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SHORT COMMUNICATION

Insulin affects sperm capacity in pig through nitric oxide

Saveria Aquila[1,2], Francesca Giordano[1], Carmela Guido[1,2], Vittoria Rago[1] and Amalia Carpino[1]

Insulin (Ins) has recently been demonstrated to have the ability to induce the capacitation process in pig spermatozoa. In various mammalian species, capacitation has been linked to the nitric oxide (NO) signalling; therefore, this study investigated NO production in Ins-treated pig spermatozoa by fluorescence-activated cell sorting. For the same samples, sperm capacitation was evaluated by chlortetracycline staining, protein tyrosine phosphorylation pattern and acrosomal status. A significant increase of the intrasperm NO level and the activation of three capacitation indices were detected in response to Ins treatment. Conversely, sperm preincubation with an NO synthase inhibitor (*N*-nitro-*L*-arginine methyl ester) or with the anti-Ins receptor β (IRβ) antibody reversed all of the Ins-related effects. These results suggest that Ins has the capacity to enhance intracellular NO concentrations in pig spermatozoa and indicate a possible NO implication upon Ins promotion of capacitation.
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INTRODUCTION

A potential new role of insulin (Ins) in sperm physiology has emerging in recent years. Ins expression and secretion have been demonstrated in human male gametes,[1] which have been found to be damaged in men affected by Ins-dependent diabetes.[2] In addition, Ins treatment can enhance motility and the extent of the acrosome reaction[3] of human spermatozoa.

In our previous study, the expression of Ins and Ins receptor β (IRβ) in pig spermatozoa and the ability of Ins to induce capacitation process were determined.[4] Furthermore, in pig spermatozoa, we recently reported the expression levels of the three isoforms of nicotinamide adenine dinucleotide phosphate-dependent nitric oxide (NO) synthases (inducibile NOS, endothelial NOS and neuronal NOS)[5] which are responsible for the synthesis of intracellular NO.[6] Because a link between capacitation and NO signalling has been suggested in some mammalian spermatozoa, the aim of the present study was to investigate the capacity of pig male gametes to produce NO in response to Ins treatment. Furthermore, a possible role of the NO free radical in the Ins-promotion of capacitation was also evaluated.

MATERIALS AND METHODS

Percoll-purified spermatozoa from five fertile male pigs (*Sus scrofa domestica*, Large White) were incubated with uncapacitating Earle's medium (Sigma Chemical, Milan, Italy) for 30 min at 39 °C and 5% CO_2 with or without 0.1 nmol l^{-1} porcine Ins (Sigma Chemical) and 0.7 mmol l^{-1} *N*-nitro-*L*-arginine methyl ester (*L*-NAME) (Vinci Biochem, Firenze, Italy). Because Ins action is mediated by its receptor, some spermatozoa were also pretreated (15 min) with the monoclonal mouse anti-IRβ antibody (C18C4 from Stressgene, Bologna, Italy) prior to the addition of 0.1 nmol l^{-1} Ins in order to perform an autocrine blockage. Untreated spermatozoa were capacitated (C) by incubation in Earle's capacitating medium for 120 min at 39 °C and 5% CO_2 as a positive control.

Intracellular NO concentrations were measured according to the protocol of Lampiao.[7] Briefly, Ins-treated sperm were loaded with 10 μmol l^{-1} of the fluorescent probe 4,5-diaminofluorescein-2/diacetate (DAF-2/DA; Vinci Biochem) and incubated (120 min and 37 °C) in the dark. Some of the samples were loaded with the NOS inhibitor, *N*-nitro-*L*-arginine methyl ester (*L*-NAME) (0.7 mmol l^{-1}) 30 min prior to DAF-2/DA loading. After incubation with DAF-2/DA, the cells were analysed by fluorescence-activated cell sorting (FACS analyser) at a single-cell level, and data were analysed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

