0021-972X/06/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 91(10):4154–4163

doi: 10.1210/jc.2006-1309

Cis-Unsaturated Fatty Acids Stimulate Reactive Oxygen Species Generation and Lipid Peroxidation in Human Spermatozoa

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Context: Defective sperm function is the largest defined cause of human infertility; however, the etiology of this condition is poorly understood. Although oxidative stress is acknowledged as a key contributor to this pathology, there are also data indicating that defective human spermatozoa contain abnormally high amounts of *cis*-unsaturated fatty acids. This study investigated whether a causative relationship exists between these two attributes of impaired semen quality.

Objective: The objective of this study was to determine whether polyunsaturated fatty acids can induce oxidative stress in human spermatozoa.

Method: Dihydroethidium and SYTOX Green were used in conjunction with flow cytometry and HPLC to investigate reactive oxygen species (ROS) generation by human spermatozoa after fatty acid exposure.

Results: Arachidonic acid (AA) induced a time- and dose-dependent increase in ROS generation by human spermatozoa that led to the promotion of peroxidative damage and a loss of sperm motility. This effect could not be blocked with inhibitors of the cyclooxygenase or lipoxygenase pathways of AA metabolism, rotenone, protein kinase C antagonists, or known inhibitors of plasma membrane redox systems. However, ROS generation could be triggered with other *cis*-unsaturated fatty acids including linoleic and docosahexaenoic acids. Saturated fatty acids, methyl esters of unsaturated fatty acids, or other amphiphiles were all ineffective. However in a cell-free system, AA could trigger a redox signal via mechanisms that were profoundly disrupted by diphenylene iodonium, a flavoprotein inhibitor.

Conclusions: The presence of excess unsaturated fatty acids in defective human spermatozoa may precipitate the oxidative stress encountered in male infertility. **(*J Clin Endocrinol Metab* 91: 4154–4163, 2006)**

INFERTILITY IS A condition that affects approximately one in 20 men of reproductive age (1). A majority of these males produce sufficient numbers of spermatozoa to fertilize the oocyte; however, the fertilizing capacity of their gametes is compromised for reasons that still remain unresolved. Indeed, defective sperm function is currently recognized as the single most important defined cause of infertility in our species (2). The functional lesions observed in such defective spermatozoa are associated with impaired motility and/or an inability to exhibit the cascade of cell-cell interactions associated with the induction of acrosomal exocytosis and sperm-oocyte fusion (3). Frequently, but not inevitably, these defective cells cannot even be induced to acrosome react and fuse with the vitelline membrane of the oocyte when stimulated with powerful divalent cation ionophores such as A23187 (4, 5). Such data suggest that whatever defects exist in the spermatozoa of infertile men, they frequently lie downstream of the calcium influx normally induced when these cells make contact with the surface of the oocyte. One of the major causes of such refractoriness appears to be oxidative stress (6). Thus, the functional incompetence exhibited by defective human spermatozoa is negatively correlated with both their redox activity and their lipid peroxidation status (7–11). Although either leukocytic infiltration or antioxidant insufficiency might occasionally create a state of oxidative stress in human spermatozoa, the most common cause of this condition is the excessive generation of reactive oxygen species (ROS) by the spermatozoa themselves (6). Although enhanced ROS generation has been observed in the spermatozoa of infertile men in a large number of independent studies (7, 8), the cause of this aberrant activity is still unknown.

One of the few biochemical signatures of defective human spermatozoa, other than high ROS generation, is a superabundance of polyunsaturated fatty acids (PUFA), such as arachidonic or docosahexaenoic acid (11, 12). Although spermatozoa normally possess extremely high levels of PUFA when they are in the testis, these molecules are lost during the functional remodeling of the sperm plasma membrane during epididymal transit. Therefore, the retention of high PUFA levels has been interpreted as representing a failure of sperm maturation commonly associated with the retention of excess residual cytoplasm by these cells and the excessive generation of ROS (12). In this study, we demonstrate for the first time that the presence of unesterified PUFA is causally associated with the induction of ROS generation and lipid peroxidation in human spermatozoa. These data have im-

First Published Online August 8, 2006

Abbreviations: AA, Arachidonic acid; COX, cyclooxygenase; DHE, dihydroethidium; DPI, diphenylene iodonium; Et^+, ethidium; NADPH, nicotinamide adenine dinucleotide phosphate; $2OHEt^+$, 2-hydroxyethidium; pCMBS, p-chloromercuribenzenesulfonic acid; PKC, protein kinase C; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

portant implications for our understanding of the origins of male infertility involving oxidative stress.

Materials and Methods

Human spermatozoa

Institutional and State Government ethical approval was secured for the use of human semen samples for the purposes of this research. Samples from unselected donors were inspected for liquefaction, consistency, debris, and volume; assessments of cell count and motility were conducted; and cell viability was measured using the eosin exclusion test (13). After allowing at least 30 min for liquefaction to occur, the spermatozoa were fractionated on a discontinuous two-step Percoll gradient, as described (14). Spermatozoa from the Percoll gradients were ultimately washed with 10 ml of Biggers-Whitten-Whittingham (BWW) medium (15), centrifuged at 600 × *g* for 15 min, and finally resuspended in HEPES-buffered BWW medium supplemented with 1 mg/ml polyvinyl alcohol at a concentration of 20×10^6/ml. All samples were tested for leukocyte contamination using a zymosan provocation assay (16) and cleared of any contaminating white cells using magnetic Dynabeads coated with a monoclonal antibody directed against the common leukocyte antigen, CD45 (Dynal, Oslo, Norway) (14).

Dihydroethidium (DHE) assay

DHE is a poorly fluorescent two-electron reduction product of ethidium (Et^+) that, on oxidation, produces DNA-sensitive fluorochromes that generate a red nuclear fluorescence when excited at 510 nm. For the assay, DHE and the vitality stain SYTOX Green (Molecular Probes, Invitrogen, Mount Waverley, Australia) were diluted in BWW/polyvinyl alcohol and added to 2×10^6 spermatozoa in a final volume of 200 μl, comprising 175 μl of purified sperm suspension, 5 μl of fatty acid resuspended in absolute ethanol (final ethanol concentration (vol/vol) maintained at 0.5%), and 20 μl of the DHE:SYTOX Green mixture to give final concentrations of 2 and 0.5 μM, respectively. The cells were then incubated in the dark at 37 C for 15 min and washed once (600 × *g* for 5 min), and the resultant red and green fluorescence was measured on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) as described (17). The HPLC profiles of the DHE reaction products were also collected as previously described (17).

