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1-*O*-Alkylglycerols Improve Boar Sperm Motility and Fertility[1]

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ABSTRACT

1-*O*-Alkylglycerols are naturally occurring ether lipids with potent biological activities. They may interfere with lipidic signaling, and they amplify platelet-activating factor (PAF) biosynthesis in a monocyte cell line. The PAF is produced by mammalian sperm and is an important activator of sperm motility. The aim of this study was to evaluate the effect of in vitro treatment of boar spermatozoa with natural 1-*O*-alkylglycerols (10 μM) on 1) boar sperm motility; 2) production of PAF and its metabolite, lyso-PAF, by spermatozoa; and 3) fertility in artificial inseminations of breeding sows. Using a computer-assisted spermatozoa analyzer, we found that 1-*O*-alkylglycerols increased percentage motility as well as velocity parameters after 24 h. These effects were partially or totally reversed by the PAF receptor-antagonist SR 27417. After [^{3}H]-1-*O*-alkylglycerol incubation with boar spermatozoa, we identified [^{3}H]lyso-PAF by high-performance liquid chromatography. Production of PAF and lyso-PAF was measured with a biological assay using [^{3}H]serotonin release from rabbit platelets. 1-*O*-Alkylglycerols significantly increased lyso-PAF production but had no effect on PAF production. The effect of 1-*O*-alkylglycerols on fertilization was also evaluated in industrial breedings: 1-*O*-alkylglycerol-treated or untreated semen dilutions were alternately used for artificial inseminations of sows on 12 farms. 1-*O*-Alkylglycerol treatment increased the number of farrows but had no effect on the mean size of the litters. This study demonstrates that 1-*O*-alkylglycerol treatment of boar spermatozoa in vitro improves their motility and fertility, and it suggests that this effect is related to PAF metabolism and function in boar spermatozoa.

fertilization, signal transduction, sperm motility and transport

INTRODUCTION

1-*O*-Alkylglycerols (alkyl-Gro) are naturally occurring lipids characterized by an ether linkage of a fatty alcohol to the 1-*sn* position of the glycerol backbone. They are present in human and cow milk and in hematopoietic organs such as bone marrow, spleen, and liver [1]. The highest content of alkyl-Gro (up to 50% by weight) has been found in shark liver oil [2]. Beneficial effects of alkyl-Gro have been reported: They reduce side effects of radiotherapy such as leukopenia and thrombocytopenia, inhibit tumor growth, and both stimulate and modulate the immune system [2].

In the human promonocyte leukemia cell line THP-1, alkyl-Gro incorporate predominantly into 1-*O*-alkyl-2-acyl-*sn*-glycerophosphocholine (RAcylGro*P*Cho), a platelet-activating factor (PAF) precursor, and amplify the production of PAF [3].

The PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a multifunctional, cellular mediator active on various cell types and systems, including circulation, inflammation, development, and reproduction [4–6]. It has been detected in mammalian spermatozoa from several species, including rabbit [7], mouse [8], and human spermatozoa [9]. It is involved in sperm motility, capacitation, and acrosome reaction in several mammalian species [10–13]. Specific PAF-receptor sites have been characterized in human spermatozoa [9, 14] as well as in uterine tissue from pregnant rabbits and humans [6]. Furthermore, sperm treatment by PAF resulted in enhancement of mouse oocyte in vitro fertilization [15].

Because boar spermatozoa contain a high percentage of ether phospholipids [16] involved in PAF synthesis, and considering the multiple roles of PAF in gamete and reproduction physiology, this study was undertaken to determine the in vitro effects of the PAF-precursor alkyl-Gro on boar sperm motility and on modulation of the synthesis of PAF and its metabolite, lyso-PAF. The effect of alkyl-Gro on fertilization was also measured in vivo by artificial insemination of breeding sows with alkyl-Gro-treated spermatozoa.

MATERIALS AND METHODS

Reagents

[^{3}H]Serotonin (5-hydroxytryptamine binoxalate [1,2-^{3}H], 30 μCi/mmol) and C18:0 [^{14}C]PAF (55 mCi/mmol) were obtained from Isotopchim (Ganagobie-Peyruis, France). Acetylsalicylic acid (Aspegic) was from Synthelabo (Toulouse, France). Essentially fatty acid-free BSA, gelatin type B, EDTA, and PAF were purchased from Sigma Chemical Co. (St. Louis, MO). The lyso-PAF (1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine) and calcium ionophore A23187 were obtained from Calbiochem (Meudon, France). Silica gel 60 Å LK6 plates were purchased from Whatman, Inc. (Clifton, NJ). The specific PAF receptor-antagonist SR 27417 [17] was kindly provided by Dr. J.M. Herbert (Sanofi Research, Toulouse, France). All solvents were obtained from Prolabo (Fontenay-sous-Bois, France).

Buffers

The seven buffers used were as follows:

Buffer 1: 44 mM NaCl, 4 mM $CaCl_2$, 1 mM sodium deoxycholate, 20 mM Tris-HCl, pH 8. Buffer 2: 44 mM glucose, 5 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1.3 mM $CaCl_2$, 0.2% (w/v) BSA, pH 7.4. Buffer 3 (Tyrode gelatin without Ca^{2+}): 137 mM NaCl, 2.6 mM KCl, 12.1 mM $NaHCO_3$, 1.05 mM $MgCl_2 \cdot 6H_2O$, 5.54 mM glucose, 4.2 mM Hepes, 0.25% (w/v) gelatin, pH 6.5. Buffer 4 (Tyrode gelatin without Ca^{2+} with EDTA): same as buffer 3, but with 0.1 mM EDTA. Buffer 5

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(Tyrode gelatin with Ca^{2+}): same as buffer 3, but with 1.15 mM $CaCl_2$, pH 7.4. Buffer 6 (acid citrate dextrose [ACD]): 13.65 g of citric acid, 25 g of disodium citrate, and 20 g of dextrose in 1 L H_2O; pH 4.4. Buffer 7 (Beltsville Thawing Solution [BTS]): 37 g of glucose, 6 g of trisodium citrate $2H_2O$, 1.25 g of $NaHCO_3$, 0.75 g of KCl, and 200 mg of gentamicin in 1 L H_2O.