The chlortetracycline (CTC) staining assay was performed according to Wang *et al.*[8] Briefly, 45 μl of the sperm suspension (5×10^6 ml^{-1}) was treated with an equal volume of a CTC solution for 30 s, followed by the addition of 8 μl of 12.5 paraformaldehyde in 0.5 mol l^{-1} Tris-HCl (pH 7.4). Ten microlitres of the fixed sperm suspension were placed on a microscope slide and examined with an epifluorescence microscope (Olimpus BX41) with multiple fluorescent filters. One hundred spermatozoa were examined and classified according to the following CTC staining patterns: uncapacitated spermatozoa (fluorescence uniformly distributed over the head), capacitated spermatozoa (a fluorescence-free band in the post-acrosome region) and acrosome-reacted spermatozoa (almost no fluorescence over the sperm head except for a thin band of fluorescence in the equatorial segment).

For western blot analysis, spermatozoa were washed and centrifuged for 5 min at 5000*g*. The pellet was resuspended in lysis buffer, and equal amounts of proteins (80 μg) were boiled for 5 min, separated by 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and probed with rabbit antiphosphotyrosine antibody

[1]Department of Pharmacy, Health Science and Nutrition, University of Calabria Arcavacata di Rende, Cosenza 87036, Italy and [2]Health Centre, University of Calabria Arcavacata di Rende, Cosenza 87036, Italy
Correspondence: Prof A Carpino (am_carpino@yahoo.it)
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(PY99; Santa Cruz, CA, USA) (1 : 500). The bound fraction of the secondary antibody (peroxidase-coupled anti-rabbit antibody; Santa Cruz) was revealed using an enhanced chemiluminescence plus western blot detection system.

The acrosome reaction assay was carried out as previously reported by Carpino *et al.*[4] Acrosomal status was monitored by the acrosome-specific fluorochrome fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin in conjunction with DNA-specific fluorochrome propidium iodide to test viability. Staining patterns: Live spermatozoa (without nuclear red propidium iodide staining) were classified into two main categories as follows: (i) acrosome-reacted cells with uniform green fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin fluorescence of the acrosomal cap; and (ii) acrosome-intact cells without any fluorescence. Values were expressed as percentage. Four replicate experiments were performed for each semen sample.

The data, presented as mean±s.e.m., were evaluated using a one-way ANOVA. The differences in mean values were calculated at significance level of $P<0.05$.

RESULTS

Incubation of spermatozoa with 0.1 nmol l^{-1} Ins induced a significant NO increase (**Figure 1a and 1b**), while sperm pre-treatment with the NOS inhibitor *L*-NAME reversed the effect of Ins (**Figure 1c**). The DAF fluorescence data are expressed as the mean fluorescence (percentage of control, control adjusted to 100%) (**Figure 1d**).

Ins treatment increased the amount of spermatozoa showing a capacitated CTC staining pattern (**Figure 2a**), induced the phosphorylation pattern of the sperm protein tyrosine (**Figure 2b**) and enhanced the percentage of acrosome-reacted cells (**Figure 3**). Sperm preincubation with *L*-NAME or anti-IRβ autocrine blockage, reversed all of the hormone-related effects (**Figure 3**).

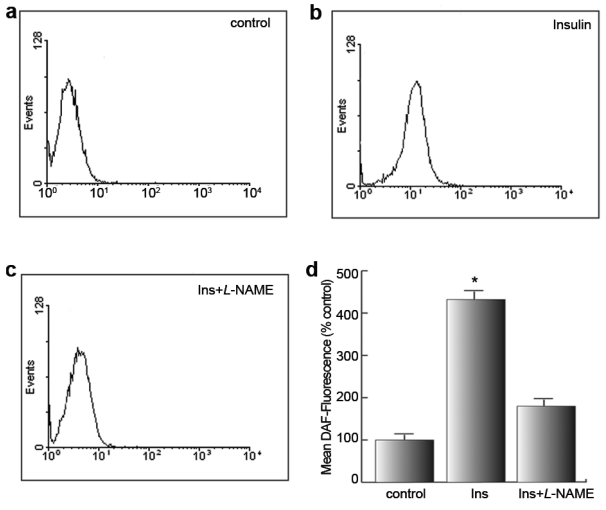

Figure 1 Representative histograms of DAF fluorescence in pig spermatozoa. (**a**) Uncapacitated spermatozoa. (**b**) Ins-treated spermatozoa. (**c**) Ins-treated sperm in presence of *L*-NAME. (**d**) Summary of the data reported above, expressed as the mean fluorescence (Mean DAF-fluorescence of the control was set to 100%. All other treatments were normalized accordingly). Values of percentage are expressed as mean ±s.d. (*$P<0.05$ *vs.* control). DAF, 4,5-diaminofluorescein-2/diacetate; Ins, insulin; *L*-NAME, *N*-nitro-*L*-arginine methyl ester.