Lipid peroxidation

Lipid peroxidation was assessed using BODIPY C11 as the probe (D3861; Molecular Probes). This probe readily incorporates into membranes where it responds to attack by peroxyl radicals by undergoing a spectral emission shift from red to green; this change can be readily monitored and quantified by flow cytometry (18). BODIPY C11 (5 μM) was added to 2×10^6 spermatozoa, incubated for 30 min at 37 C, centrifuged twice (650 × *g* for 5 min), and then treated with arachidonic acid (AA) (7.2–115.2 μM) or vehicle alone for 15 min in the presence or absence of ferrous sulfate (80 μM). At the end of this incubation period, the cells were analyzed using a FACSCalibur flow cytometer using an excitation wavelength of 488 nm. The FL-1 (530/30 nm band pass filter) was used to measure green fluorescence, and 10,000 sperm specific events were collected per data point.

Cell-free system

Percoll-purified sperm suspensions containing approximately 400×10^6 spermatozoa and treated with Dynabeads to remove all detectable traces of leukocyte contamination were gently sonicated on ice (3 × 5 sec bursts with 30 sec rest in between, followed by 4–5 × 10 sec bursts with 1 min rest in between). These broken cell preparations were centrifuged at 2000 × *g* for 5 min at 4 C, and the supernatant subsequently was centrifuged again at 10,000 × *g* for 5 min at 4 C. The supernatant from this centrifugation step was finally pelleted at 400,000 × *g* for 1 h at 4 C and resuspended in 200 μl PBS, and the protein content was determined using the bicinchoninic acid kit (Pierce Biotechnology Inc., Rockford, IL). The reaction mixture contained 10 μg of membrane protein in 380 μl PBS and 4 μl lucigenin (bis-*N*-methylacridinium nitrate at a final concentration of 250 μM). After 5 min, 10 μl of nicotinamide adenine dinucleotide phosphate (NADPH) (2.5 mM, final concentration) was added, followed at 20 min by the addition of 10 μl AA (28.8 μM final concentration) and at 40 min by 10 μl of the flavoprotein inhibitor diphenylene iodonium (DPI) to give a final concentration of 100 μM. Control incubations incorporated equal volumes of vehicle alone (PBS for NADPH, 20% ethanol for AA and 20% dimethylsulfoxide for DPI) or omitted the plasma membrane preparation. Chemiluminescence was then measured using a Berthold 953 luminometer (Berthold Detection Systems GmbH, Crown Scientific Pty Ltd., Moorebank, Australia) at 37 C.

Statistics

All experiments were repeated at least three times on independent samples, and the results were analyzed by ANOVA using the SuperANOVA program (Abacus Concepts Inc., Berkley, CA) on a Macintosh G5 computer; *post hoc* comparison of group means was by Fisher's protected least significant difference test. Differences with a P value of less than 0.05% were regarded as significant.

Results

AA initiates ROS generation and peroxidation

Addition of AA to suspensions of human spermatozoa stimulated a dose-dependent generation of ROS that was statistically significant ($P < 0.001$) at all doses tested from 7.2–115.2 μM, in the absence of any effect on sperm viability (Fig. 1A). Time-course studies established that the rise in ROS generation was rapid, apparent at the first time point tested, and sustained for the next 120 min (Fig. 1A, *inset*). To determine whether the exogenous AA had to be in direct contact with the spermatozoa for this stimulation to occur, spermatozoa were preexposed to AA for 15 min, washed twice to remove unincorporated AA, and then examined for ROS generation. This analysis revealed that preexposure to AA set in train processes that subsequently led to a progressive increase in the DHE signal over the next 120 min (Fig. 1B).

Analysis of the products of DHE metabolism by HPLC revealed two distinct peaks (Fig. 1C) corresponding to Et^+ (the two-electron oxidation product of DHE) and 2-hydroxyethidium ($2OHEt^+$, a unique reaction product generated by the interaction between DHE with superoxide anion) (17). Quantification of these peaks revealed that addition of AA was associated with a significant increase in the Et^+ peak ($P < 0.01$) and a corresponding decrease in $2OHEt^+$ (Fig. 1E). These results are in keeping with previous analyses indicating that there is competition between Et^+ and $2OHEt^+$ for binding sites in the sperm nucleus (17). As a consequence of this competition, an increase in one reaction product is associated with a decrease in the other so that the ratio of Et^+ to $2OHEt^+$ changed dramatically after AA addition (Fig. 1F). This change in the relative intensity of Et^+ and $2OHEt^+$ suggested that the ROS generated in response to AA addition includes oxidants as well as superoxide anion, and that the former predominates. In keeping with this suggestion, the AA-induced DHE signal was suppressed by the addition of catalase (Fig. 2A) but not superoxide dismutase (Fig. 2B).

To confirm that addition of AA to purified human sperm populations induced ROS generation, we sought evidence that exposure to this fatty acid was associated with the induction of oxidative stress in these cells. Using the fluorescent probe BODIPY C11, we indeed were able to demonstrate that exposure to AA resulted in significantly elevated levels of lipid peroxidation ($P < 0.001$). The induction of peroxidative damage was dose dependent, detectable within 15 min of AA addition,

