REPRODUCTION
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Leptin and leptin receptor in pig spermatozoa: evidence of their involvement in sperm capacitation and survival

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Abstract

Several studies have recently investigated the role of leptin, the adipocyte-secreted hormone, in the growth and reproduction of rodents, humans, and domestic animals. The present study was designed to explore the expression of leptin and its receptor in pig spermatozoa. Successful Western blot evidenced a 16 kDa band for leptin and six isoforms, ranging from 120 to 40 kDa, for the leptin receptor. Both leptin and leptin receptor were interestingly located at sperm acrosomal level, suggesting their involvement in the oocyte fertilization events. In fact, both capacitation indexes and acrosin activity were enhanced by leptin, and these effects were reduced by the anti-leptin receptor antibody. Afterwards, we investigated the main transduction pathways regulated by the hormone. Our results showed that, in pig sperm, leptin can trigger the signal transducer and activator of transcription 3, a classical component of cytokine signal transduction pathways, whose expression has not been previously reported in male gamete; in addition it was found constitutively activated. Besides, leptin was able to induce the activation of phosphatidylinositol phosphate kinase 3 and MAP kinase pathways as well as of BCL2, a known antiapoptotic protein. These data address to a role of leptin and its receptor on pig sperm survival. The presence of leptin and its receptor in pig sperm suggests that they, through an autocrine short loop, may induce signal transduction and molecular changes associated with sperm capacitation and survival.

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Introduction

Leptin (LEP) is known to be the hormonal link between energy stores and several vital functions, including food intake and energy homeostasis. It is a 16 kDa adipocyte-derived hormone that suppresses food intake, stimulates energy expenditure, increases metabolic rate, and ultimately causes loss of body fat (Kamohara *et al*. 1997, Rossetti *et al*. 1997, Bouloumie *et al*. 1998). The effects of LEP as a central satiety agent have been focused on, and its involvement in the regulation of fatness via feed intake was reported. Although LEP was originally thought to act largely via the central nervous system (CNS), recent studies have demonstrated that LEP exerts a wide repertoire of peripheral effects, through direct actions on target tissues. These effects include the stimulation of fatty acid oxidation in adipocytes (Muller *et al*. 1997, Bouloumie *et al*. 1998, Lord *et al*. 1998, Sierra-Honigmann *et al*. 1998, Zhao *et al*. 1998) as well as the increase of glucose uptake in skeletal muscle and brown adipose tissue (Kamohara *et al*. 1997, Yaspelkis *et al*. 1999).

The pig is emerging rapidly as an important biomedical research model, and whereas genetic influences may impact the degree of similarity between pig and human systems, the regulation of some endocrine and metabolic processes in the pig may be more similar to humans than to rodents (Tsiaoussis *et al*. 2001, Vilei *et al*. 2001). LEP plays a key role in the regulation of energy homeostasis and is also linked to the mechanisms controlling reproductive processes. However, very limited data have reported the involvement of LEP in controlling pig reproductive functions at the level of hypothalamus and pituitary. The relationship between nutrition and reproduction in swine has been extensively reviewed (Quesnel & Prunier 1995, Zurek *et al*. 1995). Nutrition may influence the reproductive performance by a number of mechanisms, including central effects on gonadotropin secretion (Booth *et al*. 1994) and local effects on ovarian function (Cosgrove *et al*. 1992, Booth *et al*. 1996). According to Zak *et al*. (1997), the different feeding regimens caused differential sow body weight changes. In pig, nutritional signals, such as LEP, are detected by the CNS and translated, by the neuroendocrine system, into signals that regulate appetite, gonadotropin-releasing hormone release,

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and subsequent luteinizing hormone (LH) secretion. Changes in body weight or nutritional status are characterized by altered adipocyte function, by a reduction of leptin expression in adipose tissue and a decrease in LH secretion.

In both male and female mice it has been shown that leptin has direct effects on fertility; in fact the hormone is able to reverse the infertility of *ob/ob* mice lacking the leptin gene (Cunningham *et al*. 1999). The biological actions of LEP on body weight homeostasis, neuroendocrine function, and fertility are carried out through interactions with its specific obese receptor (OBR, now known as leptin receptor, LEPR) in target tissues. The pattern of leptin receptor expression in the testis is species specific. There are several isoforms of OBR, which are different mainly in the cytoplasmic domain length (Lee *et al*. 1996, Takaya *et al*. 1996, Lollmann *et al*. 1997, Murakami *et al*. 1997, Bjorbaek *et al*. 1998, Dieterich & Lehnert 1998, Yamashita *et al*. 1998). The OBR, a single membrane-spanning glycoprotein, belongs to the class I cytokine receptor superfamily and shares sequence homologies for interaction with Janus kinase (JAK) as well as STATs (Tartaglia 1997). To date, the human OBR is identified as a full-length OBRb form (Tartaglia *et al*. 1995) and also as several short forms generated by alternative splicing, OBRa, OBRc, OBRd, OBRf, and OBRe (Bennett *et al*. 1996, Cioffi *et al*. 1996, Tartaglia 1997). The hypothalamus is considered the only tissue expressing predominantly the full-length OBR. However, the long form and several short isoforms are expressed in an increasing number of peripheral tissues (Cioffi *et al*. 1996, Tartaglia 1997, Glasow *et al*. 1998, Breidert *et al*. 1999), including liver, heart, kidneys, lungs, small intestine, pituitary cells, testes, ovaries, spleen, pancreas, adrenal gland, and adipose tissue (Margetic *et al*. 2002). Furthermore, studies on different OBR splice variants indicated that many tissues may contain a heterologous mix of OBR subtypes. *OBR* mRNA (long form) has been detected in a variety of porcine tissues, including adipose tissue (Lin *et al*. 2000). Using *in situ* hybridization techniques, other groups have shown that leptin receptor mRNA is expressed in Sertoli cells of adult rats (Tena-Sempere *et al*. 2001).

