Caspase 3 activation in human spermatozoa in response to hydrogen peroxide and progesterone

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Objective: To determine the role of calcium signaling on apoptosis evoked by the reactive oxygen species H_2O_2 and by the physiological agonist P in human ejaculated spermatozoa.
Design: Laboratory study.
Setting: Center for assisted human reproduction in a hospital in Spain.
Patient(s): Forty-five healthy volunteers.
Intervention(s): Spermatozoa were treated with increasing concentrations of hydrogen peroxide (H_2O_2; 10 μM, 100 μM, and 1 mM) or with 20 μM of P for 5–120 minutes.
Main Outcome Measure(s): Activation of caspase-3 and -9 as well as phosphatidylserine externalization were examined in human ejaculated spermatozoa by fluorescence methods.
Result(s): Hydrogen peroxide and P induced activation of caspase-3 and -9. In addition, the effect of H_2O_2 and P was time dependent. Dimethyl-1,2-bis (aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid loading was able to inhibit H_2O_2- and P-induced caspase-3 activation and phosphatidylserine externalization. Pretreatment of spermatozoa with Ru360, to block the calcium uptake into mitochondria, also was able to decrease the activation of caspase-3 and phosphatidylserine exposure that was stimulated by either H_2O_2 or P.
Conclusion(s): These findings suggest that H_2O_2- and P-induced mitochondrial apoptosis is dependent on calcium signaling. (Fertil Steril® 2008;90:1340–7. ©2008 by American Society for Reproductive Medicine.)

Key Words: Apoptosis, caspases, calcium, H_2O_2, progesterone, spermatozoa

One of the earliest and most consistently observed features of apoptosis is the activation of a series of cytosolic cysteine proteases, called caspases, which cleave multiple protein substrates en masse, leading to the loss of cellular structure and function and ultimately resulting in cell death (1). The caspase family consists of $\geq$14 enzymes in mammalian cells. In particular, caspases 3, 8, and 9 play a relevant role in apoptosis: caspase-9, in the mitochondrial pathway; caspase-8, in the Fas/CD95 pathway; and caspase 3, more downstream, which is an executioner caspase activated by multiple pathways (2).

Traditionally, two general pathways to apoptosis have been described. One is the *extrinsic pathway*, initiated by the binding of an extracellular death ligand, such as Factor activating Exo S ligand (FasL), to its cell-surface death receptor, such as Fas (3). The second pathway is the *intrinsic pathway,* which is mediated by mitochondrial alterations. In response to apoptotic stimuli, several proteins are released from the intermembrane space of mitochondria into the cytoplasm (4). Some of the well-characterized proteins include cytochrome *c,* which mediates the activation of caspase 9 (5), triggering a cascade of caspase activation, including caspase 3, which promotes cellular apoptosis.

Up until now, it has been unclear whether apoptosis in ejaculated spermatozoa takes place in a similar way as in somatic cells or whether spermatozoa, which are thought to have a transcriptionally inactive nucleus, undergo abortive forms of this process (6). However, whereas calcium is a key regulator of cell survival, the sustained elevation of intracellular calcium plays a role in cell death (7). Cytosolic calcium concentration ($[Ca^{2+}]_c$) is a key regulatory factor for a large number of cellular processes such as muscle contraction, metabolism, secretion, or even cell differentiation and apoptosis. Numerous hormones, neurotransmitters, and growth factors activate the plasma membrane phospholipase C, which generates inositol-1,4,5-triphosphate and diacylglycerol. Inositol-1,4,5-triphosphate, in turn, releases calcium from intracellular pools, thereby initiating the calcium signal. It has traditionally been thought that the intracellular pools of calcium are located in the endoplasmic reticulum and in the mitochondria (8). During calcium mobilization, $[Ca^{2+}]_i$ is reduced to resting level by reuptake into internal pools (9) and calcium extrusion through a plasma membrane calcium–adenosine triphosphatase pump (10). The release of calcium from intracellular pools also activates calcium entry across the plasma membrane (11).