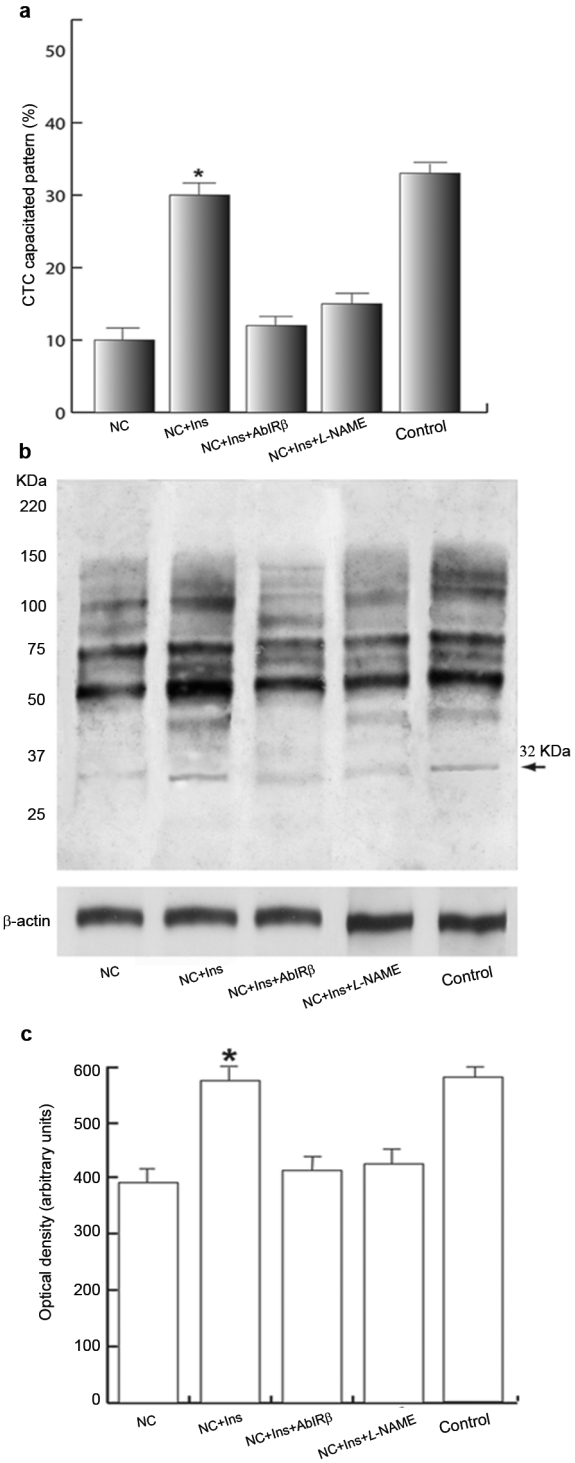

Figure 2 Effects of Ins treatment on the acquisition of CTC capacitated pattern and protein tyrosine phosphorylation in pig spermatozoa. Uncapacitated spermatozoa were incubated in the absence (NC) or presence of Ins, as well as in presence of *L*-NAME+Ins or anti-IRβ Ab+Ins. Capacitated spermatozoa (C) as positive control. (**a**) CTC pattern of percent capacitated sperm. (**b**) Western blot analysis of protein tyrosine phosphorylation from sperm lysates. (**c**) Band intensities were evaluated in term of arbitrary densitometric units. Values are as mean±s.d. (*$P<0.05$ *vs.* control). anti-IRβ Ab, insulin receptor β antibody; CTC, chlortetracycline; Ins, insulin; *L*-NAME, *N*-nitro-*L*-arginine methyl ester.