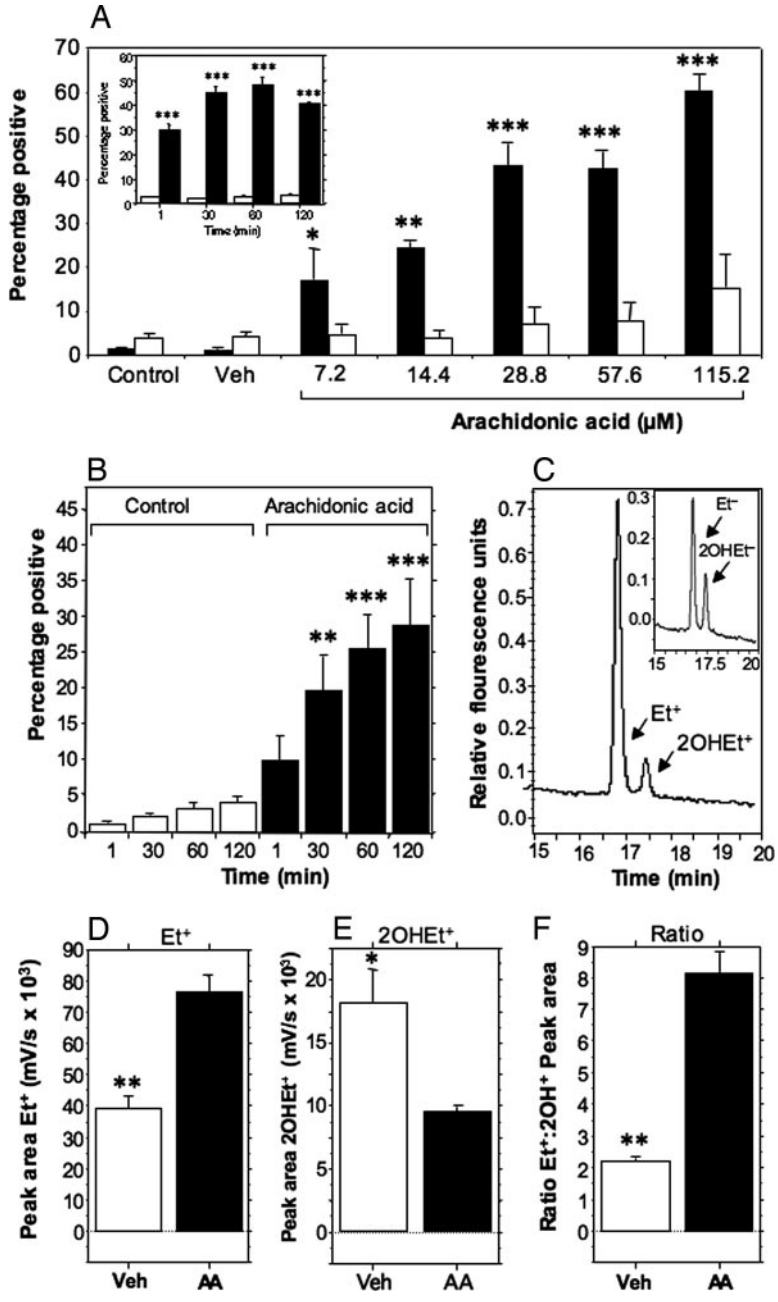

FIG. 1. Impact of AA on ROS generation by human spermatozoa. A, Dose-dependent increase in DHE signal (*closed bars*) in the absence of any significant change in sperm viability (*open bars*). AA was added in a volume of 5–175 μl of sperm suspension containing 2×10^6 cells; 20 μl of DHE/SYTOX Green solution was then added, and the mixture was incubated in the dark for 15 min at 37 C. The *inset* depicts time course studies in which, at the times indicated, DHE/SYTOX Green was added to sperm suspensions treated with AA (28.8 μM) and incubated for a further 15 min at 37 C before being analyzed by flow cytometry. B, Time-dependent increase in DHE positivity for spermatozoa exposed to 28.8 μM AA for 15 min and then washed twice before being incubated for the time specified; control incubations contained vehicle alone. C, Fluorescent chromatogram of the two products, Et^+ and $2OHEt^+$, isolated by HPLC after incubation of spermatozoa with DHE and 28.8 μM AA; the *inset* depicts control incubation with vehicle alone. Note how AA stimulation enhances the relative intensity of the Et^+ peak. D–F, Quantification of the DHE reaction products after their separation by HPLC confirmed that AA stimulation was associated with a significant increase in Et^+ peak (D), a significant decrease in the $2OHEt^+$ peak (E), and a dramatic change in the Et^+ to $2OHEt^+$ ratio (F). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and was significantly stimulated by the addition of a ferrous ion promoter ($P < 0.001$; Fig. 2D). The oxidative stress created after AA addition was also associated with a significant dose ($P < 0.01$; Fig. 2C, *inset*)- and time ($P < 0.05$; data not shown)-dependent loss of sperm motility, an aspect of sperm function that is notoriously sensitive to oxidative damage.

Inhibitors of AA metabolism

Because both lipoxygenase (19–21) and cyclooxygenase (COX) (22) pathways have been implicated in the mechanisms by which AA generates ROS in other cell types, the relative importance of these enzymes was investigated using a panel of inhibitors. Dose-dependent studies with acetylsalicylic acid (a COX 1 inhibitor), NS398 (a COX 2 inhibitor), and SC560 (COX 1 and 2 inhibitor) revealed no significant impact on the DHE signals generated in response to AA (Fig. 3, A–C). The only exception was the highest dose of SC560 used in these studies (100 μM), which did suppress the response to AA significantly ($P < 0.001$). However, contemporaneous analysis of sperm vitality with SYTOX Green revealed that this dose of inhibitor significantly ($P < 0.01$) compromised cell viability (Fig. 3C). Thus inhibition of the AA with SC560 was probably a secondary consequence of inhibitor-induced damage to the plasma membrane. In exactly the same manner, we were also able to demonstrate that addition of the lipoxygenase inhibitor (eicosatetraynoic acid) at doses from 0.5–50 μM has no significant effect on the ROS generation stimulated on exposure to AA (data not shown).