The proapoptotic effects of calcium are mediated by a diverse range of calcium-sensitive factors that are compartmentalized in various intracellular organelles, including

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endoplasmic reticulum and mitochondria (12). Excessive calcium load to the mitochondria may induce apoptosis by stimulating the release of apoptosis-promoting factors from the mitochondrial intermembrane space to the cytoplasm and by impairing mitochondrial function (13).

Apoptosis also can be stimulated by reactive oxygen species (ROS) in several cell types (14–16). It has been reported recently that ROS can mediate the apoptosis induced by either growth factors, such as transforming growth factor-β in human lens epithelial cells (17), or classical agonists, such as thrombin in human platelets (18). Interestingly, ROS-mediated apoptosis induced by hydrogen peroxide (H_2O_2) in somatic cells is Fas independent and requires the release of mitochondria-derived ROS and activation of caspase-3 (19).

Although ROS and calcium have been separately reported to be important mediators of apoptosis, no correlation between these two mediators has been reported. Here, we focused on the role of calcium on H_2O_2- or P-induced mitochondrial apoptosis in human ejaculated spermatozoa and investigated the effects of the calcium chelator BAPTA-AM and of the mitochondrial calcium uptake blocker Ru360 on H_2O_2- and P-evoked apoptosis, including caspase-3 and -9 activation, and phosphatidylserine (PS) externalization.

MATERIALS AND METHODS

Materials

Progesterone, H_2O_2, 3-[(3-chomalidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC), dithiothreitol, RPMI-1640 medium, and dimethyl BAPTA were from Sigma (Madrid, Spain). *N*-acetyl-Leu-Glu-His-Asp-7-amido-4-methylcoumarin (AC-LEHD-AMC) and z-Leu-Glu-His-Asp-7-amido-4-fluoromethylketone (z-LEHD-FMK) were from Bachem (Weil am Rhein, Germany). Ru360 was from Calbiochem (Nottingham, United Kingdom). All other reagents were of analytical grade.

Spermatozoa Preparation

Human semen was obtained from healthy volunteers at the Extremadura Center of Human Assisted Reproduction (Badajoz, Spain), as approved by local committees and in accordance with the Declaration of Helsinki. This study was approved by the institutional review board of the University of Extremadura and by the ethics committee of the Infantile Hospital (Badajoz, Spain). Each subject was ascertained to be in good health by means of their medical history and a clinical examination including routine laboratory tests and screening. The subjects all were nonsmokers, were not using any medication, and abstained from alcohol. Informed consent was obtained from all participants. Samples were collected by masturbation after 4–5 days of sexual abstinence and were allowed to liquefy at 37°C for 30 minutes. Semen was washed twice in RPMI medium (250 g, 10 min), the supernatant was discarded, and the sperm pellet was resuspended in Na-HEPES solution containing the following (in mM): NaCl, 140; KCl, 4.7; $CaCl_2$, 1.2; $MgCl_2$, 1.1; glucose, 10; and HEPES, 10 (pH 7.4). The classical semen parameters of spermatozoa concentration, motility, and morphology were examined according to the 1999 World Health Organization criteria (20). The ejaculates from donors had the following criteria: spermatozoa concentration, $>20 \times 10^6$ cells per milliliter; ratio of spermatozoa to leukocytes, >100:1; spermatozoa with normal morphology, >15% (strict criteria); and progressively motile spermatozoa, >50% (World Health Organization grades a+b, where a and b indicate rapid and slow progressively motile spermatozoa, respectively).

Caspase Activity Assay

To determine caspase activity, stimulated or resting cells were sonicated, and cell lysates were incubated with 2 mL of substrate solution (20 mM HEPES, pH 7.4; 2 mM ethylenediaminetetraacetic acid, 0.1% CHAPS, 5 mM dithiothreitol, and 8.25 μM of caspase substrate) for 1 hour at 37°C, as described elsewhere (21). Substrate cleavage was measured by using a fluorescence spectrophotometer with excitation wavelength of 360 nm and emission at 460 nm. Preliminary experiments reported that caspase-3 or -9 substrate cleaving was not detected in the presence of the inhibitors of caspase-3 or -9, DEVD-CMK or z-LEHD-FMK, respectively. The activities of caspase-3 and -9 were calculated from the cleavage of the respective specific fluorogenic substrate (AC-DEVD-AMC for caspase-3 and AC-LEHD-AMC for caspase-9). The data were calculated as fluorescence units per milligram of protein and were presented as percentage above control (untreated samples).