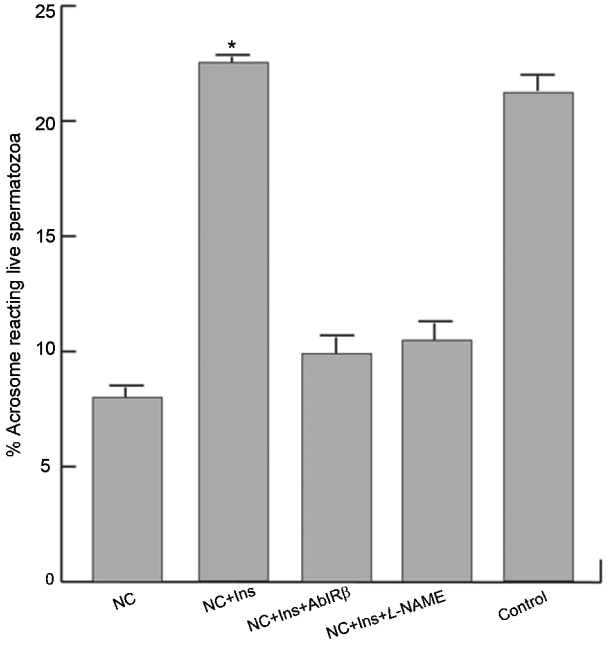

Figure 3 Acrosome reaction in Ins-treated pig sperm. Uncapacitated sperm were incubated in the absence (NC) or in the presence of Ins, as well in presence of anti-IRβ+Ins or *L*-NAME+Ins. Capacitated sperm as positive control (C). Values of percentage are expressed as mean ±s.d. (**P*<0.05 *vs.* control). anti-IRβ, insulin receptor β; Ins, insulin; *L*-NAME, *N*-nitro-*L*-arginine methyl ester.

DISCUSSION

NO is a highly reactive free radical that regulates a vast number of functional properties in mouse, hamster, bovine and human spermatozoa. Furthermore, in porcine male gametes, NO was implicated in the promotion of capacitation and/or acrosome reaction through induction by *L*-arginine, geldanamycin and leptin.[5,9,10] We previously demonstrated the ability of Ins to promote pig sperm capacitation.[4] Thus, in the present study, we have investigated NO production in Ins-treated pig spermatozoa. We used the Ins dose that induced the best stimulatory response in male gametes, based on our previous data. Our results revealed a significant increase in the level of intrasperm NO in response to Ins treatment, and this effect was reversed by sperm pre-treatment with an NOS inhibitor.

Furthermore, we have investigated the possibility of NO involvement in the Ins-mediated promotion of capacitation. Capacitation is a signal transduction-mediated event that modifies the sperm plasma membrane and leads to the acquisition of fertilizing ability. Firstly, we have observed the acquisition of a CTC capacitated pattern by the sperm membrane. We have also evaluated two cellular events associated with capacitation: protein tyrosine phosphorylation and the acrosome reaction. In fact, sequential protein tyrosine phosphorylation of the sperm compartments (midpiece, flagellum and head) occurs during capacitation and it appears to be related to the acquisition of hyperactivated motility, the capacity to bind the zona pellucida and acrosome reaction competence. In addition, capacitation prepares the sperm cells for the acrosome reaction, a process of specialized exocytosis that enables sperm cells to penetrate the female gamete. As expected, Ins treatment increased the amount of capacitated spermatozoa and induced the protein tyrosine phosphorylation pattern, including the sp-32 protein which is considered a marker of pig sperm capacitation.[11] At the same time, the acrosome reaction extent was enhanced in the male gametes stimulated by Ins. However, the inhibition of each of these indices by *L*-NAME (NOS inhibitor) and anti-IRβ autocrine blockage indicated the involvement of NO in the hormonal action.

Finally, these findings suggest that Ins can affect pig sperm capacitation through the NO pathway and provide new evidences concerning the relationships between sperm physiology and hormonal molecules involved in metabolic processes.

COMPETING FINANCIAL INTERESTS

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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