Unlabeled and Tritiated Alkyl-Gro

The alkyl-Gro were prepared from shark liver oil as described previously [3]. These compounds were mixtures of monoalkyl-Gro with the following compositions, depending on the batch: 18:1(Δ9), between 54% and 65%; 16:1(Δ7), between 5% and 15.5%; 16:0, between 5% and 10%; 14:0, 3%; 18:0, 3%; and 17:1(Δ9), 1.5%. High-field (^1H, ^{13}C) nuclear magnetic resonance experiments confirmed that these extracts were clean mixtures of monoalkyl-Gro with compositions in agreement with the gas chromatographic data. Tritiated alkyl-Gro were obtained by ^3H-labeling on the 3-*sn* C position of glycerol. A classical protection-deprotection strategy led to 1-*O*-alkyl-2-acetyl glycerol. Oxidation followed by reduction with $NaBT_3$ and saponification gave the labeled target molecule on the C-3 position. Specific radioactivity was determined by measuring the radioactivity of a weighed sample converted to moles using a molecular weight (MW) of 344 (MW of the 18:1 prominent alkyl-Gro). Stock solution of alkyl-Gro was 10 mM in ethanol:water (60:40, v/v).

Sperm Collection

Ejaculates from 2- to 3-yr-old boars (Large White Pietrain and Pen Ar Lan) were collected in insulated beakers using the gloved-hand technique. Sperm was diluted in BTS for appropriate cell count (3–3.5 × 10^7 cells/ml) and stored at 17°C. The purity of the sperm population was assessed by light microscopy.

Computer-Assisted Analysis of Motility Parameters

Sperm concentration, motility, and different movement characteristics were determined using an ATS analyzer (JC Diffusion International, La Ferté-Fresnel, France). This system, already validated for measuring specific motility parameters of mammalian spermatozoa [18], was set up as follows: frame rate, 40; number of consecutive frames to be analyzed per second, 30; minimum number of consecutive frames to be analyzed, 15; maximum velocity, 300 μm/sec; threshold velocity, 20 μm/sec; cell-size range, 10–50 pixels; and analysis temperature, 37°C. Spermatozoa were allowed to settle for 30 sec in a 10-μm deep chamber (Markler chamber), and 100–150 spermatozoa per sample were screened. Each measure indicated cell number, percentage of motile spermatozoa, and the following averages: VCL (curvilinear velocity in μm/sec), VSL (progressive velocity in μm/sec), VAP (average path velocity in μm/sec), LIN (linearity as a %), and ALH (amplitude lateral head displacement in μm). For each sample, blind measures of motility and movement parameters were performed in triplicate with the ATS system.

Effects of Alkyl-Gro on Boar Sperm Motility

Boar sperm was collected, diluted in BTS, and incubated at 17°C with or without alkyl-Gro (10^{-5} M), or with vehicle (0.02% [v/v] ethanol) for control, for indicated periods of time.

To study the concentration-response relationship, increasing alkyl-Gro concentrations were used (from 10^{-8} to 5 × 10^{-5} M), and sperm motility was measured after 72 h.

Measurement of Boar Sperm Viability

Sperm viability was assessed by eosin exclusion test. Five microliters of sperm dilution were mixed with 5 μl of eosin solution (1% [w/v] in BTS). Immediate counting of the fraction of uncolored cells was used to calculate the percentage sperm viability.

Incorporation of [^{3}H]Alkyl-Gro into Boar Sperm Lipids

The [^{3}H]alkyl-Gro (10^{-5} M, 92.5 mCi/mmol) were dissolved in BTS, and boar spermatozoa (1.5 × 10^8 cells) were diluted in 2 ml of this solution and incubated at 35°C under 95% air + 5% CO_2 for indicated periods of time. Cells were washed three times in BTS with 0.2% (w/v) BSA. The supernatant was removed, and total lipids were extracted according to the method of Bligh and Dyer [19]. Lipid extract was dried under a nitrogen stream and separated by thin-layer chromatography (TLC) on silica gel plates using chloroform:methanol:acetic acid (35:14:2.7, v/v) as mobile phase. Radioactive zones were detected by a radiochromatographic scanner (Bioscan, Washington, D.C.), and phospholipid classes were identified by their retention factor (R_f). Radioactive contents on the silica gel were scraped off, and their radioactivity was quantified in a liquid scintillation counter (Packard, Rungis, France).

The silica containing nonpolar lipids from the above TLC was scraped off, extracted with 0.1% ethyl acetate:acetic acid (1:0.025, v/v). The neutral lipids were further analyzed on silica gel TLC (hexane:diethyl ether:acetic acid, 40:10:0.2, v/v). The zones comigrating with alkyldiacylglycerols or alkyl-Gro standards were scraped off, and their radioactivity was measured by liquid scintillation counting.

Synthesis of [^{14}C]Lyso-PAF from [^{14}C]PAF

The C18:0 [^{14}C]PAF (10^{-7} Ci, 55 mCi/mmol) was hydrolyzed by the action of phospholipase A_2 (PLA_2 from *Naja naja*, 10 μg/ml, 8.6 U) to form [^{14}C]lyso-PAF. Incubation with PLA_2 was performed at 37°C in 3 ml of buffer 1 and 3 ml of diethyl ether for 60 min under constant stirring. Lipids were then extracted according to the method of Bligh and Dyer [19], and the product obtained was dried under a nitrogen stream. The [^{14}C]lyso-PAF was isolated by TLC using chloroform:methanol:acetic acid:water (25:12.5:4:2, v/v) as solvent and was scraped off and extracted according to the method of Bligh and Dyer [19].

Incorporation of [^{3}H]Alkyl-Gro into PAF and Lyso-PAF and Analysis by High-Performance Liquid Chromatography

Spermatozoa (2 × 10^8 cells) were washed in BTS two times and incubated for 24 h with BTS containing [^{3}H]alkyl-Gro (10^{-5} M, 92.5 mCi/mmol) at 35°C under 95% air + 5% CO_2 and then washed three times in buffer 2. Spermatozoa were resuspended in the same buffer and total lipids were extracted according to the method of Bligh and Dyer [19] after addition of PAF and lyso-PAF (50 μg, respectively) as carriers. The samples were purified using straight-phase high-performance liquid chromatography (HPLC) on a 30-cm × 10-μm Microporasil column (Interchim, Montluçon, France) with the following linear-gradient solvent (1 ml/min): from 63.9:33.6:2.5 (v/v) chloroform:methanol:water to 63.1:33.6:3.3 chloroform/methanol/water over 40 min, and then to 61.4:33.6:5 chloroform:methanol:water over 20 min. The retention time of PAF and lyso-PAF was determined using an authentic standard of C18:0 [^{14}C]PAF and [^{14}C]lyso-PAF synthesized as described above. The peaks corresponding to PAF and lyso-PAF were collected, dried under vacuum/centrifuge (Jouan, St. Herblain, France), and their radioactivity quantified in a liquid scintillation counter.

Effects of Alkyl-Gro on Lyso-PAF and PAF Production

Boar spermatozoa (1.5 × 10^8 cells) were washed two times in BTS and incubated at 35°C under 95% air + 5% CO_2 with alkyl-Gro (10^{-5} M), or with vehicle (BTS + 0.02% ethanol) for control, for the indicated periods of time. The reactions were stopped by addition of 80% ethanol. After 1 h at room temperature, the precipitated material was removed by centrifugation. The supernatant was evaporated by vacuum/centrifuge, and PAF and lyso-PAF were isolated by TLC as described above, scraped off, and extracted according to the method of Bligh and Dyer [19] and dried. Quantification of PAF and lyso-PAF produced was performed using a bioassay of [^{3}H]serotonin release by rabbit platelets. The lyso-PAF was measured as PAF after its chemical acetylation [20]: dry residue of lyso-PAF was treated overnight at room temperature with 100 μl of acetic anhydride and 100 μl of pyridine, and then reagents were evaporated. The yield of this reaction (57%) was determined using the PAF bioassay of known amounts of synthetic lyso-PAF after acetylation. The dry extracts containing PAF or acetylated lyso-PAF were dissolved in 450 μl of buffer 5 supplemented with the ADP scavenger complex phosphocreatine/creatine kinase (10^{-3} M and 10 U/ml, respectively). Meanwhile, [^{3}H]serotonin-labeled platelets were prepared as previously described by Ardlie et al. [21]. Briefly, six volumes of blood from male New Zealand White rabbits were collected from the marginal vein into one volume of ACD and centrifuged at 375 × *g* for 20 min. The top layer of platelet-rich plasma was collected and incubated at 37°C for 45 min with 10^{-6} Ci/ml of [^{3}H]serotonin and Aspegic (10^{-4} M) for inhibition of cyclooxygenase. The platelets were then sedimented at 1400 × *g* for 20 min and washed first in buffer 4 and then in buffer 3. The pellet was gently resuspended in the latter buffer at a dilution of 1.25 × 10^9 cells/ml. For PAF quantification,