Determination of PS Externalization

The PS externalization of resting and stimulated cells was determined according to a procedure published elsewhere (18). Briefly, cells were stimulated in HEPES-buffered saline. Samples of cell suspensions (500 μL) were transferred to 500 μL of ice-cold 1% (wt/vol) glutaraldehyde in phosphate-buffered saline for 10 minutes. Cells then were incubated for 10 minutes with annexin V– fluorescein isothiocyanate conjugate (0.6 μg/mL) in phosphate-buffered saline that was supplemented with 0.5% (wt/vol) bovine serum albumin and 2 mM $CaCl_2$. After incubation, the cells were collected by centrifugation for 60 seconds at 10,000 $\times$ *g* and were resuspended in phosphate-buffered saline. Cell staining was measured by using a Hitachi spectrofluorometer (Tokyo, Japan). Samples were excited at 488 nm, and emission was recorded at 516 nm. The data were calculated as fluorescence units per milligram of protein and presented as percentage above control (untreated samples).

Measurement of Intracellular Free Calcium Concentration ($[Ca^{2+}]_c$)

Cells were loaded with fura-2 by incubation with 4 μM fura-2-acetoxymethyl ester for 30 minutes at room temperature, according to a procedure published elsewhere (22). Once

loaded, the cells were washed and used within the next 2–4 hours. Fluorescence was recorded from 2-mL aliquots of magnetically stirred cellular suspension (2×10^8 cells/mL) at 37°C by using a Hitachi spectrophotometer with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were monitored by using the fura-2 340:380 nm fluorescence ratio and were calibrated according to the method of Grynkiewicz et al. (23). The experiments were performed in a calcium-free medium, and ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (1 mM) was added.

Statistical Analysis

Analysis of statistical significance was performed by using the Student's *t*-test. $P<.05$ was considered to indicate a statistically significant difference. For multiple comparisons, one-way analysis of variance was used.

RESULTS

Hydrogen Peroxide and P Induce Activation of Caspase-3 and -9 in Human Spermatozoa

To examine the effect of H_2O_2 on caspase-3 activation, human spermatozoa were treated with increasing concentrations of H_2O_2 (10 μM, 100 μM, and 1 mM) for 5–120 minutes. As shown in Figure 1A, treatment of spermatozoa with H_2O_2 for 30 minutes induced an activation of caspase-3, without a clear relationship to dose response. Cell stimulation with H_2O_2 caused the maximum effect at a dose of 10 μM (± SEM; 20.1% ± 0.8% above control, $P<.05$). In addition, the treatment with 20 μM P for 30 minutes also increased the caspase-3 activity (Fig. 1A). Activation of caspase-3 by P was significantly greater ($P<.05$) in magnitude than were the increases obtained with H_2O_2.

As shown in Figure 1B, the effect of H_2O_2 on caspase-3 activation was time dependent, with maximal caspase activity reached after 60 minutes of stimulation with the dose of 10 μM H_2O_2 (30.6% ± 0.9% above control, $P<.05$). After this point, caspase activity decreased. Therefore, 60 minutes of incubation and 10 μM H_2O_2 were the incubation time and concentration that were used throughout this study. The effect of 20 μM P also was time dependent, as shown in Figure 1C, reaching the maximal effect after 120 minutes of incubation.

Caspase-9 is an initiator caspase that is involved in the initial steps of mitochondrial apoptosis (5). To investigate whether the activation of caspase-3 by H_2O_2 is a mitochondrial apoptosis, we checked the caspase-9 activity in the presence of H_2O_2. As shown in Figure 2A, treatment of spermatozoa with 10 μM H_2O_2 for 60 minutes induced a significant increase of caspase-9 activity. In addition, P, at 20 μM for 60 minutes, also was able to induce activation of caspase-9. We also investigated whether the activation of caspase-3 is dependent on caspase-9 activity by using the specific inhibitor of caspase-9, z-LEHD-FMK (24). