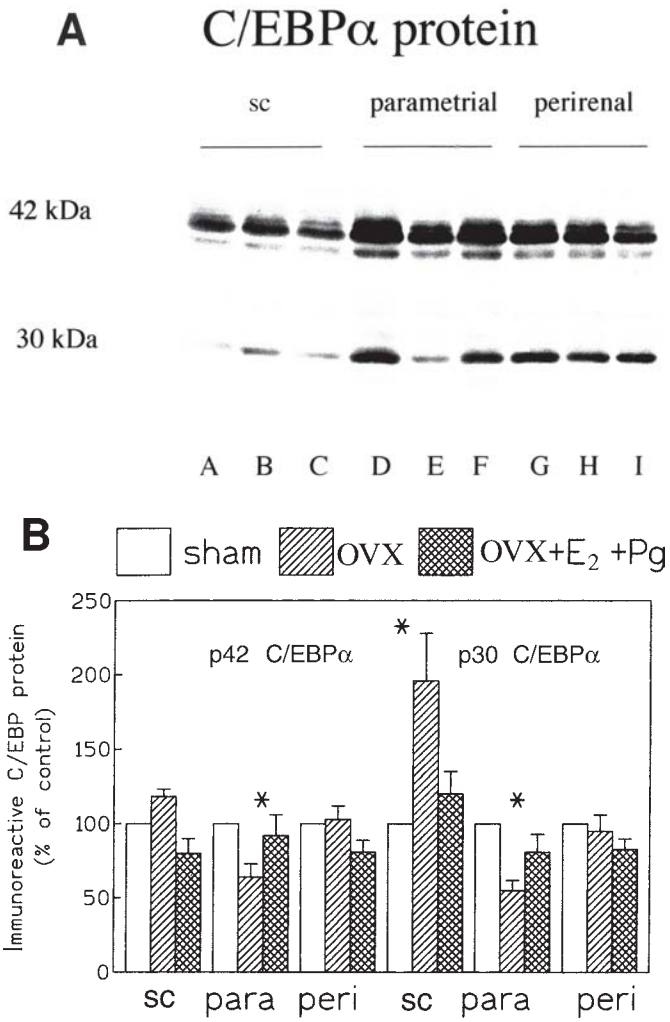

Fig. 4. Influence of ovarian status on C/EBPα protein expression in rat LDPs from femoral sc, parametrial, and perirenal fat depots. Cellular extracts of LDPs from femoral sc (lanes A, B, and C), parametrial (lanes D, E, and F), and perirenal (lanes G, H, and I) fat depots from sham (lanes A, D, and G), OVX (lanes B, E, and H) and OVX+E_2+Pg (lanes C, F, and I) rats were probed with C/EBPα polyclonal antiserum. (**A**) Representative Western blot of C/EBPα proteins. (**B**) Densitometric analysis of C/EBPα Western blots. The data are means ± SEM of seven separate experiments and are expressed as percentage-of-control values (one hundred percent is assigned to each type of preadipocyte from sham rats). *$p < 0.05$, OVX vs sham and OVX+E_2+Pg rats.

the transcriptional repressor liver-enriched inhibitor protein (LIP) *(34)*. As shown in Fig. 3, the steady-state levels of LAP and LIP were found unaffected by the ovarian status whatever the anatomic origin of the EDPs.

Next, we studied the C/EBPα expression, which is activated by C/EBPß and positively autoregulated *(35)*. The C/EBPα mRNA gives rise to two major translation products, p42 and p30 C/EBPα *(36)*. As shown in Fig. 4, ovariectomy specifically decreased (–40%) p42 and p30 isoforms in parametrial LDPs, whereas it promoted p30 C/EBPα expression (+100%) in sc LDPs. Moreover, these effects of ovariectomy on C/EBPα levels were prevented by in vivo estradiol and progesterone treatment (Fig. 4).

Finally, the expression of C/EBPζ, a dominant inhibitor of C/EBPα and -β that appears later during adipogenesis *(35)*, was compared in LDPs from the different experimental groups. In sham rats, the steady-state levels of C/EBPζ protein exhibited a strong site specificity since sc LDPs expressed about fivefold higher levels of this protein than deep intraabdominal LDPs (Fig. 5A,B). Furthermore, C/EBPζ protein levels were significantly reduced (–45%) by ovariectomy in sc LDPs but unaltered in parametrial and perirenal LDPs.

These experiments demonstrate that C/EBPα and -ζ expressions are affected differently by ovariectomy depending on the anatomic origin of the preadipocytes.

Discussion

We have previously reported that the differentiation capacities of rat parametrial and perirenal preadipocytes are either unchanged or enhanced by ovariectomy, respectively *(15)*. By contrast, both parametrial and perirenal preadipocytes elicit increased proliferation capacities after ovariectomy *(15)*. The purpose of the present study was to test the hypothesis that these changes in adipogenesis following ovariectomy could be linked to modified expressions of some of the key transcriptional factors controlling the adipoconversion process.

The first factor that we investigated was the growth-associated gene, c-*myc*. Our experiments show that induction of c-*myc* mRNA by serum growth factors is either depressed

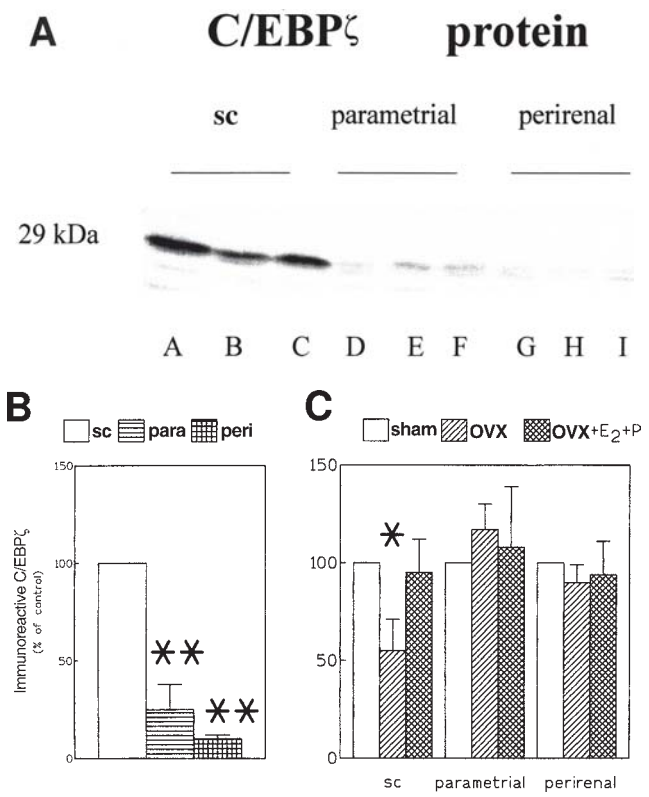

Fig. 5. Influence of ovarian status on C/EBPζ protein expression in rat LDPs from femoral sc, parametrial, and perirenal fat depots. Cellular extracts of LDPs from femoral sc (lanes A, B, and C), parametrial (lanes D, E, and F), and perirenal (lanes G, H, and I) fat depots from sham (lanes A, D, and G), OVX (lanes B, E, and H), and OVX+E_2+Pg (lanes C, F, and I) rats were probed with C/EBPζ polyclonal antiserum. (**A**) Representative Western blot of C/EBPζ proteins. (**B**) Densitometric analysis of C/EBPζ Western blots. The data are means ± SEM of four separate experiments and are expressed as percentage-of-control values (one hundred percent is assigned to sc preadipocyte from sham rats). $^{**}p <$ 0.01, parametrial and perirenal vs sc preadipocytes from sham rats. (**C**) Densitometric analysis of C/EBPζ Western blots. The data are means ± SEM of four separate experiments and are expressed as percentage-of-control values (one hundred percent is assigned to each type of preadipocyte from sham rats). $^{*}p < 0.05$, OVX vs sham rats.

in parametrial or unchanged in perirenal preadipocytes after ovariectomy. These findings together with similar observations reported in Swiss 3T3 fibroblasts *(37)* and rat hepatocytes *(38)*, suggest that only marginal induction of c-Myc is probably required for DNA synthesis in these cells.

Concerning the early events of adipogenesis, our results clearly indicate that the expression of LPL, an early marker of adipogenesis that controls the uptake of free fatty acids in adipocytes, is insensitive to ovariectomy in parametrial and perirenal preadipocytes. By contrast, in sc preadipocytes that do not exhibit altered adipogenesis after ovariectomy *(15)*, LPL expression is increased by ovariectomy or normal after supplemental estradiol and progesterone treatment.

From patterns of marker gene expressions (FAS, LPL), it has been suggested that fat accumulation in sc depots depends more on free fatty acid capture than on lipogenesis, whereas the opposite situation appears to prevail in intraabdominal fat tissues *(39)*. Thus, the peculiar effects of ovariectomy on LPL expression in sc cells could play a significant role in the sc depot growth observed in this physiopathologic situation. Note that a similar effect on LPL expression in sc preadipocytes was observed in another situation of gonadal deficiency. In fact, we have recently reported that castration also promoted LPL expression in sc cells, an effect that was abolished by in vivo testosterone treatment *(40)*.

Concerning the influence of ovariectomy on C/EBP factors, the protein expression of C/EBPβ, which is induced early during differentiation, was not affected by ovariectomy whatever the anatomic origin of the preadipocytes. A different situation was observed with the expressions of p42 and p30 C/EBPα isoforms, which appear later than C/EBPβ during the course of adipogenesis. As a matter of fact, expressions of p30 and p42 C/EBPα were decreased in parametrial cells or unchanged in perirenal preadipocytes after ovariectomy. Consistent with these findings is a recent report showing that C/EBPα gene expression is decreased by metabolic signals such as glucose in epididymal fat but not in omental adipose tissue *(41)*. Thus, the situation of ovariectomy provides another example in which C/EBPα is regulated in divergent ways in perigonadal and intraabdominal fat cells.

Cell proliferation and terminal differentiation are often mutually exclusive events. C/EBPα induces growth arrest mainly through increased expression of the CDK inhibitor p21 *(28)* and promotes differentiation. Thus, the reduced expression of C/EBPα found in parametrial preadipocytes after ovariectomy could influence the growth-arrest process. However, in perirenal preadipocytes that also elicit increased proliferation capacities after ovariectomy, C/EBPα expression was unaltered. It appears, therefore, that the increased proliferation of perirenal and parametrial preadipocytes is more likely related to the positive effects of ovariectomy on the MAPK and c-*fos* signaling pathways in these cells *(15)*. Since expression of the CDK inhibitor p21 is also regulated by estrogens and progesterone in some cell types *(42,43)*, it would be interesting to establish whether p21 expression is differently affected by ovariectomy in parametrial and perirenal cells.

C/EBPζ, which is expressed later than C/EBPα, is generally considered to play an important regulatory role in the very late differentiation steps of adipogenesis *(23)*. This factor, which is regulated by nutriments, could also intervene in the control of fat cell metabolism *(23)*. C/EBPζ is a negative modulator of the transcriptional activities of C/EBPα and -β because it heterodimers with these C/EBPs and thus prevents their binding to the CCAAT enhancer sequences. As shown in the present study, the levels of C/EBPζ were about fivefold higher in sc than in intraabdominal preadipocytes from normal rats. The physiologic consequence of

the strong site-specific expression of C/EBPζ could be a much weaker expression of C/EBP transactivated genes in sc than in intraabdominal adipocytes. This hypothesis is supported by the findings that compared with deep intraabdominal fat cells, sc adipocytes are less metabolically active, and express fewer C/EBP transactivated gene products such as aP2, GLUT4, insulin receptor, and Ob *(39, 44–46)*. Conversely, the reduced C/EBPζ expression found in sc cells after ovariectomy could have, by consequence, enhanced transcription of C/EBP transactivated genes in these cells. C/EBPζ also forms with C/EBPβ heterodimer, which binds to novel DNA sequence to activate transcription of stress-inducible genes *(47)*. Hence, site-specific expression of C/EBPζ could be important in the regulation of cellular events in stressful conditions. Therefore, the marked differences in C/EBPζ protein levels between sc and deep intraabdominal adipocytes are an interesting observation with respect to our current knowledge of the site specificities of adipogenesis and regulation of fat cell metabolism.

Besides the C/EBP factors, PPARγ is another master transcriptional regulator of adipogenesis *(48)*. This factor is induced earlier than C/EBPα and cooperates with it in the promotion of differentiation. Further experiments will be needed to establish whether the adipogenic changes observed in perirenal cells after ovariectomy can be accounted for by altered expression or activity of PPARγ.

Finally, our study shows that ovarian status site specifically influences the expression of transcriptional factors, particularly C/EBPα and -ζ. The molecular basis of this site specificity, which remains unsettled, is often discussed in terms of receptor density. For example, estrogen and progesterone receptor numbers are lower in superficial than in deep intraabdominal fat cells *(6,11)*. Several studies have led to the identification of coactivators and corepressors that interact with nuclear receptors and play an important role by enhancing or repressing the transcriptional activity of these receptors. All the coregulators identified so far are ubiquitously expressed at mRNA levels *(49)*. Nevertheless, some important coactivators (SRC-1 and CBP/p300) and corepressors (SMRT and N-CoR) have distinct tissue-specific expressions *(50)*. Interestingly, in vivo and in vitro exposure of pituitary cells to estradiol decreases and increases mRNA levels of coactivator SRC-1 and corepressor SMRT, respectively *(50)*. Thus, the relative expression of coregulator genes may contribute to the heterogeneity of hormonal responses in tissues. Further experiments will be needed to determine whether the site-specific effects of ovariectomy on adipogenesis are also related to differences in coregulator expressions between preadipocytes from various anatomic origins.

In summary, our study strongly suggests that the mechanisms whereby ovariectomy has a site-specific influence on adipose conversion of rat preadipocytes involves, at least in part, modified expressions of some of the C/EBP family members that are important transcriptional regulators of fat cell differentiation and metabolism.

Materials and Methods

Materials

Fetal bovine serum (FBS) was obtained from Gibco-BRL (Grand Island, NY). Phenol red–free Dulbecco's modified Eagle's medium (DMEM) and DMEM-Ham's F12 (50:50 mix) were obtained from Sigma (St. Louis, MO). The antisera and synthetic peptides specific for C/EBPα (SC 61), C/EBPβ (SC130), and gadd153/CHOP (SC793) were from Santa Cruz Biotechnology (Santa Cruz, CA). Western blotting protocols and random sequence hexanucleotide primers for DNA labeling (Megaprime kit) were from the Radiochemical Centre (Amersham, Buckinghamshire, UK). The polymerase chain reaction (PCR) purification kit (QUIAquick) was obtained from Quiagen (Santa Clarina, CA). Serum estrogen and progesterone radioimmunoassay kits were from Ciba Corning.

Animals

Procedures with experimental animals were authorized and followed the guidelines of the Ministery of Agriculture (France) (authorization 006614). Female Sprague-Dawley rats (125–150 g) were OVX and treated as described previously *(14,15)*. Two weeks after the operation, half of the OVX rats received one sc injection of estradiol benzoate (5 μg/animal) and oxyprogesterone caproate (5 μg/animal) every other day for 2 wk (OVX+E_2+Pg), while the other half of the OVX and cycling sham-operated rats (sham) received only the vehicle. Rats were killed by decapitation 1 d after the last injection. Femoral sc, parametrial, and perirenal fat pads were removed aseptically. Characteristics of the adipose tissues of these animals were previously described *(14,15)*. Serum steroid levels of the animals, at the time of death, were as follows: estrogens, 10 ± 5, undetectable, and 15.6 ± 2.3 pg/mL; and progesterone, 4.6 ± 0.3, 2.5 ± 0.05, and 3.2 ± 0.2 ng/mL in sham, OVX, and OVX+E_2+Pg rats, respectively (n = 10).

Cell Culture

Cell preparation and culture were performed as described in ref. *15.* Briefly, preadipocytes obtained from the stromavascular fraction of adipose tissue by collagenase digestion were plated at a density of 1 to 2×10^4 cells/cm^2 in 8% FBS-DMEM. After 12 h, cultures were washed and fed with 8% FBS-DMEM. Medium was changed every other day. At confluence (3 d postplating), cells were allowed to differentiate in DMEM-Ham's F12 containing 5 μg/mL of insulin, 10 μg/mL of transferrin, and 200 p*M* T3 (ITT medium) in the absence of serum as described in ref. *15*. Whatever the anatomic origin, at least 80% of the control cells in culture were fully differentiated at d 8–10 postconfluence as assessed by measurements of GPDH activity.

c-*myc* expression was examined in proliferating preadipocytes cultured for 2 d postplating in 8% FBS-DMEM and then serum starved for 18 h. Preliminary experiments showed that c-*myc* expression could not be dected in serum-starved cells, and induction of c-*myc* mRNA was examined 1 h after serum exposure. EDPs (3 d in ITT medium) were used to study LPL and C/EBPβ expressions while C/EBPα and -ζ expressions were investigated in LDPs (8 d in ITT medium).

Preparation of Cellular Extracts

Preadipocyte extracts were prepared as follows. Proliferating or differentiated preadipocytes were scraped and sonicated in cold buffer containing 50 m*M* Tris (pH 8.0), 120 m*M* NaCl, 1 m*M* EDTA, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 0.57 m*M* phenylmethylsulfonyl fluoride, 0.5 m*M* sodium deoxycholate, 1 m*M* orthovanadate, 30 m*M* β-glycerophosphate, 25 mg/mL of aprotinin, and 20 mg/mL of leupeptin. After centrifuging at 100,000*g* for 15 min at 4°C, the resulting supernatant was denaturated with Laemmli buffer (v/v) and stored at –20°C.

Western Blot Analysis

Equal amounts of protein (20–100 µg) and prestained molecular weight markers were subjected to SDS–polyacrylamide gel electrophoresis (12.5%–15% acrylamide). Proteins were transferred to polyvinylidene difluoride membranes. The filters were subsequently stained by Ponceau red to verify equal protein loading and transfer. After blocking by TBS containing 0.1% Tween-20 (TBS-T) and 2.5% gelatin for 2 h, filters were incubated overnight with the primary antibody diluted in TBS-T/2.5% gelatin (0.5 µg/mL). Membranes were washed and incubated with the secondary antiserum coupled to peroxidase (1:10,000 dilution in TBS-T) for 1 h and extensively washed with TBS-T. Filters were next incubated with the enhanced chemiluminescent (ECL) detection solution and then exposed to X-ray films. Reprobing of the membranes gave identical results. Specificity of the immunoreactive proteins was verified by loss of the sample immunoreactivity when incubated with the antiserum neutralized with the corresponding specific peptide. Signals were quantified by densitometry. Control experiments with various amounts of protein (10–100 µg) were conducted to ensure that the densitometric signal intensity was proportional to the loaded amount of protein.

Northern Blot Analysis

Total RNAs were extracted following the acid-guanidinium-isothiocyanate protocol. RNA samples (20–40 µg) were separated on denaturing gels containing 1% agarose and 12.5% formaldehyde. The electrophoresed RNAs were capillary transferred onto a Hybond N^+ membrane in 0.05 *N* NaOH and crosslinked to this membrane (1 h at 80°C). Prehybridizations were carried out for 3 h at 68°C in Church solution (0.5 *M* sodium phosphate, 1 m*M* EDTA, and 7% SDS). Hybridizations with [^{32}P]-cDNA probes labeled by random priming ($2–5 \times 10^8$ dpm/µg) were performed overnight at 68°C. The hybridized membranes were then washed twice in 2X saline sodium citrate (SSC), 0.1% SDS at room temperature for 15 min and once in 0.5X SSC, 0.1% SDS at 65°C for 5 min before being exposed to an X-ray film at –80°C. The band intensities were quantified by densitometry. Membranes were next stripped of probe by boiling 0.5% SDS and then hybridized with probe specific for the ribosomal acidic protein PO chosen as an internal control because its expression is sex steroid independent *(51)*.

To verify the integrity of the loaded RNA, parallel gels were run and stained with ethidium bromide to visualize 28S and 18S ribosomal RNAs.

The probes were specific for rat ribosomal acidic protein PO and c-*myc* as described in ref. *51*and *52*; respectively. The oligonucleotide primer pairs specific for rat LPL were as follows: sense: 5' GCGGATTTCGTAGATGTC 3'; antisense 5' TGCCTTGCTGGGGTTTTC 3'. These probes were prepared by reverse transcriptase–PCR methods using rat proliferating preadipocyte RNA (c-*myc*, ribosomal acidic protein PO) and rat adipocyte RNA (LPL). The PCR products were purified with a PCR purification kit.

Other Determinations

Protein concentrations were measured following the dye-binding procedure *(53)* using bovine serum albumin as standard. Serum estrogen and progesterone levels were determined after ether extraction as previously described *(15)*. All results are expressed as means ± SEM from at least three individual experiments. Comparisons between groups were made using analysis of variance with Bonferroni *p* values.

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