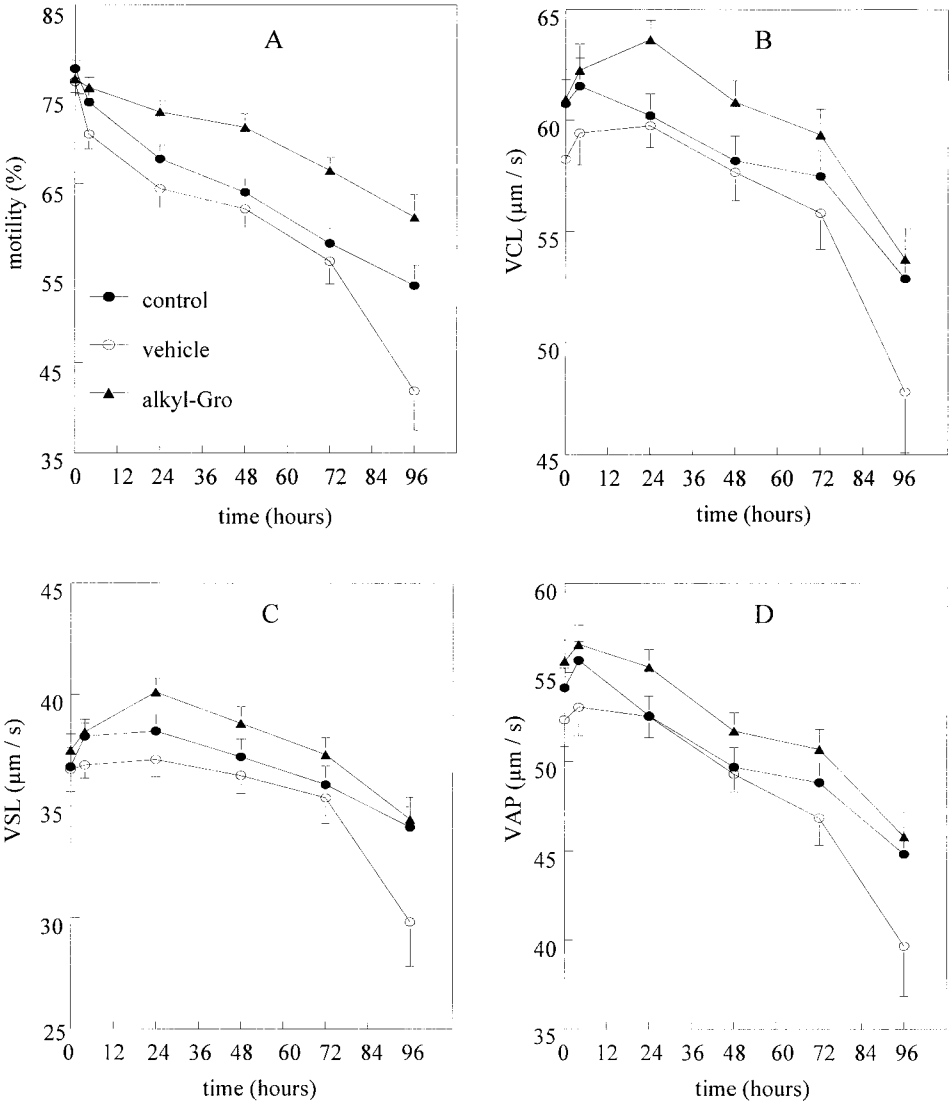

FIG. 1. Effects of alkyl-Gro on boar sperm motility. Spermatozoa were incubated without (control) or with alkyl-Gro for the indicated times, and percentage motility (**A**), VCL (**B**), VSL (**C**), and VAP (**D**) were measured. Significance of differences between alkyl-Gro and control: percentage motility, $P < 0.001$; VCL, $P < 0.001$; VSL, $P < 0.05$; and VAP, $P < 0.001$ (three-way ANOVA, n = 18).

50 μl of labeled platelets were added into samples and stirred at 37°C for 10 min. The reaction was stopped by addition of 9.25% (w/v) formaldehyde (20 μl), and platelets were sedimented at 4°C at 2500 × *g* for 15 min. Then, 200 μl of supernatants were collected for radioactivity measurement. A standard curve using synthetic C16:0 PAF was performed for each assay. In parallel, the specific PAF receptor-antagonist SR 27417 [17] was added to identical samples to quantify unspecific [^{3}H]serotonin release, which was then subtracted for [PAF] calculation.

Effects of Alkyl-Gro and PAF-Antagonist SR 27417 on Boar Sperm Motility

Spermatozoa were collected, diluted in BTS, and incubated with alkyl-Gro (10^{-5} M), or with vehicle for control (0.02% ethanol). The SR 27417 (5×10^{-7} M), or vehicle for control (0.05% HCl 1N), was added for the indicated periods of time.

Effect of Alkyl-Gro on Boar Sperm Fertility

Fertility was studied on 12 factory farms. Each farm received and used, at the same time, alternately and without knowing, alkyl-Gro-treated and untreated semen. Every other sow showing estrus was inseminated with alkyl-Gro-treated semen. Number of nulliparous sows was equilibrated in treated and untreated sow groups. Boar sperm was diluted in BTS containing, or not containing, alkyl-Gro (10^{-5} M). Depending on the farm, sperm dilutions (3–3.5 × 10^7 cells/ml) were used either within 3 days or after 4–5 days for artificial inseminations (two or three inseminations per sow at 12- to 18-h intervals). The semen dose containing 85 ml was inseminated through an ordinary catheter. Success of fecundations was controlled by echography, and number of farrows was used to establish the success rate.

Statistical Analysis

Data are presented as the mean ± SEM of the indicated number of experiments, each performed in triplicate. The significance of the difference between each treatment was tested by two-way ANOVA (factors: treatment and time) or three-way ANOVA (factors: treatment, time, and animal), followed by individual paired *t*-test for each time-point.

Significance of the effect of alkyl-Gro on fertility was tested by calculation of chi-square.

RESULTS

Effect of Alkyl-Gro (10^{-5} M) on Motility of Boar Spermatozoa

Boar spermatozoa were incubated with or without alkyl-Gro (10^{-5} M), or with vehicle, and percentage motility and movement parameters were measured at the indicated times. Treatment with alkyl-Gro resulted in a significant (three-way ANOVA, $P < 0.001$, n = 18, followed by individual paired *t*-test) increase in percentage motility compared with untreated spermatozoa after 24-h (9.887 ± 4.04% over control) and up to 96-h treatments (15.05 ± 3.61% over control) (Fig. 1A). The maximum difference

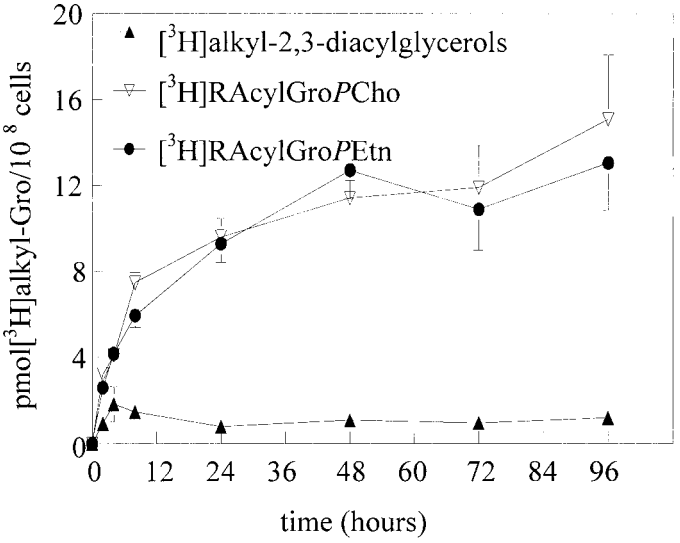

FIG. 2. Incorporation of [3H]alkyl-Gro into lipid classes. Spermatozoa were incubated with [3H]alkyl-Gro for the indicated times. Lipid classes were resolved by TLC, and the radioactive peak of each class was scraped and counted (n ≥ 3).

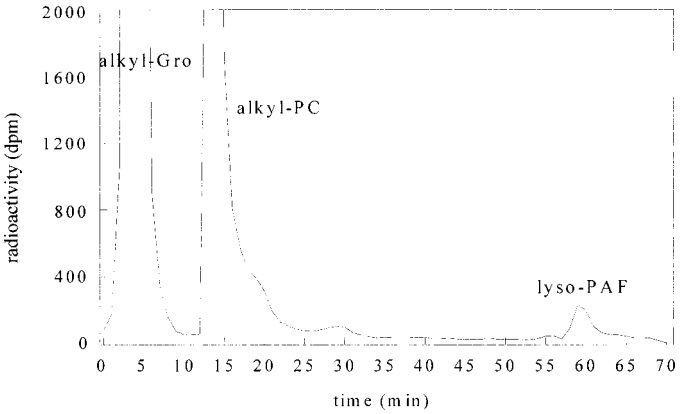

FIG. 3. Incorporation of [3H]alkyl-Gro into [3H]lyso-PAF. Spermatozoa were incubated with [3H]alkyl-Gro as in Figure 2 for 24 h. Lipids were extracted and resolved by straight-phase HPLC, and effluent fractions of 1 min were collected and counted. Representative profile from four experiments. Alkyl-PC, 1-*O*-Alkyl-2-radyl-*sn*-glycero-3-phosphocholine.

between spermatozoa incubated with or without alkyl-Gro was after 72-h treatment (18.26 ± 4.68% over control). Sperm viability measured after 72-h treatment was not increased in alkyl-Gro-treated sperm compared with untreated sperm (82.9 ± 2% and 82.5 ± 4.8%, respectively; n = 4). After 72 h of incubation, we also performed a concentration-related measurement of the alkyl-Gro effect on sperm motility and found a median effective concentration (EC_{50}) of 4 ± 0.9 × 10^{-7} M. Furthermore, 10^{-5} M alkyl-Gro induced the maximum effect, and we used this concentration for later experiments. Significant increases in several movement parameters were also observed in 10^{-5} M alkyl-Gro-treated spermatozoa compared to untreated spermatozoa: VCL, VSL, and VAP (three-way ANOVA; $P < 0.001$, $P < 0.05$, and $P < 0.001$, respectively) (Fig. 1, B–D), whereas while LIN and ALH were not significantly changed (data not shown). No significant differences between untreated and ethanol-treated spermatozoa were observed, except after 96 h, when ethanol significantly decreased percentage motility, VAP, VCL, and VSL (*t*-tests; $P < 0.01$, $P = 0.05$, $P = 0.05$, and $P < 0.05$, respectively).

Incorporation of [3H]Alkyl-Gro into Sperm Lipids

Incubation of spermatozoa in the presence of [3H]alkyl-Gro (10^{-5} M, 92.5 mCi/mmol) resulted in an incorporation of radioactivity into their lipids, mainly as free alkyl-Gro. After 4 h, 11.4% of initial radioactivity was found as free alkyl-Gro, a percentage that did not vary significantly with longer (24, 48, 72, and 96 h) incubation times, suggesting a fast and stable incorporation into and/or binding equilibrium to spermatozoa. We also observed incorporation of [3H]alkyl-Gro into RAcylGro*P*Cho and [3H]-1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (RAcylGro*P*Etn), to a much smaller extent, and, in contrast, with a steady increase depending on time up to 48 h, followed by a near plateau for longer times (Fig. 2), suggesting slow and saturable mechanisms. This incorporation reached 9.28 ± 0.86 pmol per 10^8 cells at 24 h and 13.05 ± 2.21 pmol per 10^8 cells at 96 h for RAcylGro*P*Etn and 9.59 ± 0.86 pmol per 10^8 cells at 24 h and 15.10 ± 2.99 pmol per 10^8 cells at 96 h for RAcylGro*P*Cho. The percentage of initial [3H]alkyl-Gro incorporated into RAcylGro*P*Etn and RAcyl*P*Cho after 96 h was 0.098 ± 0.016% and 0.113 ± 0.022%, respectively (n ≥ 3). No significant amount of radioactivity was detected in 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoinositol or in 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoserine. We also observed a light incorporation of [3H]alkyl-Gro into [3H]-1-*O*-alkyl-2,3-*sn*-diacylglycerols (Fig. 2).

Formation of [3H]Lyso-PAF after [3H]Alkyl-Gro Incorporation

To demonstrate that alkyl-Gro incorporated into phospholipids were used for PAF or lyso-PAF synthesis, we incubated spermatozoa with [3H]alkyl-Gro (10^{-5} M, 92.5 mCi/mmol, 24 h) and measured [3H]PAF and [3H]lyso-PAF after straight-phase HPLC separation. We detected a production of [3H]-1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine ([3H]lyso-PAF) in the pmol range (1.48 ± 0.43 pmol per 10^8 cells, n = 4, no triplicate) (Fig. 3), indicating that alkyl-Gro could be used for lyso-PAF biosynthesis. However, no [3H]PAF formation could be detected by this method.

Production of PAF and Lyso-PAF by Boar Spermatozoa after Alkyl-Gro Treatment

Boar spermatozoa were incubated with or without alkyl-Gro (10^{-5} M) for indicated periods of time, and PAF production was measured using a biological method of [3H]serotonin release by rabbit platelets in the presence and the absence of the PAF receptor-antagonist SR 27417 (see *Materials and Methods*). This method permits detection of PAF amounts in the fmol range. A time-dependent decrease of PAF production was noted. We detected 115.16 ± 10.79, 102.55 ± 12.69, 90.23 ± 9.66, and 88.42 ± 13.96 fmol per 10^8 spermatozoa, respectively, at 24, 48, 72, and 96 h (Fig. 4A).

Alkyl-Gro treatment had no significant effect on PAF production by boar spermatozoa (n = 4) compared to control.