Rodent testis revealed the OBR expression but with a differential cellular site; in fact, the OBR immunoreactivity was confined in Leydig cells of rat but in germ cells of mouse (El-Hefnawy *et al*. 2000, Caprio *et al*. 2003). This discrepancy may be due to interspecies difference. LEP and OBR were found in human seminiferous tubules (Soyupek *et al*. 2005), human seminal plasma (Jope *et al*. 2003), human sperm (Aquila *et al*. 2005) and, recently, OBR was also identified in boar sperm (De Ambrogi *et al*. 2007). In the present study, we investigated LEP and OBR expression in pig sperm, evaluating their potential role on sperm capacitation and survival. Besides, we investigated the main pathways involved in LEP/OBR signaling in pig sperm.

Results

LEP expression in pig sperm

To determine whether mRNA for LEP is present in pig spermatozoa, RNA isolated from Percoll-purified sperm was subjected to reverse PCR. The primer sequences were based on the pig gene sequence and the RT-PCR amplification revealed the expected PCR product size of 280 bp (Fig. 1A). This product was sequenced and found to be identical to the classical pig gene sequence.

The presence of LEP protein in pig spermatozoa was also investigated by Western blotting (WB) using an antibody raised against the carboxyl terminus of the protein. One immunoreactive band was observed at 16 kDa in the lysates from pig sperm samples at the same mobility of the adipocyte extract used as positive control (Fig. 1B).

OBR expression in pig sperm

To evaluate mRNA for OBR in pig spermatozoa, RNA isolated from Percoll-purified sperm was subjected to reverse PCR. The primer sequences were based on the *OBR* pig gene sequence of the transmembrane region and the RT-PCR amplification revealed the expected PCR product size of 460 bp (Fig. 2A). This product was sequenced and found to be identical to the pig gene sequence considered.

WB of pig sperm extracts showed six different immunoreactive *OBR* isoforms (120, 90, 80, 65, 60, and 40 kDa) (Fig. 2B), such as the WB of pig epididymal tissue used as positive control (Fig. 2B). The bands were not detected by non-immune rabbit serum indicating that these proteins are specific for OBR (Fig. 2B1).

Immunolocalization of LEP and OBR in pig sperm

An intense red fluorescence localized leptin in the acrosome of pig sperm while the other cellular regions were unlabeled (Fig. 3A). In addition, a brilliant green

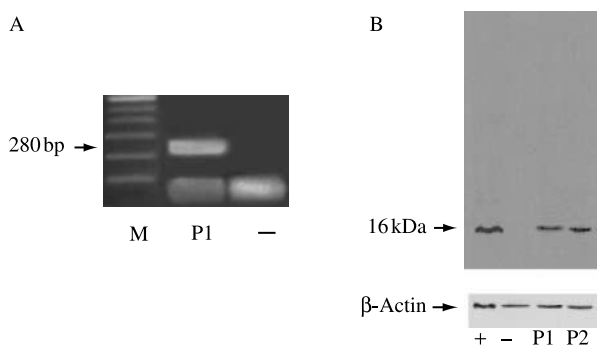

Figure 1 Leptin expression in pig sperm. (A) RT-PCR result in one representative pig sperm sample (lane P1), negative control (lane−), and markers (lane M). (B) Immunoblot of leptin from representative pig sperm samples (lanes P1 and P2), adipocyte cells used as positive (lane+), negative (lane−) controls. β-Actin served as loading control.

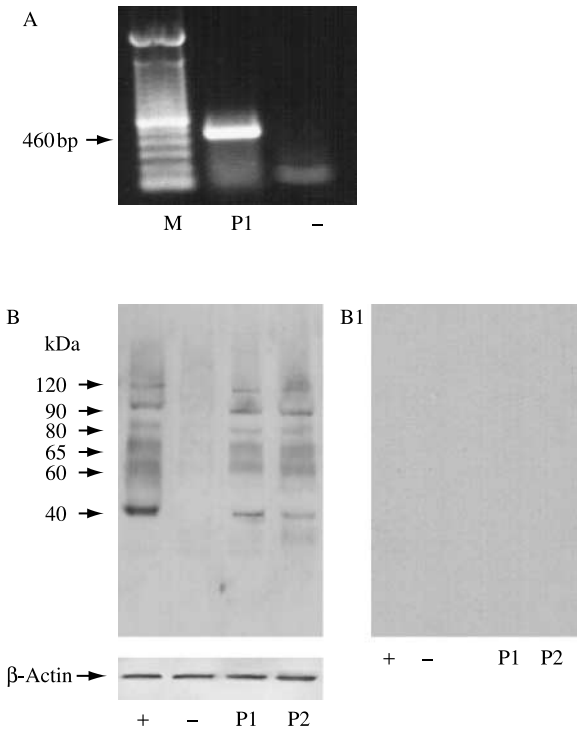

Figure 2 OBR expression in pig sperm. (A) RT-PCR result in one representative pig sperm sample (lane P1), negative control (lane−), and markers (lane M). (B) Immunoblot of OBR from two representative pig sperm samples (lanes−P1, P2), pig epididymis extract (lane+), and negative control (lane−). (B1) Immunoblot of the negative control (membrane incubated with normal rabbit serum). β-Actin served as loading control.

light revealed that OBR was confined in the apical portion of sperm acrosome (Fig. 3B). No fluorescent signal was obtained when primary Abs (anti-LEP Ab or anti-OBR Ab) were omitted (Fig. 3 inserts) thus confirming the specificity of the Ab binding.

LEP influences pig sperm capacitation enhancing both cholesterol efflux and protein tyrosine phosphorylation

One of the early events associated with the capacitation of mammalian spermatozoa is the cholesterol efflux, followed by protein phosphorylation. Our results showed a significant increase in cholesterol efflux upon

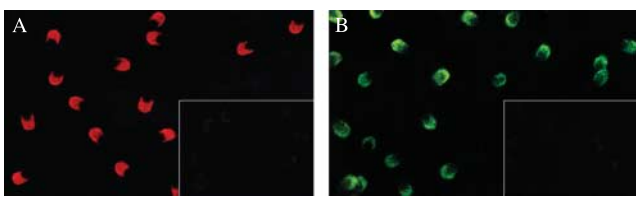

Figure 3 Representative immunofluorescence labeling of leptin and leptin receptor (OBR) in pig spermatozoa. (A) A red intense fluorescence localized leptin in the sperm acrosomal region. (B) A green brilliant light showed OBR in the apical acrosome. Inserts: immunonegative controls. Scale bars: 5 μm.

1 and 10 nM LEP (Fig. 4A). Protein tyrosine phosphorylation pattern of pig sperm was also induced by 1 and 10 nM LEP treatment (Fig. 4B), but 60 nM LEP did not produce any effect. Further, anti-OBR Ab was able to abolish 10 nM LEP effect.

LEP stimulates pig sperm acrosin activity

Acrosin is a sperm representative acrosomal enzyme. Acrosin activity showed that both 1 and 10 nM LEP were able to stimulate the enzymatic activity, while no effect was observed with 60 nM LEP (Fig. 5). Anti-OBR Ab combined with 10 nM LEP was able to abolish LEP effect (Fig. 5).

STAT3 is activated by LEP/OBR in pig sperm

STAT3 is a critical mediator of LEP action. Our results showed the expression of STAT3 (Fig. 6A) in pig sperm

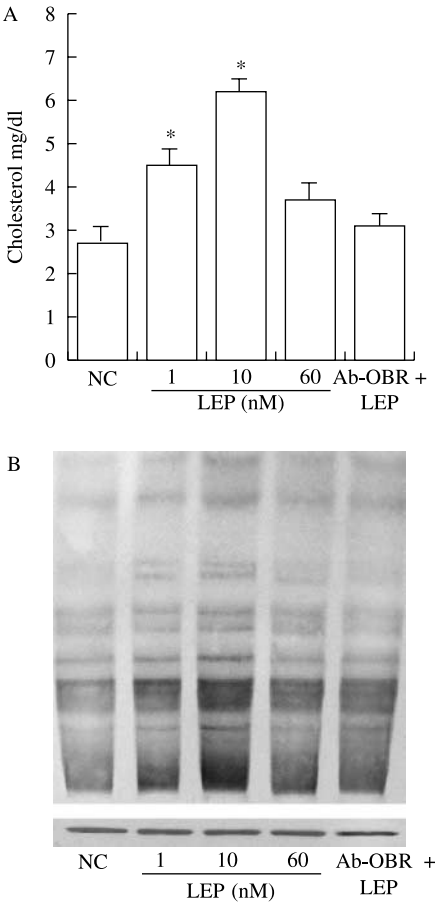

Figure 4 Leptin affects cholesterol efflux and protein tyrosine phosphorylation of pig sperm. Spermatozoa were incubated in the absence (NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin. (A) Cholesterol in culture medium from pig sperm. Results are presented as mean±S.E.M. and are given per 10×10^6 spermatozoa. *$P<0.05$ versus control. (B) Western blot analysis of protein tyrosine phosphorylation from sperm lysates.

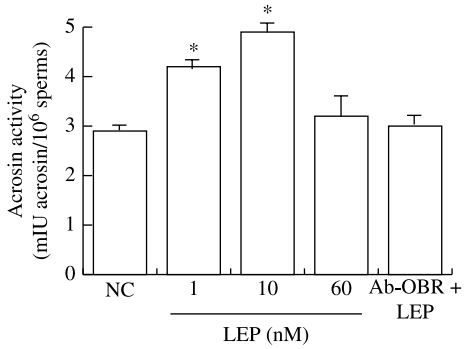

Figure 5 Leptin affects acrosin activity of pig sperm. Spermatozoa were incubated in the absence (NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody + 10 nM leptin. Values are mean ± S.E.M. *$P<0.05$ versus control.

and that it is constitutively activated (phosphorylated) (Fig. 6B). Furthermore, 1 and 10 nM LEP treatments induced a significant increase in STAT3 activation that was reversed by the anti-OBR Ab (Fig. 6B and C).

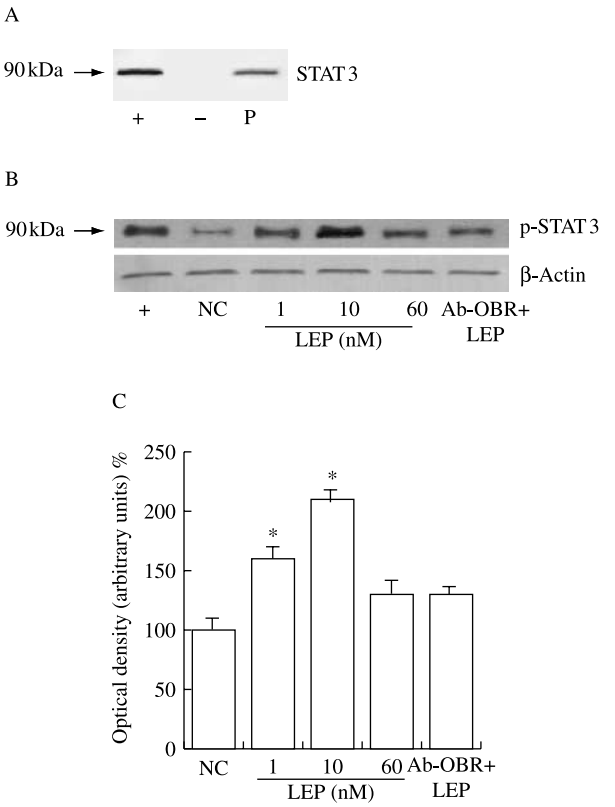

Figure 6 Western blot analysis of STAT3 from one representative pig sperm lysate. (A) Immunoblot of STAT3 in the pig sample (lane P), MCF7 used as positive (lane +) and negative (lane −) controls. (B) p-STAT3 band in sperm incubated in the absence (lane NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin (Ab OBR + LEP). MCF7 cell lines were used as positive controls (lane +). β-Actin served as loading control. (C) Band intensities were evaluated in term of arbitrary densitometric units. Values are as mean ± S.E.M. *$P<0.05$ versus control.

ERK1/2 is activated by LEP/OBR in pig sperm

Components of the ERK family of MAPK are involved in sperm motility and capacitation. Our results showed the expression of ERK1/2 in pig sperm (Fig. 7A) and evidenced that LEP, through its receptor, is able to activate ERK1/2 (Fig. 7B and C). The maximal effect was observed with 1 and 10 nM LEP, while 60 nM LEP did not result in further activation (Fig. 7B and C).

LEP/OBR signaling modulates pig sperm survival

Our data revealed that pig sperm express BCL2, a key protein in sperm survival signaling (Fig. 8A). Furthermore, leptin induced the Serine 70 phosphorylation of BCL2, necessary for its full antiapoptotic function, and this effect was inhibited by the anti-OBR Ab (Fig. 8B and C).

PI3-K plays also an important role in sperm survival, and its main downstream effector is the Akt. Our results showed the expression of Akt (Fig. 8D) and phosphorylated Akt (S473 and T308 phosphorylations) in pig sperm (Fig. 8E). In addition, LEP induced an increase in the kinase phosphorylation, which was reduced by using anti-OBR Ab (Fig. 8E and F). The specific PI3-K inhibitor, 10 μM

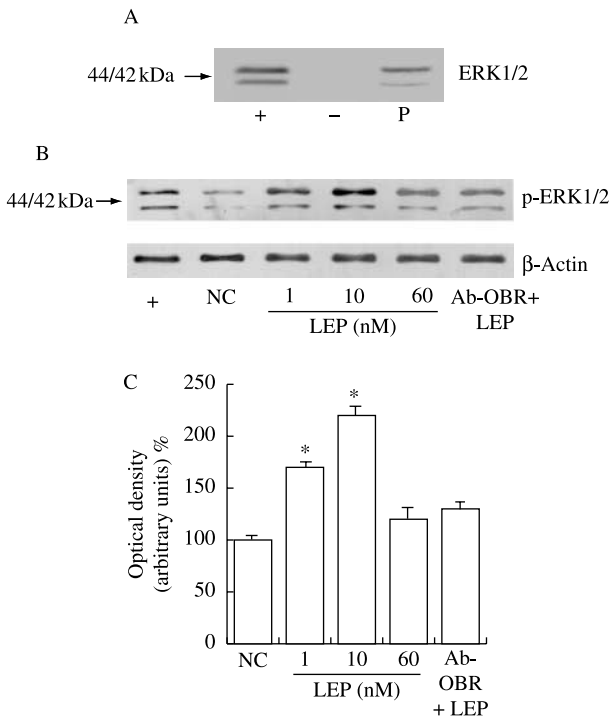

Figure 7 Western blot analysis of ERK1/2 from one representative pig sperm lysate. (A) Immunoblot of ERK1/2 in the pig sample (lane P), MCF7 used as positive (lane +) and negative (lane −) controls. (B) p-ERK1/2 band in spermatozoa incubated in the absence (lane NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin (Ab OBR + LEP). MCF7 cell lines were used as positive controls (lane +). β-Actin served as loading control. (C) Band intensities were evaluated in term of arbitrary densitometric units. Values are as mean ± S.E.M. *$P<0.05$ versus control.

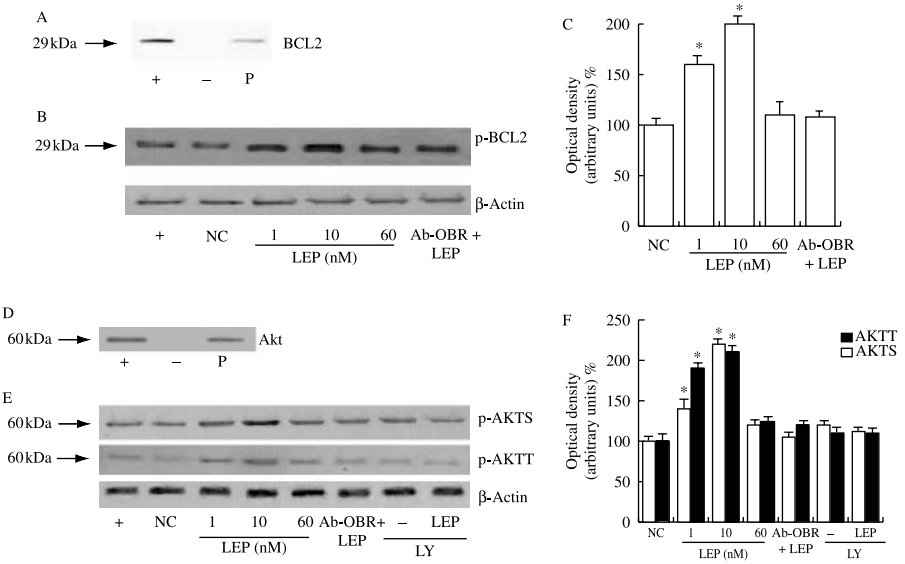

Figure 8 Western blot analysis of BCL2 and Akt from one representative pig sperm lysate. (A and D) Immunoblots of BCL2 and Akt, in the pig sample (lane P), MCF7 used as positive (lane+) and negative (lane−) controls. (B) p-BCL2 band when sperm cells were incubated in the absence (lane NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin (Ab OBR+ LEP). (E) p-AKTS (Akt ser) and p-AKTT (Akt thr) bands in sperm incubated in the absence (lane NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin (Ab OBR+LEP) or LY294002 (LY). (C and F) Band intensities were evaluated in terms of arbitrary densitometric units. Values are as mean±S.E.M. $*P<0.05$ versus control. β-Actin served as loading control.

LY294002, abolished LEP-induced Akt phosphorylations (Fig. 8E and F), confirming the PI3K/Akt activation. Also in the PI3K/Akt pathway, the 60 nM LEP did not induce a significant effect (Fig. 8E and F).

Discussion

In recent years, it has been suggested that LEP is a metabolic signal to the reproductive system (Barash *et al.* 1996). To date, the mechanisms by which LEP regulates reproductive function remain to be determined. LEP and OBR were recently found in human sperm (Jope *et al.* 2003, Aquila *et al.* 2005) and the OBR in boar sperm (De Ambrogi *et al.* 2007). However, LEP and its signaling in the male gamete function are still unknown in non-human species. In the present study, we have investigated the presence of LEP and OBR in pig sperm evaluating, for the first time, their potential action on capacitation, and sperm survival. The main aim of our study was also to identify the potential signaling molecules that are set in motion by LEP in pig sperm.

First, we have demonstrated the presence of LEP and OBR in pig sperm at different levels: mRNA expression, protein expression, and immunolocalization. Pig sperm LEP, as protein, was evidenced at the same size as human sperm leptin (Aquila *et al.* 2005). In addition, we detected the *LEP* and *OBR* mRNAs in pig perm. Other authors reported the presence of different mRNAs in mammalian ejaculated spermatozoa; however, the significance of mRNA in these cells is currently under investigation (Miller 2000, Andò & Aquila 2005). WB analysis revealed six different OBR isoforms, among these the120 kDa band is consistent with the OBRb isoform while the other bands could correspond to short isoforms. A partially similar pattern of OBR has been detected in the adult mouse testis (El-Hefnawy *et al.* 2000), but the pattern of OBR expression is species specific.

Interestingly, immunofluorescence assays localized both LEP and OBR exclusively at acrosomal level of pig sperm. Spermatozoa are highly polarized cells, thus they compartmentalize specific metabolic and signaling pathways where they are necessary. Therefore, a role of LEP/OBR in the events leading to the sperm becoming able to fertilize the oocyte can be hypothesized. Our data on OBR agree with the De Ambrogi *et al.* (2007) findings, showing the receptor prevalently located on the acrosome of boar sperm, whereas in human sperm the receptor was visualized at the tail (Jope *et al.* 2003). These differences may be due to sample management or they could be species specific. Despite the use of the same antibody, the different leptin localization in human and pig sperm is particularly intriguing. In fact, the hormone was specifically compartmentalized in the equatorial segment and in the mid-piece of human sperm (Aquila *et al.* 2005). We can only hypothesize that leptin is differently involved in the regulation of sperm events in the two species.

In order to fertilize oocyte in the female genital tract, the mammalian spermatozoa must undergo the capacitation process that is a prerequisite for the acrosome reaction. Capacitation induces various biochemical and biophysical changes in the sperm plasma membrane, such as the efflux of cholesterol (Visconti *et al.* 1999*a*, 1999*b*, Shadan *et al.* 2004). Cholesterol efflux from spermatozoa destabilizes lipid raft structures in the plasma membrane initiating the protein phosphorylation and the acquisition of a capacitated status. Tyrosine phosphorylation of sperm proteins during capacitation has been reported in mouse, human, bull, hamster (Visconti *et al.* 1995, Leclerc *et al.* 1996, Galantino-Homer *et al.* 1997), and also in pig (Kalab *et al.* 1998,

Flesch *et al*. 1999, Tardif *et al*. 2003). Our data have shown that, in pig sperm, leptin increased cholesterol efflux, protein tyrosine phosphorylation, and the acrosin activity. Therefore, LEP, through its receptor, appears to affect both capacitation and acrosome reaction, suggesting its role in the acquisition of fertilizing ability of pig sperm.

The signaling events, derived from LEP binding to its receptor, have been recently investigated at biochemical and molecular level. The JAK/STAT pathway is one of the main signaling cascades activated by LEP (Thomas 2004). The OBR, which belongs to the class I cytokine receptor superfamily, binds cytoplasmic kinases, mainly JAK2 (Ghilardi & Skoda 1997). Activated JAK2 auto-phosphorylates numerous tyrosine residues and, at the same time, it phosphorylates tyrosine residues on the functional OBR. Then the phosphorylated intracellular domain of the receptor provides a binding site for STAT proteins (particularly STAT3), which are activated and translocated to the nucleus where they stimulate transcription of target genes. In our study, for the first time, we have demonstrated that STAT3 is expressed in the pig sperm, it is constitutively activated, and its phosphorylation increases following LEP binding to its receptor. Therefore, we provide new information to indicate that LEP can stimulate the JAK-STAT pathway in pig sperm.

Since capacitation is a crucial step in the acquisition of sperm fertilizing ability, it is likely controlled by redundant mechanisms, with cross-talks between different pathways (de Lamirande *et al*. 1997, Leclerc *et al*. 1998). The components of the extracellular signal-regulated kinase family of MAPK have been detected in spermatozoa affecting their motility and capacitation (Naz *et al*. 1992, Luconi *et al*. 1998, de Lamirande & Gagnon 2002). Our results have shown the expression of ERK1/2 in pig male gamete and have evidenced that LEP, through its receptor, was able to positively activate ERK1/2, suggesting that MAPK-dependent processes are involved in the hormone action.

Several pathways are also activated by JAKs including PI3K/Akt. PI3K plays an important role in the survival and metabolism of somatic and sperm cells (Fisher *et al*. 1998, Luconi *et al*. 2001, Aquila *et al*. 2004, 2005, Aparicio *et al*. 2006). The PI3K main downstream effector is the Akt, identified as a serine/threonine protein kinase, which was found in human ejaculated spermatozoa (Aquila *et al*. 2004) and in boar spermatozoa (Aparicio *et al*. 2006).

From our results it emerges that PI3K and Akt are implicated in mediating LEP signals in pig sperm, suggesting that this hormone may be involved in sperm survival. Concomitantly, BCL2 (Ito *et al*. 1997), a key protein in survival signaling, is enhanced upon LEP exposure and this effect was reduced by the anti-OBR Ab. Previous works have shown that LEP attenuates apoptosis of different cell types, such as osteoblasts, granulosa cells, and pancreatic islet cells (Almong *et al*. 2001, Okuya *et al*. 2001, Gordeladze *et al*. 2002). Moreover, recently, LEP has been found to inhibit stress-induced apoptosis of T lymphocytes *in vivo* (Fujita *et al*. 2002). Data presented here clearly demonstrate that LEP, by inducing the phosphorylation of classical key survival proteins, such as ERK1/2, PI3K, Akt, and BCL2, aids the pig sperm survival process.

It is important to point out that in all our experiments, we obtained different responses by using low or high LEP doses; in fact 1 nM and 10 nM were stimulatory, whereas the higher LEP concentration seemed to be ineffective (similar to the control). The outcome of signaling activation can depend on differences in ligand concentration as it was demonstrated in human sperm (Aquila *et al*. 2005). Besides, recently it was hypothesized that the net effect of LEP upon male reproductive function may depend on its circulating level (Caprio *et al*. 2001, Tena-Sempere & Barreiro 2002). The effect obtained with the higher LEP dose may be also due to the down-regulation or internalization of the OBR depending on the ligand concentration as previously demonstrated (Uotani *et al*. 1999).

In addition, it has been demonstrated that pig seminal plasma contains a significant amount of LEP, which decreases considerably in the follicular fluid (Lackey *et al*. 2002). As sperm leave seminal plasma during their transit in the female reproductive tract, they are exposed to decreased LEP concentrations. From our results, it may be speculated that the high LEP in seminal plasma may contribute to maintain sperm in a quiescent metabolic condition. Instead, the low LEP concentrations in the pig female reproductive tract (Gregoraszczuk *et al*. 2004) secretions could contribute to sperm activation, by facilitating their capacitation and acquisition of fertilizing ability.

Fatness in pigs has economical importance due to market incentives for the production of lean pork and also because elevated fatness increases feed costs. LEP was identified as a metabolic signal affecting central regulation of reproduction in the pig. Our data have shown that this hormone affects pig sperm acquisition of fertilizing ability. Therefore, further studies, addressed to the knowledge of the balance between local and systemic leptin, will clarify whether the manipulation of LEP concentration, as a strategy to alter body composition, may affect pig reproduction.

Materials and Methods

Chemicals

BSA protein standard, Laemmli sample buffer, pre-stained molecular weight marker, Percoll (colloidal PVP coated silica for cell separation), sodium bicarbonate, dimethyl sulfoxide, Earle's balanced salt solution, triethanolamine buffer, $MgCl_2$, propidium iodide, LY294002 (PI3K inhibitor), and all other chemicals were purchased from Sigma. Recombinant porcine leptin was purchased from Protein Laboratories Rehovot

(Rehovot, Israel) and acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, ECL Plus WB detection system, Hybond ECL were purchased from Amersham Pharmacia Biotech. Antibodies (Abs) polyclonal rabbit anti-leptin (A-20), rabbit anti-OBR (H-300), rabbit anti-p-Akt1/Akt2/Akt3 (Ser473), rabbit anti-p-Akt1/Akt2/Akt3 (Thr308), rabbit anti-phosphotyrosine (PY99), mouse ant-p-signal transducer and activator of transcription-3 p-STAT3 (B-7), peroxidase-coupled anti-rabbit, and FITC/Texas Red conjugated anti-rabbit IgG were from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit anti-p-BCL2 and p-ERK1/2 (p42/44 kDa) Abs were from Cell Signaling (Milan, Italy). Cholesterol-oxidase (CHOD)–peroxidase (POD) enzymatic colorimetric kit was from Inter-Medical (Biogemina Italia Srl, Catania, Italy). Total RNA Isolation System kit, enzymes, buffers, nucleotides 100 bp ladder used for RT-PCR were purchased from Promega. Moloney murine leukemia virus (M-MLV) was from Gibco-Life Technologies Italia. Oligonucleotide primers were made by Invitrogen.

Animals and semen samples

The investigation has been conducted on semen from seven fertile male pigs (*Sus scrofa domestica*, Large White) kept at the 'Swine Artificial Insemination Centre' (Rende, Cosenza, Italy). The animals were 24 to 30-month old and their weights were from 280 to 320 kg. Individual fresh ejaculates were collected by the gloved-hand method and filtered immediately by Universal Semen bags (Minitub, Tiefenbech, Germany). Semen was transported within half an hour to the laboratory, diluted 1:10 with TBS buffer, and centrifuged on a discontinuous Percoll density gradient (72%/90%) to remove bacteria and debris (Kuster *et al.* 2004). Epididymides were obtained from two out of the seven animals after castration at local animal hospitals. All surgical procedures followed approved guidelines for the ethical treatment of animals. Epididymides were brought immediately to the laboratory, then were carefully dissected, freed from sperm, homogenized, and lysed for WB analysis.

Evaluation of sperm viability

Viability of pig sperm was assessed using the DNA-specific fluorochrome PI. Sperm suspension (1×10^6 ml) was exposed to PI (12 μmol/l) for 5 min at room temperature. Then spermatozoa were fixed by adding 1 ml of 12.5% (w/v) paraformaldehyde in 0.5 mol Tris/l (pH 7.4) and the slides were immediately examined under an epifluorescence microscope (Olympus BX41) observing a minimum of 200 spermatozoa×slide (100× objective).

Sample treatments

Percoll-purified sperm were washed with unsupplemented Earle's medium (uncapacitating medium) and were incubated for 30 min at 39 °C and 5% CO_2 without (control) or with increasing concentrations of LEP (1, 10, and 60 nM). These doses were chosen on the basis of physiological concentrations to reproduce the environment of the sperm journey. Some cells were also pretreated (15 min) with the inhibitor, LY294002, or with the anti-OBR Ab (autocrine blockage). No adverse effects among the different treatments have been observed on pig sperm viability.

Then, samples were centrifuged (3000 ***g***×5 min) using the upper phase for further determinations and sperm pellet for WB analysis.

Western blot analysis of sperm proteins

WB analysis was used to identify LEP and OBR in sperm samples obtained from all the seven animals. Sperm samples were washed twice with Earle's balanced salt solution (uncapacitating medium) and then centrifuged for 5 min at 5000 ***g***. The pellet was resuspended in lysis buffer as previously described (Aquila *et al.* 2002). Equal amounts of proteins (80 μg) were boiled for 5 min, separated by 10% PAGE, transferred to nitrocellulose sheets, and probed with an appropriate dilution of the (indicated) specific Ab. The bound of the secondary antibody was revealed with the ECL Plus WB detection system according to the manufacturer's instructions. The negative control was performed using a sperm lysate that was immunodepleted of LEP or OBR (i.e., lysates pre-incubated with anti-leptin Ab or anti-OBR Ab for 1 h at room temperature and immunoprecipitated with Protein A/G-agarose).

To further validate the results for OBR, as negative control, non-immune rabbit serum, instead of the first Ab, was used at the same dilution ratio (1:1000). Epididymal extract was used as pig control tissue.

WB was also performed to identify STAT3, BCL2, ERK1/2, Akt1/Akt2 in pig sperm extracts and evaluate the cell signaling induced by LEP (p-Akt1/Akt2/Akt3, p-STAT3, p-BCL2, and p-ERK1/2). MCF7 cell lines were used as positive controls while negative controls were performed as indicated above. The blots were stripped (glycine 0.2 M (pH 2.6) for 30 min at room temperature) and reprobed with anti-β-actin Ab as loading control.

The experiments were repeated four times for each sample.

RNA isolation, reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from pig spermatozoa purified as previously described (Aquila *et al.* 2002). Before RT-PCR, RNA was incubated with RNase-free DNase (DNase) I in single-strength reaction buffer at 37 °C for 15 min. This was followed by heat inactivation of DNase I at 65 °C for 10 min. Two micrograms of DNase-treated RNA samples were reverse transcribed by 200 IU M-MLV reverse transcriptase in a reaction volume of 20 μl (0.4 μg oligo-dT, 0.5 mM deoxy-NTP, and 24 IU RNasin) for 30 min at 37 °C, followed by heat denaturation for 5 min at 95 °C. PCR amplification of cDNA was performed with 2 U of Taq DNA polymerase, 50 pmol primer pair for both LEP and OBR in 10 mM Tris–Hcl (pH 9.0) containing 0.1% Triton X-100, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.25 mM each dNTP. The applied PCR primers and the expected lengths of the resulting PCR products are the following: 5′ATTCCTGGCTTGGCCC 3′ and 5′ AAGGCAGACTGGTGAGGATCTGTT 3′ for LEP with a product size of 248 bp; 5′ ACTTCCTCTTGCCTGCTGGAATCT 3′ and 5′ GACACAGGCACATGGCATTCACAA 3′ for OBR with a

product size 460 bp. Cycling conditions were: 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min for LEP; 95 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min for OBR. For all PCR primer amplifications, negative RT-PCR was performed without M-MLV reverse transcriptase. The PCR-amplified products were subjected to electrophoresis in 2% agarose gels stained with ethidium bromide and visualized under u.v. transillumination.

Immunofluorescence assay

Following Percoll separation, sperm cells were rinsed three times with 0.5 mM Tris–Hcl buffer (pH 7.5); then 10 µl of concentrated cell suspension was added to 250 µl drop of warm (37 °C) TBS and allowed to settle onto slides in a humid chamber. The overlying solution was carefully pipetted off and replaced by absolute methanol for 7 min at −20 °C. After methanol removal, sperm cells were washed in TBS, containing 0.1% Triton X-100 and were treated for immunofluorescence.

Leptin and OBR stainings were carried out, after blocking with normal goat serum (10%), using anti-leptin (1:100) and anti-OBR (1:100) as primary Abs, followed by anti-rabbit IgG Texas Red-conjugated/FITC-conjugated Abs (1:200) respectively. Sperm cells incubated without the primary Abs were utilized as negative controls. The slides were immediately examined under an epifluorescence microscope (Olympus BX41), observing a minimum of 200 spermatozoa×slide (100×objective).

Measurement of cholesterol in the sperm culture medium

Cholesterol was measured (in duplicate) in the incubation medium from pig spermatozoa obtained from all the seven animals. The CHOD–POD enzymatic colorimetric method was used according to the manufacturer's instructions. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control, NC) for 30 min at 39 °C and 5% CO_2. Other samples were incubated in the presence of 1, 10, and 60 nM LEP concentrations. Some samples were incubated with anti-OBR Ab combined with 10 nM LEP. At the end of the sperm incubation, the culture media were recovered by centrifugation, lyophilized, and subsequently dissolved in 1 ml of the buffer reaction. The samples were incubated for 10 min at room temperature and then the cholesterol content was measured with the spectrophotometer at 505 nm. Cholesterol standard used was 200 mg/dl. The limit of sensitivity for the assay was 0.05 mg/dl. Inter- and intra-assay variations were 0.71% and 0.57% respectively.

The experiments were repeated four times for each sample.

Acrosin activity assay

Acrosin activity was assessed by the method of Glogowski *et al.* (1998). Briefly, sperm samples from all the seven animals were washed in Earle's medium and centrifuged at 800 ***g*** for 20 min, then they were resuspended (final concentration of 100×10^3 sperm/ml) in different tubes containing no treatment (control) or the indicated treatments (experimental). One milliliter of substrate–detergent mixture (23 mmol/l N alpha-benzoyl-DL-arginine p-nitroanilide in DMSO and 0.01% Triton X-100 in 0.055 mol/l NaCl, 0.055 mol/l HEPES (pH 8.0) respectively) was added for 1 h at room temperature. After incubation, 0.5 mol/l benzamidine was added (0.1 ml) to each of the tubes and then they were centrifuged at 1000 ***g*** for 30 min. The supernatants were collected and the acrosin activity measured spectrophotometrically at 410 nm. In this assay, the total acrosin activity is defined as the amount of the active (non-zymogen) acrosin associated with sperm plus the amount of active acrosin that is obtained by pro-acrosin activable. The acrosin activity was expressed as mIU/10^6 sperms. The experiments were repeated four times for each sample.

Statistical analysis

Data, presented as mean±S.E.M., were evaluated by the one-way ANOVA. The differences in mean values were calculated at a significance level of $P\leq0.05$.

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