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Evidence for a regional-specific control of rat preadipocyte proliferation and differentiation by the androgenic status

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In the rat, castration induces a decreased weight of fat depots. One possible explanation for these alterations could be that the capacities of preadipocytes to proliferate and differentiate are reduced by castration. Considering the regional specification of adipose tissue metabolism, these capacities and their eventual modulation by the androgenic status were presently compared in cultured preadipocytes from rat subcutaneous (SC) and epididymal fat depots.

In epididymal preadipocytes, castration induced an increase in their proliferative capacity and conversely, a decrease in their adipogenesis. *In vivo* treatment by testosterone reversed the proliferative alteration but not the defective adipogenesis caused by castration. *In vitro*, no direct effect of testosterone on the proliferative capacities of epididymal preadipocytes could be observed suggesting that testosterone acts indirectly or needs the presence of other cofactors such as insulin, dexamethasone and growth hormone. Surprisingly, testosterone partly counteracted the inhibitory effect of growth hormone on preadipocyte differentiation.

In contrast to these observations, SC preadipocytes were completely insensitive to the androgenic status in terms of proliferation and differentiation.

This study showing site-specific effects of castration on preadipocyte proliferation and differentiation suggests that part of the decreased fatness induced by castration in the rat is related to the modulatory effect of androgenic status on adipogenesis in some deep fat depots.

Introduction

Various pathophysiological situations suggest that fat distribution and accumulation are under the control of sex steroid hormones. Android obesity, which is an important risk factor for cardiovascular diseases (Vague, 1956; Kissabah *et al.*, 1982), leads to excessive accumulation of adipose tissue in subcutaneous abdominal and visceral regions. In man, testosterone treatment reduces abdominal fatness (Rebuffé-Scrive *et al.*, 1991).

The molecular basis underlying the influence of sex hormones on the regional distribution of adiposity is still poorly understood. The presence of specific receptors for androgens in both mature and precursor fat cells (Miller *et al.*, 1990; De Pergola *et al.*, 1990) suggests a direct influence of these hormones on preadipocytes and mature fat cells. Indeed, testosterone was shown to up-regulate both androgen receptors (De Pergola *et al.*, 1990) and β-adrenoceptors (Xu *et al.*, 1990) in preadipocytes.

In vivo, adipose tissue growth is variable according to anatomical localization (Faust *et al.*, 1978) and this observation can be confirmed by *in vitro* studies on cultured precursor fat cells (Djian *et al.*, 1983; Lau *et al.*, 1987). For example, comparison between different deep-intraabdominal fat depots has revealed that perirenal fat depots contained more replicating and differentiating preadipocytes than epididymal fat depots (Djian *et al.*, 1983; Lau *et al.*, 1987). In contrast, fat cell precursors from the latter depot express more extensive maturation than those from subcutaneous depots (Grégoire *et al.*, 1990).

Primary culture of preadipocytes provides a convenient model to study the influence of both the anatomical origin (Faust *et al.*, 1978; Djian *et al.*, 1983) and the hormonal status on the ability of these cells to proliferate and differentiate (Kirkland *et al.*, 1992). By using this experimental approach, it has been shown that hypophysectomy reduces preadipocyte replicative capacity whatever the fat localization (perirenal and epididymal). In a recent study, we have observed that castration decreases the weight of both femoral subcutaneous (SC) and epididymal fat depots (Lacasa *et al.*, 1993). One possible explanation for these weight reductions could be that the capacities of preadipocytes to proliferate and differentiate are reduced by castration. The aim of the present study was to test this hypothesis by comparing the capacities of proliferation and differentiation of fat cell precursors isolated from femoral SC and epididymal fat depots removed from sham-operated, castrated and testosterone treated castrated rats.

Results

Cell growth

The growth rate of precursors from both femoral SC and epididymal fat depots were determined from day 1 to 3 after plating (Figure 1). Wells were initially seeded with 1×10^5 cells. The plating efficiency (10–15%) was identical in both experimental cell groups. The growth rate of femoral SC precursors was unaltered by androgenic status. In contrast, the growth rate of precursor cells from the epididymal fat

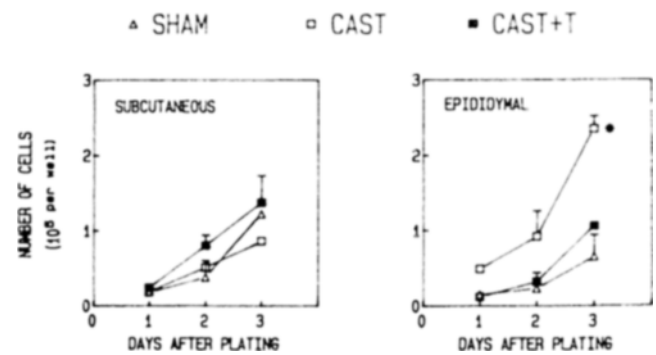

Figure 1 Influence of androgenic status on the growth curve of preadipocytes from femoral SC and epididymal fat deposits. Femoral SC and epididymal preadipocytes from SHAM (Δ), CAST (□) and CAST + T (■) rats were prepared and plated in 8% FBS-DMEM. At the indicated days, cells were collected and counted. Data from one of four experiments are presented. Each experiment was performed in duplicate. $^*P < 0.05$ comparison between preadipocytes from CAST and SHAM rats using analysis of variance (ANOVA)

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depots was increased by a factor of at least two by castration ($P < 0.05$), an effect which was prevented by testosterone treatment. Thus, the androgenic status seems to influence site-specifically the growth of preadipocytes.

However, these experiments do not establish whether such site-specific modulation of fat cell precursor growth is due to a direct or indirect effect of androgens on these cells. This prompted us to compare preadipocyte proliferation rates in the presence or absence of testosterone in the culture medium, by following [^{3}H]-thymidine incorporation by cells. As shown in Table I, *in vitro* addition of testosterone alone failed to alter cell proliferation from normal rats during the 24–48 h exposure. Since, in cultured hepatocytes at least, testosterone was reported to require GH in the culture medium to induce the expression of estrogen binding protein (Smirnova *et al.*, 1992), various experimental conditions were used testing testosterone in combination with GH (55 ng/ml), insulin (850 nM), dexamethasone (5 nM) or treatment of FBS by charcoal (to eliminate steroids). However, these experiments failed to reveal any significant effect of testosterone on preadipocyte proliferation. Furthermore, cell counting did not show any effect of long-term exposure to testosterone (up to 10 days) on preadipocyte proliferation (data not shown).

Cell differentiation

Next, we investigated the influence of androgenic status on the differentiation capacity of preadipocytes isolated from the two fat depots. For this purpose, preadipocytes were allowed to differentiate in ITT medium for 8–10 days. It can be seen from Figure 2 that preadipocytes exhibit various differentiation status depending on both the anatomical origin of the cells and the androgenic status of the rats. As already reported by another group (Grégoire *et al.*, 1990), the epididymal precursors differentiate more extensively than the SC ones. However, androgenic status did not influence the differentiation process in femoral SC preadipocytes. In contrast, epididymal preadipocytes from CAST rats failed to express significant levels of differentiation, since only few cells accumulated lipid droplets. Moreover, *in vivo* testosterone treatment did not reverse this alteration.

To provide a biochemical confirmation of these data, we measured GPDH activity, a late marker of differentiation, (Ailhaud *et al.*, 1992). Data presented in Table 2 confirmed that epididymal cells from CAST rats failed to correctly differentiate since GPDH activity was reduced by a factor of 10 in these cells, in comparison with cells from sham rats. In addition, testosterone administration was unable to correct the defective differentiation process caused by castration. To further validate these observations, LDH activities were also measured. Data in Table 2 show that these activities remained stable throughout the differentiation process (Deslex *et al.*, 1987) and were unaffected by the androgenic status. Moreover, even during longer culture periods (up to 18–20 days), epididymal cells from CAST rats did not differentiate. The presence of ITT medium immediately after plating did not restore to normal the GPDH activity of preadipocytes from CAST and CAST + T rats (data not shown).

Table I *In vitro* effect of testosterone on [^{3}H]-thymidine incorporation in preadipocytes from SC and epididymal fat deposits from control rats

	SC preadipocytes	*Epididymal preadipocytes*
Control	8416 ± 1495	12740 ± 320
Testosterone 1 nM	8998 ± 110	12801 ± 308
10 nM	7492 ± 838	13486 ± 18
100 nM	8860 ± 1009	13312 ± 82

Femoral SC and epididymal preadipocytes from control rats were cultured as indicated in Materials and methods. Testosterone and [^{3}H]-thymidine were added in the culture medium at day 1. Cells were allowed to incorporate [^{3}H]-thymidine during 24 h. Data are means ± SEM of four experiments performed in triplicate and are expressed as c.p.m. per well

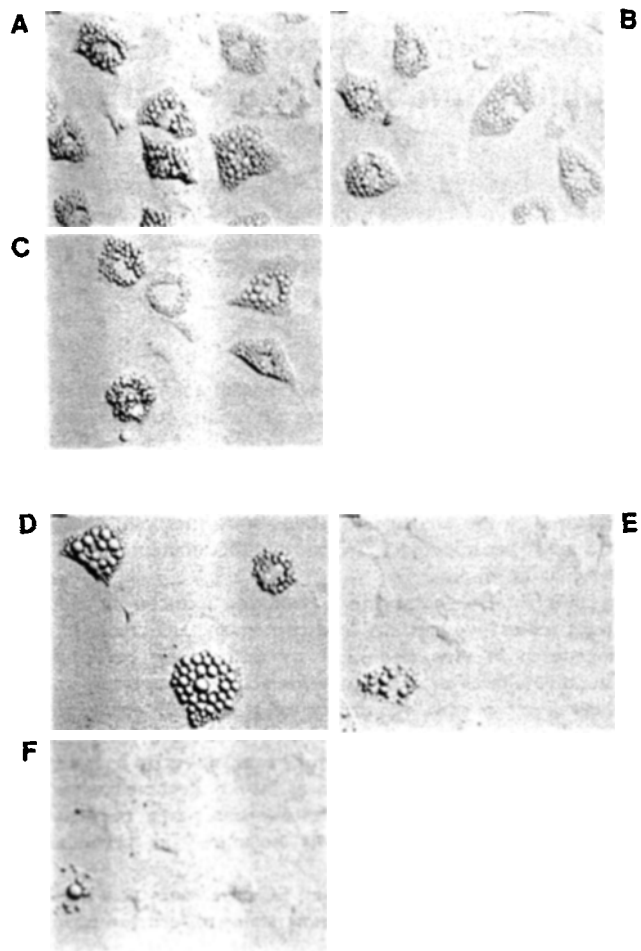

Figure 2 Influence of androgenic status on the differentiation capacity of preadipocytes from femoral SC and epididymal fat deposits. Femoral SC (A, B and C) and epididymal (D, E and F) preadipocytes from SHAM (A and D), CAST (B and E) and CAST + T (C and F) rats were cultured as indicated in Materials and methods. After 8 days in ITT medium, cells were photographed. Magnification × 200

Finally, to see if testosterone could directly influence preadipocyte differentiation, epididymal cells from sham rats were cultured in the presence of testosterone alone or in combination with dexamethasone or growth hormone. As shown in Figure 3, the GPDH activity was not modified by testosterone with or without dexamethasone. In contrast, testosterone partly counteracted the inhibitory effect of growth hormone on differentiation, an effect already reported in (Wabitsh *et al.*, 1994).

Discussion

In rats, we have previously reported that castration induced a decreased tissue weight in both femoral SC and epididymal fat depots (Lacasa *et al.*, 1993). One possible explanation for these reduced adiposities could be that castration restrains the capacities of precursor fat cells to proliferate and/or to differentiate.

In the present work, we show indeed that castration affects dramatically and oppositely these capacities (increased proliferation, almost complete abolition of differentiation) in preadipocytes from epididymal but not from SC fat depots.

Table II Influence of androgenic status on the GPDH and LDH activities in differentiated preadipocytes from femoral SC and epididymal fat deposits

	GPDH *(mU/10⁵ cells)*	*LDH* *(mU/10⁵ cells)*
Femoral SC		
SHAM	34 ± 5	158 ± 15
CAST	21 ± 3	170 ± 32
CAST +	39 ± 12	199 ± 30
Epididymal		
SHAM	47 ± 11	210 ± 30
CAST	3.6 ± 1.4*	171 ± 15
CAST + T	5.7 ± 1.9*	200 ± 23

Femoral SC and epididymal preadipocytes from SHAM, CAST and CAST + T rats were prepared and allowed to differentiate in ITT medium. After 10 days, cells were collected and the enzymatic activities were measured in cytosolic fractions. Data are means ± SEM of 6–8 separate experiments performed in duplicate. Comparisons were made using the one way ANOVA. $*P<0.01$ comparisons between CAST or CAST + T. *vs* SHAM

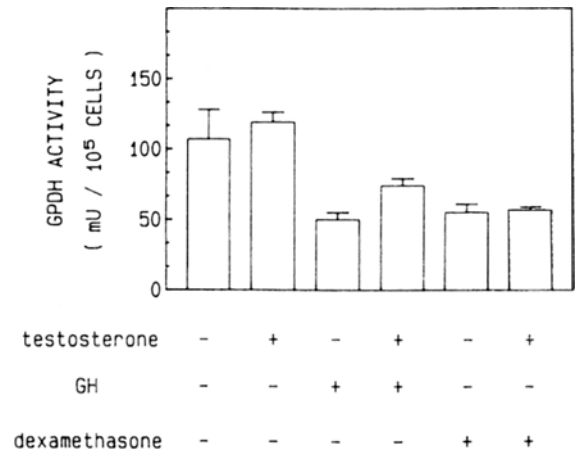

Figure 3 *In vitro* effect of testosterone on GPDH activity of epididymal preadipocytes from control rats. Epididymal preadipocytes from control rats were cultured as indicated in Materials and methods. Testosterone (10 nM) was added in the culture medium at day 1 after plating. Dexamethasone (10 nM) or growth hormone (55 ng ml) were present in ITT medium. After 8 days in this medium, cells were collected and GPDH activity was measured. Data from one of three experiments are presented. Each experiment was performed in triplicate

The insensitivity to castration of the proliferation and differentiation capacities of SC preadipocytes could be related in part to their low androgen receptor number in comparison with epididymal preadipocytes (Jaubert *et al.*, 1993).

These site-specific effects of castration being observed in fat cell precursors cultured during 2–3 weeks, seem to be most probably linked to modifications of intrinsic properties of the cells.

Proliferation and differentiation processes of adipose precursor cells are regulated by positive and negative factors. Among the positive factors are glucocorticoids, insulin and IGF-1 and among the negative factors are growth factors such as TGF-β, bFGF, EGF and TNFα (Smyth *et al.*, 1993; Butterwith, 1994). Epididymal adipose precursor cells from castrated rats could thus exhibit reduced sensitivity to positive factors and/or higher sensitivity to any of the negative factors. Alternatively, castration may restrain adipose tissue development by altering the production of some of these positive and negative factors including local factors known to influence preadipocyte proliferation and differentiation such as prostaglandins and endothelin-1 (Vassaux *et al.*, 1992; Shinoara *et al.*, 1992).

On the other hand, castration could also alter the receptors and/or the associated transducing pathways of some of these positive and negative factors in preadipocytes from a deep-intraabdominal fat depot such as the epididymal adipose tissue.

Although epididymal preadipocytes from castrated rats have increased ability to proliferate, they do not differentiate. This is not surprising considering that in different cell types, the proliferation and differentiation pathways are often exclusive. Overexpression of genes promoting cell proliferation could favor this pathway over differentiation. One candidate gene could be c-*myc*. As a matter of fact, overexpression of this gene was shown to prevent adipogenesis by promoting proliferation and inhibiting expression of the adipocyte differentiation promoter C/EBPα (Freytag & Geddes, 1993). In addition, TNF which induces c-*myc* expression, inhibits adipocyte differentiation, as well (Ninomiyia-Tsuji *et al.*, 1993). Overexpression of c-*myc* and consequently underexpression of C/EBPα could thus be suggested to occur in epididymal cells from castrated rats. Experiments are in progress to test this hypothesis.

As shown in the present study, testosterone treatment was able to reverse the effects of castration on the proliferation but not on the differentiation capacities of preadipocytes. The reasons for these discrepant observations are unclear. It must be noted, however, that testosterone treatment was recently reported to prevent the changes caused by castration on the adenylate cyclase system of mature adipocytes (Xu *et al.*, 1993; Dieudonné *et al.*, 1993) but not again of preadipocytes (Lacasa *et al.*, 1994).

It has been proposed that preadipocytes could be target cells for androgens (De Pergola *et al.*, 1990; Xu *et al.*, 1990). Supporting this view is the finding that within the same fat depot, the androgen receptor number in preadipocytes is about two times greater than in mature adipocytes whatever the anatomical localization of adipose tissue (Jaubert *et al.*, 1993). Furthermore, this parameter was shown to decrease during differentiation of preadipocytes from subcutaneous and epididymal fat depots (Dieudonné *et al.*, 1995).

In the present study and under various conditions, we failed to observe any direct effect of testosterone '*per se*' on the proliferation and differentiation processes in preadipocytes. It cannot be excluded that, to elicit direct biological effects, testosterone needs the presence of other cofactors such as insulin and dexamethasone in the culture medium and/ or more prolonged exposure. In fibroblasts (Levine *et al.*, 1993) and bone cells (Kaspeck *et al.*, 1990), androgens have been shown to influence proliferation and differentiation primarily by modulating the production of growth factors such as FGF and TGF-β, the actions of which may require longer culture periods.

In conclusion, this study demonstrates for the first time that preadipocyte proliferation and differentiation are site-specifically controlled by the androgenic status in the rat. The data presented suggest that the loss of differentiation capacity characterizing epididymal preadipocytes after castration may contribute, at least in part, to explaining how castration reduces the fat mass in rats. In some way, androgenic status leads to alterations of transcriptional activation of specific genes implied in preadipocyte proliferation and differentiation processes. Studies on the expression of genes known to regulate these processes in adipose precursor cells are currently under investigation.

Materials and methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), DMEM-Ham's F12 (50:50 mix) and fetal bovine serum (FBS) were

obtained from Gibco-BRL (Grand Island, NY, USA). [^{3}H]-thymidine, specific activity 28 Ci/mmol, was purchased from the Radiochemical Centre (Amersham, Bucks, UK), collagenase from Boehringer (Mannheim, FRG) and NADH, H^+, dihydroxyacetone phosphate, growth hormone (porcine pituitaries), dexamethasone, triiodothyronine, transferrin, trypsin, testosterone and porcine insulin were from Sigma Chemical Co. (St. Louis, MO, USA). Testosterone RIA tests were provided by Biomérieux (Marcy l'étoile, France).

Animals

Male Sprague-Dawley rats (125–150 *g*) were castrated as described (Lacasa *et al.*, 1993) and treated as follows. Five days after the operation, half of the castrated rats received one SC injection of testosterone propionate (0.5 mg/100 *g* body weight) every other day for 10 days (CAST + T) while the other half (CAST) and the sham-operated rats (SHAM) received the vehicle only. One day after the last injection, rats were killed by decapitation. Serum testosterone levels were on the day of sacrifice: 1.5 ± 0.2, 0 and 13.5 ± 3.7 nmol/l in SHAM, CAST and CAST + T rats, respectively. Femoral SC and epididymal fat samples were removed aseptically. Characteristics of the adipose tissues of these animals were previously described (Lacasa *et al.*, 1993).

Cell culture

Cell preparation and culture were performed as described (Deslex *et al.*, 1987). Briefly, preadipocytes were obtained by collagenase digestion followed by a centrifugation at 600 *g* for 5 min. After two washings, cells were plated into cell culture dishes precoated with polylysine at a density of $1-2 \times 10^4$ cells/cm^2 in 2 ml 8% FBS-DMEM. Cultures were washed twice with DMEM after 12 h and fed with 8% FBS-DMEM. Medium was changed every other day. At confluence (2–3 days post plating), cells were allowed to differentiate in DMEM-Ham's F12 containing 5 μg/ml insulin, 10 μg/ml transferrin, 2.5 μg/ml sodium ascorbate and 200 pM T3 (ITT) (Deslex *et al.*, 1987). Whatever the anatomical origin, 80% of the control cells in culture were fully differentiated at day 8–10 post confluence. When added to the medium, testosterone was dissolved in ethanol (final ethanol concentration never exceeding 0.01%).

Cell counting

Cell number was determined at day 1, 2 and 3 post plating. Cell cultures were washed three times with saline, then trypsinized with calcium and magnesium free Hank's solution containing 0.2% trysin and finally counted in a hemocytometer. Cell viability was assessed by trypan blue exclusion. Whatever their anatomical origin and the animal treatment, 95% of the cells excluded trypan blue.

[^{3}H]-thymidine incorporation

At day 1 post plating, cells were incubated with 1 μCi/ml [^{3}H]-thymidine and the different effectors for 24–48 h. Incubations were stopped by aspiration of the medium and extensive washings with saline. Cells were treated with SDS 1% and then with TCA 10% for 1 h at 4°C. After filtration on GF/C glass fiber filters, radioactivity was measured. Results were expressed as counts per min of [^{3}H]-thymidine incorporated per well. This incorporation was linear up to at 48 h.

Glycerol 3-phosphate dehydrogenase (GPDH) assay

Cell differentiation was assessed by following GPDH activity. After 10–12 days, ITT media were removed and cells were scrapped in cold buffer containing 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA and 1 mM dithiotreitol. Cells were sonicated in the same buffer and centrifuged at 100 000 *g* for 20 min at 4°C. The GPDH activity was measured in the supernatant according to Wise & Green (1979) and expressed in mU (nmol NAD/min) per mg protein or per 10^5 cells.

Other determinations

Protein concentration was measured according to Bradford (1976) with BSA as standard. Lactate dehydrogenase activity was measured as previously described (Lacasa *et al.*, 1988). All results are expressed as means ± SEM from at least three individual experiments. Comparison between groups were made using analysis of variance (ANOVA) with Bonferroni *P* values.

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References

Ailhaud, G., Grimaldi, P. & Negrel, R. (1992). *Ann. Rev. Nutr.*, **12**, 207–233.
Bradford, M.M. (1976). *Anal. Biochem.*, **72**, 248–254.
Butterwith, S.C. (1994). *Pharmacol. Therap.*, **61**, 399–411.
De Pergola, G., Xu, X., Yang, S., Giorgino, R. & Bjorntorp, P. (1990). *J. Steroid Biochem. Mol. Biol.*, **37**, 553–558.
Deslex, S., Negrel, R. & Ailhaud, G. (1987). *Exp. Cell Res.*, **168**, 15–30.
Dieudonne, M.N., Pecquery, R., Dausse, J.P. & Giudicelli Y. (1993). *Biochim. Biophys. Acta.*, **1176**, 123–127.
Dieudonne, M.N., Pecquery, R., Leneveu, M.C., Jaubert, A.M. & Giudicelli, Y. (1995). *Endocrine*, **3**, 537–541.
Djian, P., Roncari, D.A.K. & Hollenberg, C.H. (1983). *J. Clin. Invest.*, **72**, 1200–1208.
Faust, I.M., Johnson P.R., Stern, J.S. & Hirsh, J. (1978). *Am. J. Physiol.*, **235**, E279–E286.
Freytag, S.O. & Geddes, T.J. (1992). *Science*, **256**, 379–382.
Gregoire, F., Todoroff, G., Hauser, N. & Remacle, C. (1990). *Biol. Cell.*, **69**, 215–222.
Jaubert, A.M., Pecquery, R., Sichez, I., Dieudonne, M.N., Cloix, J.F. & Giudicelli, Y. (1993). *Endocrine*, **1**, 535–540.
Kaspeck, C., Fitzsimmons, R., Strong, D., Mohan, S., Jenning, S.J. & Bayline, D. (1990). *J. Clin. Endocrinol. Metab.*, **71**, 1322–1329.
Kirkland, J.L., Hollenberg, C.H., Gillon, W. & Kindler, S. (1992). *Endocrinology*, **131**, 2769–2773.
Kissabah, A.H., Vydelingum, N., Murray, R., Evans, D.J., Hartz, A.J., Kalkoff, R.H. & Adams, P.W. (1982). *J. Clin. Endocrinol. Metab.*, **54**, 254–260.
Lacasa, D., Agli, B. & Giudicelli, Y. (1988). *Biochem. Biophys. Res. Commun.*, **153**, 489–497.
Lacasa, D., Agli, B., Mur, M., Dieudonne, M.N. & Giudicelli, Y. (1993). *J. Endocrinol.*, **138**, 493–501.
Lacasa, D., Denis, D., Agli, B., De Mazancourt, P. & Giudicelli, Y. (1994). *Biochim. Biophys. Acta.*, **1224**, 527–532.
Lau, D.C.W., Wang, K.L. & Tough, S.C., (1987). *Clin. Exp. Metab.*, **36**, 631–636.
Levine, A.C., Ren, M., Huber, G.K. & Kirshenbaum, A. (1993). *Endocrinology*, **130**, 2413–2419.
Miller, L.K., Kral, J.G., Strain, G.W. & Zumoff, B. (1990). *Steroids*, **55**, 410–415.
Ninomiyia-Tsuji, J., Morti, F.M. & Ringold, G.M. (1993). *Proc. Natl. Acad Sci. USA.* **90**, 9611–9615.

Rebuffe-Scrive, M., Marin, P. & Bjorntorp P. (1991). *Int. J. Obesity*, **15,** 791–795.
Shinoara, O., Murata, Y. & Shimizu, M. (1992). *Endocrinology*, **130,** 2031–2036.
Smirnova, O.V., Vishyakova, T.G., Bocharov, A.V., Kovtun, I.V. & Rozen, V.B. (1992). *J. Steroid. Biochem. Mol. Biol.* **42,** 243–249.
Smyth, M.J., Sparks, R. & Wharton, W. (1993). *J. Cell Science*, **106,** 1–9.
Vague, J. (1956). *Am. J. Clin. Nutr.*, **4,** 20–34.
Vassaux, G., Gaillard, D., Ailhaud, G. & Negrel, R. (1992). *J. Biol. Chem.*, **267,** 11092–11097.
Wabitsch, M., Ilondo, M.M., Heinze, E., Shymko, R.M., Teller, W.M. & De Meyts, P. (1994). *J. Cell. Biochem.*, **18A,** 173.
Wise, S. & Green, H. (1979). *J. Biol. Chem.*, **254,** 273–275.
Xu, X., De Pergola, G. & Bjorntorp P. (1990). *Endocrinology*, **126,** 1229–1234.
Xu, X., De Pergola, G., Eriksson, P.S., Fu, L., Carlsson, B., Yang, S., Eden, S. & Bjorntorp, P. (1993). *Endocrinology*, **132,** 1651–1657.