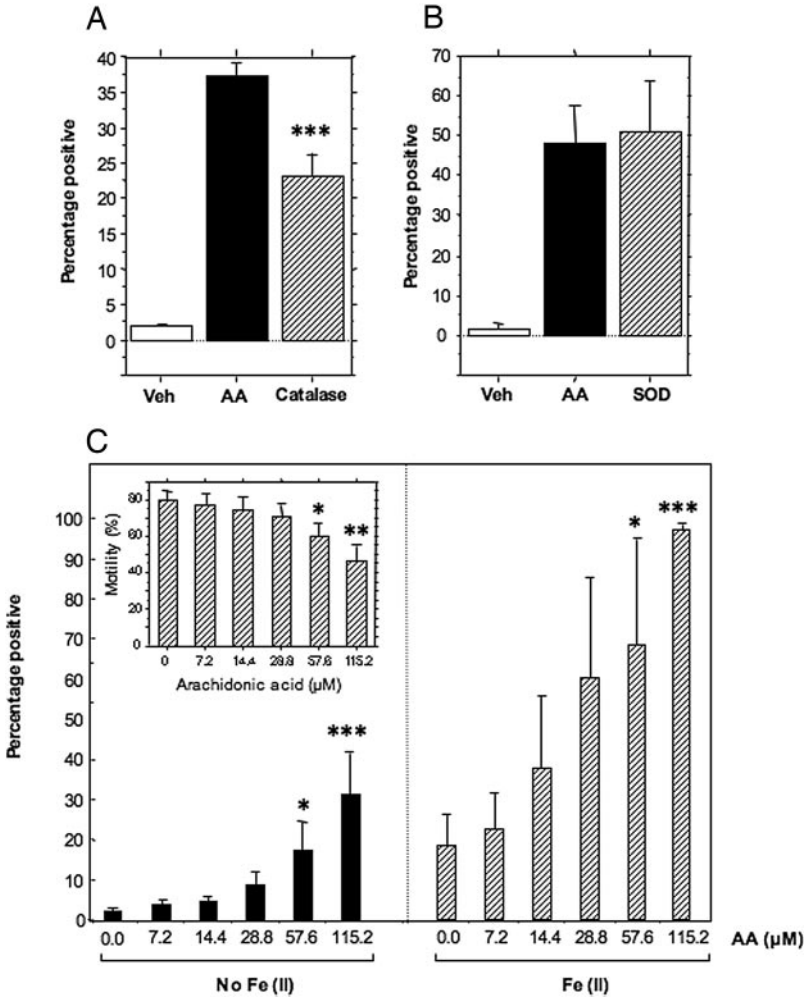

FIG. 2. AA-induced ROS generation and oxidative stress. A, Catalase (3000 U) had a suppressive effect on the generation of ROS in response to AA ($P < 0.001$); whereas superoxide dismutase (SOD) (B) (300 U) has no significant impact. C, Exposure to AA also induced a significant dose-dependent effect on lipid peroxidation in both the presence and absence of a ferrous ion promoter. BODIPY C11 was used as the probe, and the incubation time was 15 min. *Inset*, the addition of AA also induced a significant dose (7.2–115 μM)- and time (15, 60, and 120 min)-dependent loss of motility. Only the dose-dependent data are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Impact of different fatty acids

Because the metabolism of AA via the COX or lipoxygenase pathways did not appear to be relevant to the induction of ROS generation, we investigated whether this property was possessed by the other two major unsaturated fatty acids that are present in human spermatozoa (11, 12), linoleic acid (an omega 6 PUFA possessing two double bonds per molecule) or docosahexaenoic acid (DHA; an omega 3 PUFA possessing six double binds per molecule). Both of these PUFA were effective at stimulating ROS generation by human spermatozoa (Fig. 4, A and B). This effect was observed in the absence of any significant impact on sperm viability, with the exception of the highest dose tested (115 μM), which was clearly cytotoxic, particularly in the case of DHA (Fig. 4, A and B). In contrast, the dominant saturated fatty acids in human spermatozoa, palmitic (Fig. 4C) and stearic acid (Fig. 5A), had no effect on ROS generation by these cells. Direct comparison of the ability of different PUFA to stimulate ROS at a single dose of 10 μM (Fig. 5A) indicated that the more highly unsaturated fatty acids (AA and DHA, four and six double bonds, respectively) were significantly more active than either stearic acid (no double bonds) or linoleic acid (two double bonds per molecule). However, the presence of numerous double bonds was not, of itself, sufficient to stimulate ROS generation because the methyl ester of AA was completely inactive in this regard (Fig. 5B). These results suggested that it must be the amphiphilic properties of the unsaturated fatty acids that are critical for their ROS generating activity. The fact that other, nonspecific, amphiphiles such as cholic acid (Fig. 5C) could not stimulate ROS generation by human spermatozoa emphasized the existence of structural constraints on the ability of amphiphilic molecules to create oxidative stress in the male germ line. This relative specificity distinguishes these cells from phagocytic leukocytes, which can respond to a wide variety of amphiphiles, including sodium dodecyl sulfate (SDS), with the enhanced production of ROS, via mechanisms that can be significantly enhanced by the protein phosphatase inhibitor calyculin A (23). In the case of human spermatozoa, no stimulation of ROS generation was observed with SDS doses from 7.2–115.2 μM, whereas concentrations above this level led to a dramatic loss of cell viability (data not shown). Furthermore, calyculin A (0.1 μM) had no impact on the ability of sublethal concentrations of SDS (50 μM) to stimulate ROS generation by these cells (data not shown).

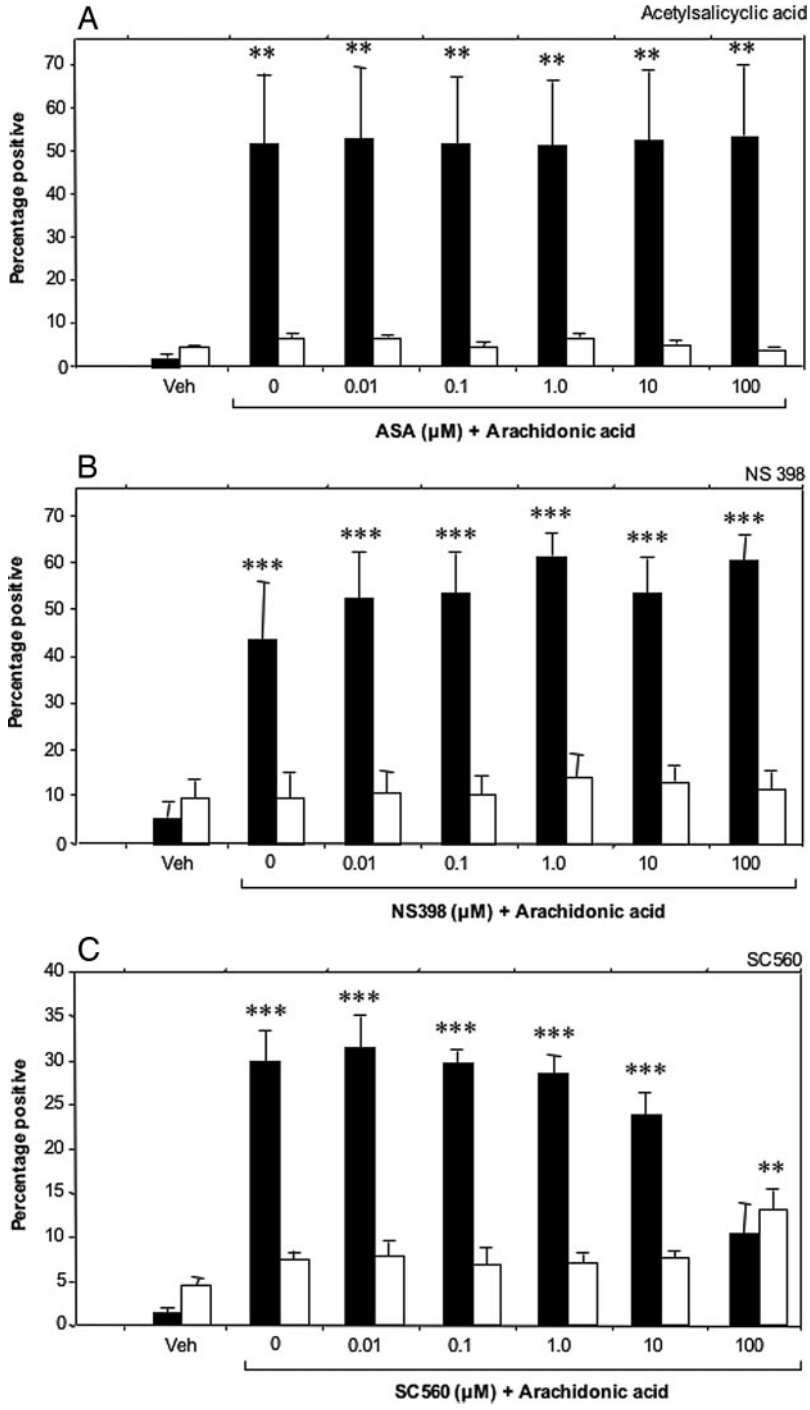

FIG. 3. Impact of AA metabolism inhibitors on ROS generation by human spermatozoa. In all cases, 2×10^6 cells in 170 μl were preincubated with inhibitor for 10 min at 37 C. AA was then added in a volume of 5 μl to produce a final AA concentration of 28.8 μM. DHE/SYTOX Green was then added, and the mixture was incubated in the dark for 15 min at 37 C before the cells were washed once ($600 \times g$ for 5 min) and analyzed by flow cytometry. Inhibitors used were acetylsalicylic acid (ASA) (A) (a COX 1 inhibitor), NS398 (B) (a COX 2 inhibitor), and SC560 (C) (COX 1 and 2 inhibitor). **, $P < 0.01$; ***, $P < 0.001$. *Open bars*, Sperm viability as determined by SYTOX Green; *closed bars*, ROS generation as determined by DHE.

Inhibitors of ROS generating systems

From the foregoing, it is clear that the amphiphilic properties of PUFA are critical for their ROS-inducing potential. However, these data provide no information concerning the mechanisms by which the ROS are generated. To address this issue, inhibitors of known free radical-generating systems were assessed for their ability to suppress the AA response. The reagents tested included the vanilloid inhibitors of nicotinamide adenine dinucleotide plasma membrane electron transport systems, capsaicin (24, 25) and resiniferatoxin (25), as well as inhibitors of the NADPH oxidase system in various cell types such as retinoic acid (26) and two alkylating agents [*N*-ethyl maleimide and p-chloromercuribenzenesulfonic acid (pCMBS)] (27, 28). This analysis revealed that none of these reagents were capable of suppressing the stimulation of ROS generation by AA, although pCMBS did significantly compromise cell viability (Fig. 6A). Similarly, the mitochondrial inhibitor rotenone (10 μM) was unable to inhibit the redox response to AA (data not shown). A possible role for protein kinase C (PKC) was also investigated, because this

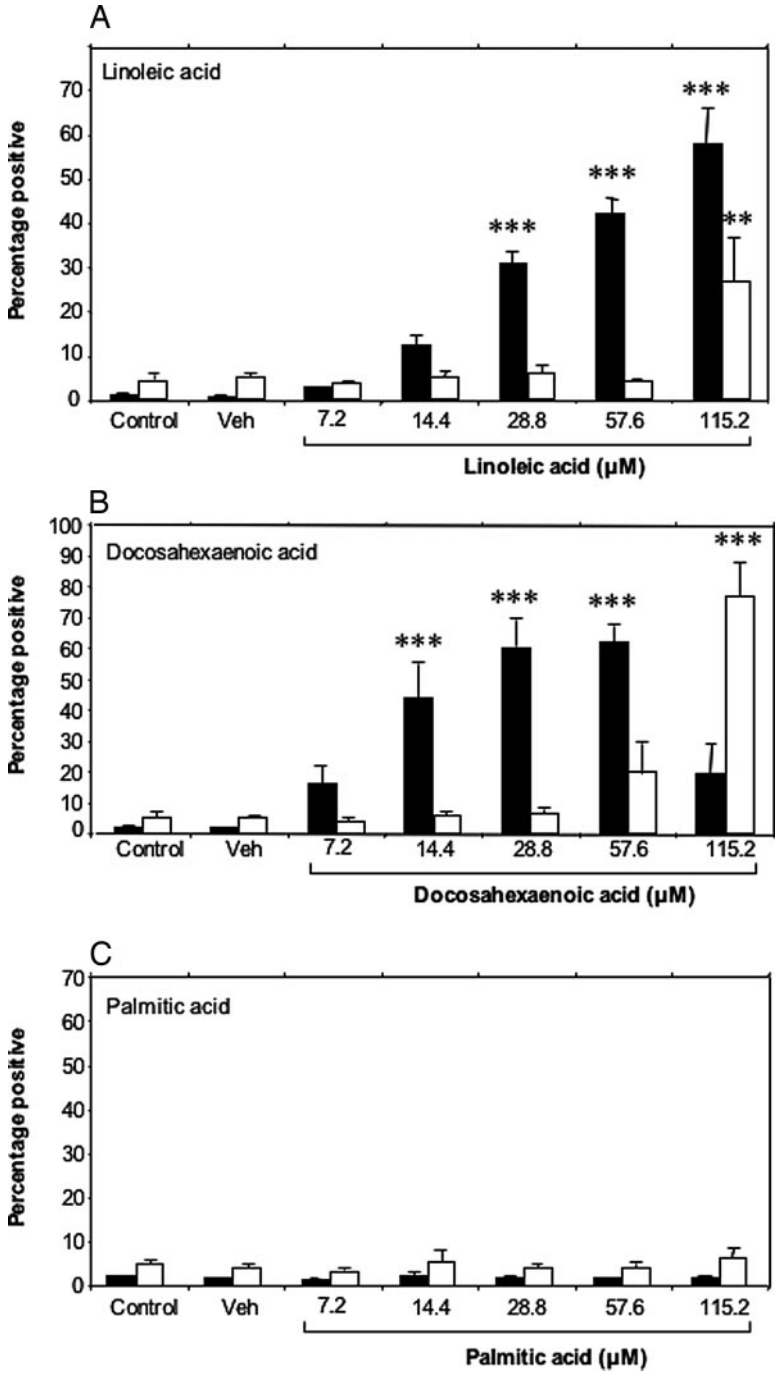

FIG. 4. Analysis of the ability of different fatty acids to stimulate ROS generation by human spermatozoa. Fatty acids (5 μl) were added to 2×10^6 spermatozoa in 175 μl medium followed by DHE/SYTOX Green. These cells were then incubated in the dark for 15 min at 37 C before being washed once (600 $\times$ *g* for 5 min) and analyzed by flow cytometry. A, Linoleic acid; B, docosahexaenoic acid; C, palmitic acid. **, $P < 0.01$; ***, $P < 0.001$. *Open bars*, Sperm viability as determined by SYTOX Green; *closed bars*, ROS generation as determined by DHE.

kinase has been shown to activate NADPH oxidase activity in human leukocytes via mechanisms that can be inhibited by rottlerin (29). In the case of human spermatozoa, high doses of rottlerin (10 μM) actually stimulated ROS generation ($P < 0.001$) and had no significant effect on the response to AA (Fig. 6B). Other PKC inhibitors such as bisindolylmaleimide (1 and 10 μM) and staurosporine (1 and 10 μM) had no significant effect on the ability of AA to stimulate ROS generation by human spermatozoa (data not shown).

To gain more insights into the mechanisms by which ROS were generated in response to AA, a cell-free system was developed using an isolated sperm membrane preparation as a source of putative oxidase, NADPH as a source of electrons and lucigenin as a detection reagent. Under these circumstances, addition of NADPH induced a highly significant redox response that was dramatically enhanced by the presence of AA in a manner than could be readily reversed by the presence of DPI, a flavoprotein inhibitor (Fig. 6C).

Discussion

Spermatozoa are extremely susceptible to ROS because they are richly endowed with substrates for free radical attack and highly deficient in protective enzymes, such as

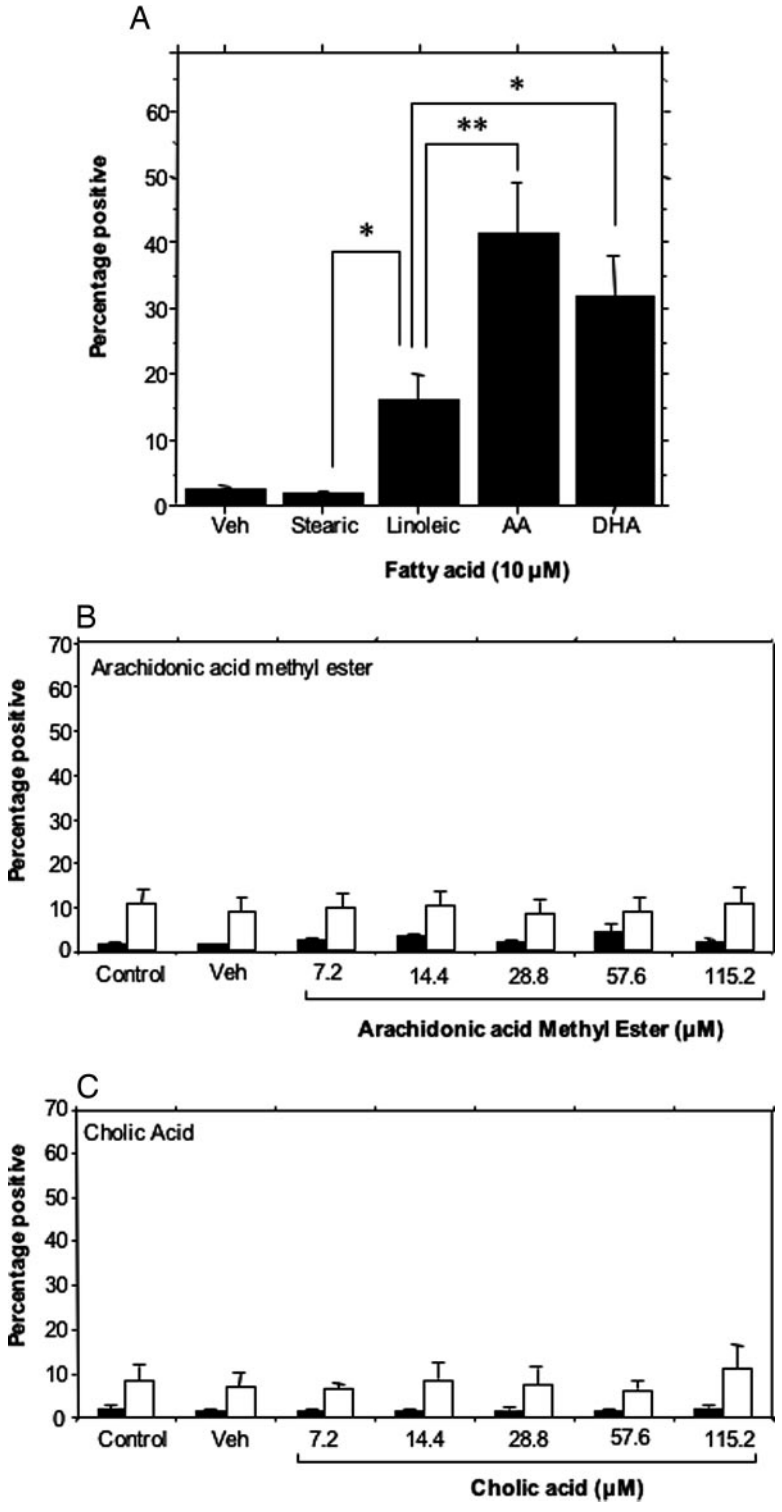

FIG. 5. Comparative analysis of the ability of fatty acids and amphiphiles to stimulate ROS generation by human spermatozoa. A, Comparative analysis of the impact of stearic, linoleic, arachidonic, and docosahexaenoic acids on the DHE signal (10 μM for 15 min), illustrating the greater effectiveness of the more highly unsaturated fatty acids. ANOVA revealed a highly significant overall difference due to treatment ($P < 0.001$); differences between group means are indicated as determined by Fisher's protected least significant difference test. B, The methyl ester of AA lacked the ability to stimulate ROS generation. C, A nonspecific amphiphile, cholic acid, also lacked any prooxidant activity. *, $P < 0.05$; ** $P < 0.01$. *Open bars*, Sperm viability as determined by SYTOX Green; *closed bars*, ROS generation as determined by DHE.

superoxide dismutase and catalase, because they have discarded most of their cytoplasm during spermiogenesis. The only other biochemical signature of these defective cells is that they contain a superabundance of PUFA, particularly 20:4, 22:5, and 22:6 (11, 12). It is believed that such high concentrations of PUFA reflect a state of developmental immaturity on behalf of the spermatozoa. The spermatozoa released from the germinal epithelium of the testes are loaded with unsaturated fatty acids (30) and enter the epididymis in a functionally incompetent state, lacking the capacity for both coordinated movement and interaction with the oocyte. In many ways the phenotype of these immature spermatozoa is reflective of the defective gametes encountered in infertile males. These cells are also functionally in-

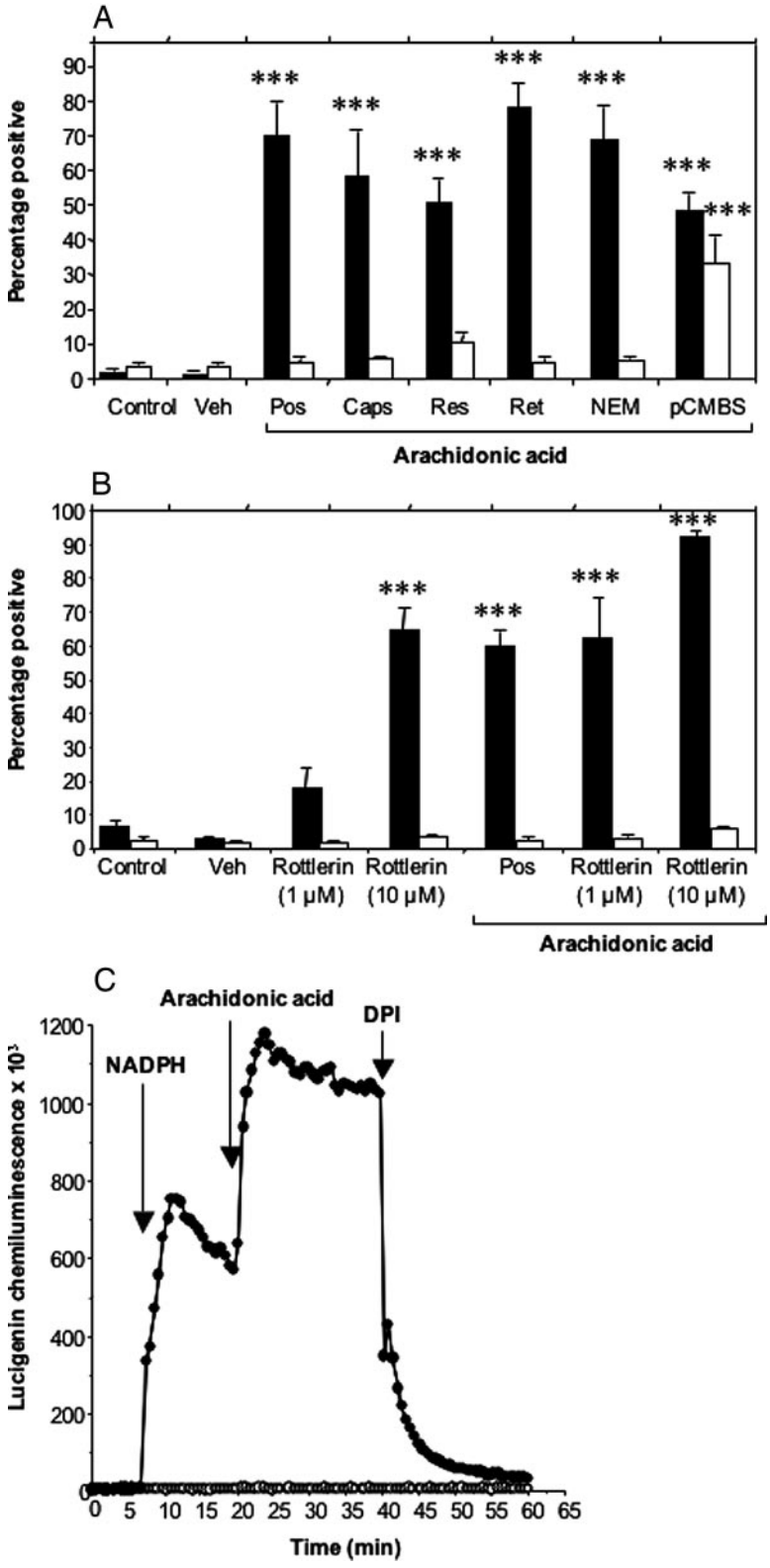

FIG. 6. Inhibitors of the ROS generating system. A, The ability of AA to stimulate ROS generation in human spermatozoa was not disrupted by a number of known inhibitors of plasma membrane redox systems, when used at doses that have proven effective in other cell types, and including capsaicin (Caps; 100 μM), resiniferatoxin (Res; 10 μM), retinoic acid (Ret; 20 μM) *N*-ethyl maleimide (NEM; 100 μM), and pCMBS (100 μM). B, Impact of the protein kinase C inhibitor, rottlerin, on human spermatozoa in the presence and absence of AA (28.8 μM). C, Cell-free system illustrating the impact of AA (28.8 μM) on the NADPH-induced redox activity recorded in sperm membrane preparations, illustrating the stimulatory impact of this fatty acid on putative oxidase activity and the powerful suppressive effect of DPI (10 μM), a flavoprotein inhibitor. Representative of three replicate experiments. Control, No treatment; Veh, vehicle alone; Pos, positive control containing AA alone. ***, $P < 0.001$. *Open bars*, Sperm viability as determined by SYTOX Green; *closed bars*, ROS generation as determined by DHE.

competent and loaded with PUFA such as AA and DHA. During epididymal transit the acquisition of functional competence is accompanied by a remodelling of the plasma membrane that involves the selective depletion of phospholipids, a loss of PUFA and an increase in the sterol to phospholipid ratio (30). Indeed, the total fatty acid content of testicular spermatozoa is around six times higher than cells recovered from the epididymis whereas the DHA content is

around three times higher. The observation that defective human spermatozoa recovered from the low density region of Percoll gradients are also characterized by a high cellular content of DHA (11, 12) suggests that these functionally impaired cells are in a developmentally immature state. This conclusion is also supported by the fact that these spermatozoa exhibit several additional signs of immaturity including poor chromatin compaction (31), the presence of excess residual cytoplasm (32), and high levels of HspA2 chaperone expression (33). Because defective spermatozoa are also characterized by the excessive generation of ROS (34), we examined whether there was a causative relationship between this activity and the fatty acid composition of these cells.

In our initial experiments, addition of AA to human spermatozoa was found to induce a state of oxidative stress characterized by the generation of ROS, a significant increase in lipid peroxidation, and a concomitant loss of sperm motility. Analysis of the products of DHE oxidation by HPLC indicated that the major product generated in the presence of AA is an oxidant capable of effecting the two-electron oxidation of DHE to Et^+. Although human spermatozoa can clearly generate superoxide anion under certain circumstances, such as exposure to redox cycling quinones (17), in the presence of PUFA other oxidants appear to predominate. The specific nature of this oxidant is unclear although the suppressive effect of catalase suggests that hydrogen peroxide is involved, even though DHE is relatively insensitive to direct oxidation by this particular oxidant (35).

These results are similar to those recorded with vascular smooth muscle cells, which also respond to an unsaturated (oleic acid) but not saturated (stearic acid) fatty acids, with hydrogen peroxide production (36). Unsaturated fatty acids have also been shown to stimulate ROS generation by phagocytic leukocytes; however, in this case, the primary target is the NADPH oxidase, NOX2, which effects the one-electron reduction of oxygen to generate superoxide anion (29, 37). In both smooth muscle cells and leukocytes, PKC was shown to be an important mediator of ROS generation. However, in spermatozoa, AA-induced ROS was not suppressed by the concomitant presence of several different PKC inhibitors and was even stimulated by rottlerin, a potent inhibitor of NADPH oxidase activity in monocytes (29). This difference, together with the inability of SDS to activate ROS generation in spermatozoa, the lack of response to calyculin A, and the inactivity of a range of nicotinamide adenine dinucleotide oxidoreductase inhibitors (Fig. 6), suggests that whatever pathways are driving ROS production in PUFA-treated spermatozoa, they are not those encountered in somatic cell types.

Alternative biochemical routes for ROS generation, particularly in the case of AA, involve metabolism of this fatty acid through the COX or lipoxygenase pathways (19–22). However, inhibitors of these enzymes failed to have any impact on AA-induced ROS production by human spermatozoa (Fig. 3). The involvement of alternative pathways was also suggested by the observation that ROS generation could be stimulated by other unsaturated fatty acids, including linoleic acid and DHA, the two dominant unsaturated fatty acids in defective human spermatozoa (11, 12). The kinked, amphiphilic configuration of these *cis*-unsaturated fatty acids appeared to be critical for ROS generation because both saturated fatty acids (palmitic, stearic) and unsaturated fatty acid esters were without effect. The amphiphilic properties of AA have also been shown to be involved in the ability of this fatty acid to stimulate ROS generation by the NADPH oxidase of phagocytic leukocytes (37). In this case, the targets for this amphiphile action appear to be p47 (phox) (38) and the small GTPase, Rac (23). In addition, AA has been shown to bind to the cytochrome b558 flavoprotein, inducing a conformational change in this molecule (39) and increasing its affinity for oxygen (40). Intriguingly, the methyl ester of AA is incapable of inducing these critical conformational changes in the cytochrome b558 constituent of phagocyte NADPH oxidase (39), reflecting the situation observed with spermatozoa. Notwithstanding this similarity, the impact of amphiphilic compounds on ROS generation in spermatozoa differs from the effects observed in phagocytes in being more selective. Thus, whereas a range of amphiphilic compounds can stimulate ROS generation in leukocytes (40), reagents such as SDS or cholic acid are without effect in spermatozoa (Fig. 5).

In conclusion, these data are consistent with the hypothesis that defective sperm function is associated with oxidative stress triggered by the presence of abnormally high concentrations of unsaturated fatty acid. Research into the mechanisms by which spermatozoa remodel their lipid profiles during posttesticular maturation may shed light on the etiology of defective sperm function and facilitate the clinical management of this condition.

Acknowledgments

Received June 19, 2006. Accepted July 31, 2006.

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We gratefully acknowledge the financial support provided by the Australian Research Council Centre of Excellence in Biotechnology and Development.

Disclosure summary: The authors have nothing to disclose.

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