Production of lyso-PAF also was measured with this biological method after chemical acetylation of lyso-PAF to form PAF. The amounts of lyso-PAF detected were approximately 100-fold higher than PAF. In untreated spermato-

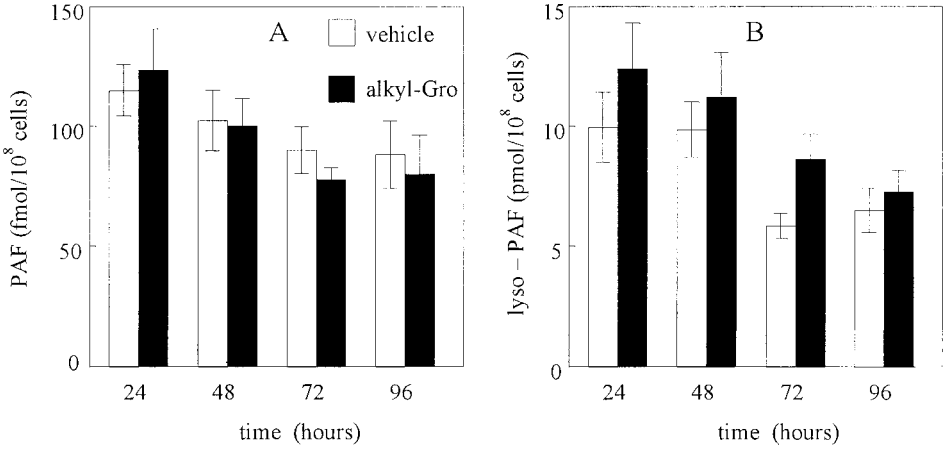

FIG. 4. Production of PAF and lyso-PAF by boar spermatozoa. Spermatozoa were incubated with alkyl-Gro or vehicle for the indicated times. The PAF and lyso-PAF were extracted in ethanol and separated by TLC. **A**) PAF activity was measured using the [^{3}H]serotonin release from rabbit platelets. **B**) Lyso-PAF was acetylated and measured as in **A**. Overall difference of lyso-PAF production between alkyl-Gro-treated and untreated spermatozoa: $P = 0.02$ (two-way ANOVA). Difference at 72 h: $P < 0.001$ (paired *t*-test, n = 4).

zoa, we measured 9.97 ± 1.48, 9.87 ± 1.15, 5.85 ± 0.52, and 6.51 ± 0.93 pmol per 10^8 spermatozoa, respectively, at 24, 48, 72, and 96 h (Fig. 4B). Alkyl-Gro treatment resulted in an overall increase in lyso-PAF production (n = 4, two-way ANOVA, $P = 0.02$); the highest alkyl-Gro-induced rise in lyso-PAF was observed after 72 h and represented 53.5 ± 14.6% of the control (paired *t*-test, $P < 0.001$) (Fig. 4B).

Effect of PAF Receptor-Antagonist SR 27417 on Alkyl-Gro-Induced Rise in Boar Sperm Motility

When boar spermatozoa were incubated with SR 27417 at 5×10^{-7} M, we observed that this PAF-receptor antagonist reduced significantly the alkyl-Gro-induced rise in percentage motility (three-way ANOVA, $P < 0.01$, n = 9); furthermore, total inhibition of the alkyl-Gro effect was ob-

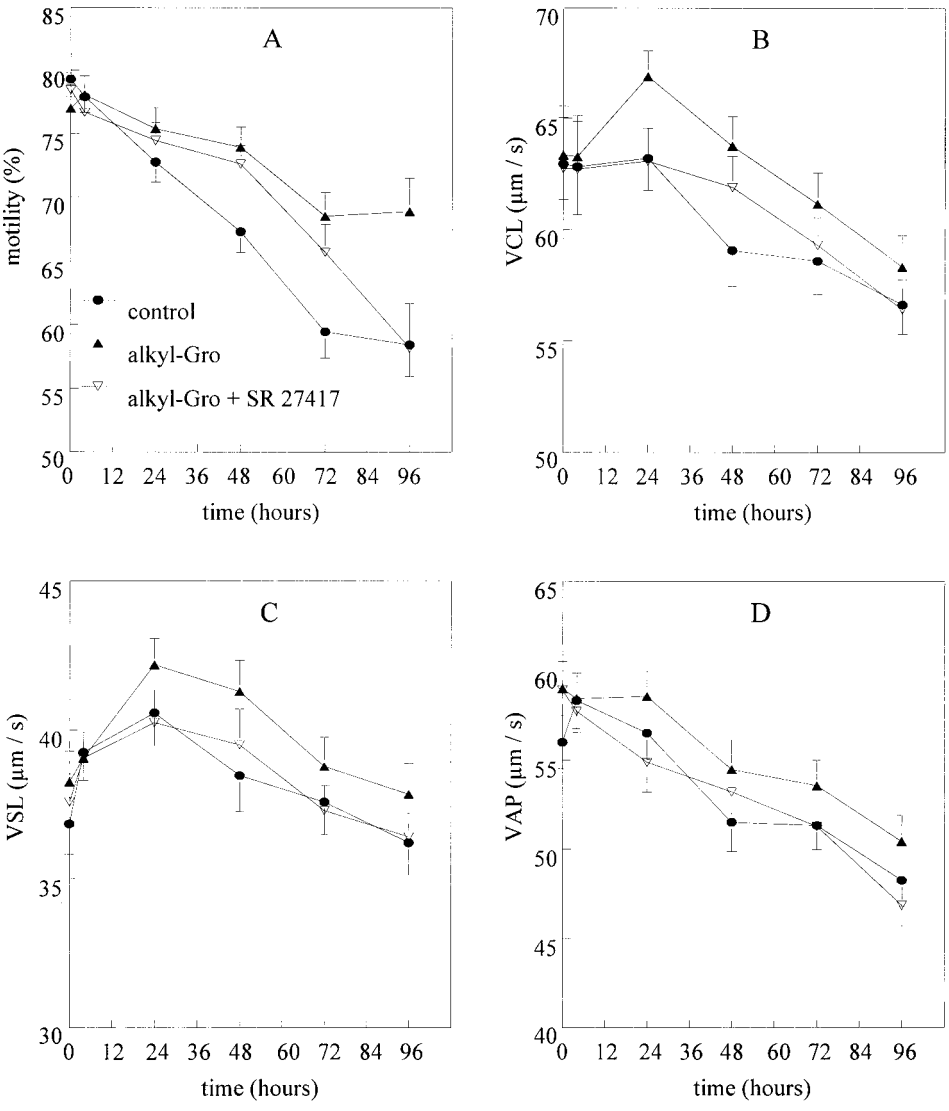

FIG. 5. Effects of SR 27417 on motility of alkyl-Gro-treated spermatozoa. Spermatozoa were incubated without (control) or with alkyl-Gro or alkyl-Gro + SR 27417 for indicated times, and percentage motility (**A**), VCL (**B**), VSL (**C**), and VAP (**D**) were measured. Significance of difference between alkyl-Gro-treated and alkyl-Gro + SR 27417-treated cells: percentage motility, $P < 0.01$; VCL, VSL, and VAP, $P < 0.001$ (three-way ANOVA, n = 9).

TABLE 1. Effect of alkyl-Gro on boar sperm fertility.[a]

Time after treatment	No. of inseminations		% Farrows[b]		No. of living piglets/farrows	
	Control	alkyl-Gro	Control	alkyl-Gro	Control	alkyl-Gro
1–3 days	179	178	83.8%	85.39%	12.2 ± 0.26	11.64 ± 0.25
4–5 days	177	182	72.88%	84.06%[c]	11.19 ± 0.40	11.55 ± 0.27
Total	356	360	78.37%	84.72%[d]	11.74 ± 0.19	11.59 ± 0.17

[a] Boar sperm was diluted in BTS (Control) or BTS supplemented with 10 μM alkyl-Gro (alkyl-Gro). Fertility was studied on 12 factory farms. Each farm used alternately alkyl-Gro-treated and untreated semen for artificial insemination. Inseminations were performed either 1–3 days or 4–5 days after treatment.
[b] Percentage of pregnant sows was calculated as 100 × farrows/inseminated sows.
[c] $P < 0.01$, chi-square test.
[d] $P < 0.05$, chi-square test.

served at 96 h (Fig. 5A). In the same way, we observed that VCL, VSL, and VAP of spermatozoa incubated with alkyl-Gro and SR 27417 significantly decreased (three-way ANOVA, $P < 0.001$, n = 9) compared to those of spermatozoa treated with alkyl-Gro after 24 h and up to 96 h (Fig. 5, B–D). This inhibition of alkyl-Gro effects by the PAF-receptor antagonist was almost total, because no significant differences of VCL, VSL, and VAP were observed between control cells and alkyl-Gro- and SR 27417-treated cells. The LIN and ALH were not modified after treatment either by alkyl-Gro or by alkyl-Gro and SR 27417. Interestingly, treatment by SR 27417 alone did not notably modify sperm percentage motility, VCL, VSL, LIN, or ALH (data not shown), and it only decreased slightly VAP (three-way ANOVA, $P < 0.001$, n = 9) (Fig. 6).

Effect of Alkyl-Gro on Boar Sperm Fertility

To find out if sperm treatment with alkyl-Gro could have beneficial effects on fertilization in vivo, we studied the effect of alkyl-Gro treatment of spermatozoa on breeding sow fertilization rates. We separated data in two groups of sows, which were inseminated with treated semen either 1–3 days or 4–5 days after treatment. Overall, in sows inseminated with alkyl-Gro-treated spermatozoa, the percentages of pregnancies and farrows significantly increased by 6.35% (chi-square test, $P < 0.05$) compared with control sows that were inseminated with untreated spermatozoa (Table 1). This increase reached 11.18% (chi-square test, $P < 0.01$) in the group inseminated after a longer time. No significant change in size of the litters was observed.

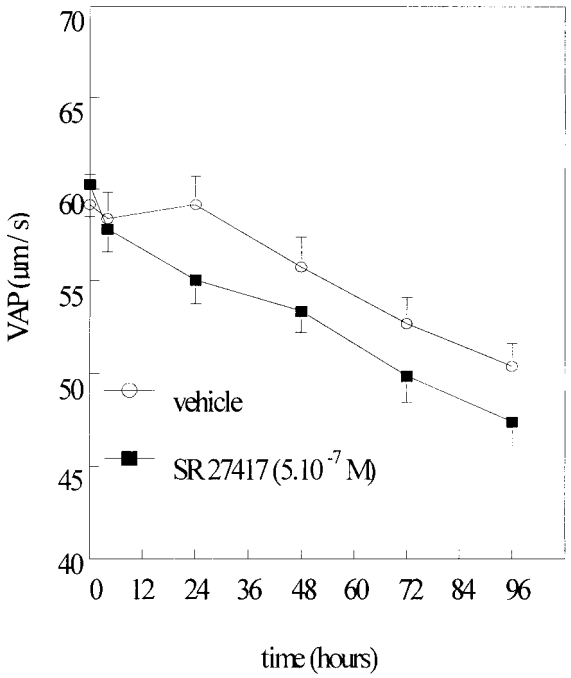

FIG. 6. Effect of SR 27417 on VAP. Spermatozoa were incubated with SR 27417 or vehicle for the indicated times, and VAP was measured. Significance of the difference between control and treated spermatozoa: $P < 0.001$ (three-way ANOVA, n = 9).

DISCUSSION

In this study, we report that boar spermatozoa incubated with naturally occurring alkyl-Gro increased their percentage motility and the motility parameters VCL, VSL, and VAP after 24 h and up to 96 h. This effect was antagonized by the PAF receptor-antagonist SR 27417. Both PAF and lyso-PAF were produced by boar spermatozoa in the fmol and pmol ranges, respectively. We also observed that alkyl-Gro induced a rise in lyso-PAF, but did not modify PAF production. Finally, treatment of spermatozoa with alkyl-Gro resulted in increased fertilization rates obtained by artificial insemination in breeding sows.

Phospholipids from porcine [16, 22] spermatozoa contain a high percentage of ether-linked phospholipids, among which RAcylGro*P*Cho is the main one. The significance of a high proportion of ether-linked phospholipids with respect to sperm functions is unclear; however, in human spermatozoa, RAcylGro*P*Cho could serve as a precursor for PAF synthesis through the remodeling pathway, which involves the PLA_2-dependent production of lyso-PAF and its acetylation by acetyltransferase [23]. In rat spermatozoa, PAF is also produced through the de novo pathway [24]. Additionally, PAF has been detected in rabbit [7], bovine [25], and mouse [8] spermatozoa. The PAF acetylhydrolase produces lyso-PAF, which is both a metabolite and a precursor for PAF, and PAF acetylhydrolase activity has been reported in human, bull, stallion, rabbit, rooster [26], and bovine [27] seminal plasma. However, the concentration of lyso-PAF in spermatozoa is unknown.

Receptors for PAF have been detected in human spermatozoa [9, 14], and convergent literature confirms the stimulating effect of PAF on motility, capacitation, and/or acrosome reaction in humans [10, 11], mice [12], and rabbits [13]. Additionally, lyso-PAF stimulates human sperm motility and other functions [10, 28, 29].

Because it has been demonstrated in THP1 cells that alkyl-Gro incorporate into the PAF precursor RAcylGro-*P*Cho and increase PAF production [3], we hypothesized that alkyl-Gro might improve sperm functions and fertility in mammals.

We first demonstrated that boar spermatozoa incubated with alkyl-Gro (10^{-5} M) increased their percentage motility

and motility parameters VAP, VCL, and VSL after 24 h and up to 96 h. This effect did not result from an increased viability of treated sperm. We further studied the lipid classes in which alkyl-Gro were incorporated and found that most [^{3}H]alkyl-Gro remained in its free form, and that only a minor fraction (0.113 ± 0.022%) was found in the PAF precursor RAcylGro*P*Cho at the plateau. This is in contrast with THP1 cells, in which the fraction of [^{3}H]alkyl-Gro incorporated into [^{3}H]RAcylGro*P*Cho reached 3% [3]. A detectable fraction of [^{3}H]alkyl-Gro was also found in [^{3}H]-1-*O*-alkyl-2,3-diacylglycerols. Because only traces of triglycerides were found in boar spermatozoa [22], the production of 1-*O*-alkyl-2,3-diacylglycerols after alkyl-Gro incubation could result from a specific metabolism of these ether lipids in spermatozoa.

We further investigated whether alkyl-Gro could be used to produce lyso-PAF and PAF. Following [^{3}H]alkyl-Gro incubation, [^{3}H]lyso-PAF in the pmol range was detected in spermatozoa, whereas [^{3}H]PAF was not. We also measured PAF and lyso-PAF in spermatozoa at resting conditions after incubation both with and without alkyl-Gro. The PAF concentration was 1.15 fmol per 10^6 cells after 24 h and slowly decreased over time. Alkyl-Gro had no effect on PAF concentration. To our knowledge, these are the first data available regarding the production of PAF by boar spermatozoa. Most published data concern human spermatozoa and show wide discrepancies in PAF production (expressed in fmol per 10^6 cells): 3 [8], 23 [23], 36 [30], 91 [31], and 7200 [32]. Our data are within the range of PAF concentrations found in spermatozoa from other species: 0.8 fmol per 10^6 cells in mice [8], and 0.35 fmol per 10^6 in rabbits [7]. Under the same conditions, we measured lyso-PAF and found that this PAF metabolite was produced in quantities 50- to 100-fold higher than PAF in boar spermatozoa. The lyso-PAF also decreased slowly over time from 24 to 96 h. Furthermore, in contrast to PAF, lyso-PAF production was increased by alkyl-Gro treatment. The lyso-PAF displays stimulating actions on sperm functions [10, 28, 29], and because lyso-PAF was found in much higher quantities than PAF and was increased by alkyl-Gro treatment, one may hypothesize that a weak agonist effect of lyso-PAF on PAF receptors [33] might explain, at least partially, the stimulating effects of alkyl-Gro on spermatozoa. Failure to observe an alkyl-Gro-induced rise in PAF might result from fast hydrolysis of PAF into lyso-PAF by PAF acetylhydrolase. Indeed, high activity of this enzyme was found in seminal plasma of several species [24, 26, 27]. Involvement of PAF receptor in alkyl-Gro effects was further supported by experiments showing that the PAF receptor-antagonist SR 27417 reversed, either partially or totally, the stimulating effects of alkyl-Gro on spermatozoa motility. On the other hand, the PAF-receptor antagonist alone had no notable effect on sperm motility, except a slight decrease in VAP, suggesting that PAF-receptor activation is not essential in long-lasting motility.

Other mechanisms might be mentioned with respect to the effects of alkyl-Gro. For example, alkyl-Gro are in vivo and in vitro protein kinase C (PKC) inhibitors [34], whereas the derivative 1-*O*-alkyl-2-acyl-Gro is a diacylglycerol analogue with specific inhibiting or activating actions on PKC [35–36]. Although low PKC activity was found in mature boar spermatozoa [37], PKC might be involved in several human sperm functions, including flagellar motility [38] and acrosome reaction [39]. Nevertheless, the role of PKC in long-lasting motility is poorly documented. The physiological consequences of high alkyl-phospholipid levels found in spermatozoa [16, 22] is also poorly understood. The low rate of alkyl-Gro incorporation into alkyl-phospholipids would not raise notably (<1%) the total content of alkyl-phospholipids in boar spermatozoa in our experimental conditions. Therefore, this minimal change could hardly explain the effect of alkyl-Gro observed in spermatozoa.

Finally, we demonstrated by in vivo experiments that treatment of boar spermatozoa with alkyl-Gro resulted in increased rates of pregnancies and farrows in sows artificially inseminated with treated semen. This effect was stronger when treated semen were used for inseminations after 4–5 days. These in vivo effects correlate with the results of our in vitro studies and may find interesting applications in breeding. The mechanism of such a beneficial effect on in vivo fertility might relate to the in vitro results showing a rise of lyso-PAF, because lyso-PAF has been shown to increase oocyte sperm penetration in humans [29].

Based on our data showing that, in boar spermatozoa, alkyl-Gro induced a rise in lyso-PAF and a stimulating effect on motility, which was reversed by a PAF-receptor inhibitor, we suggest that the alkyl-Gro effect is related, as least partially, to PAF-receptor activation. Furthermore, a beneficial effect of alkyl-Gro on sperm fertility was confirmed in breedings.

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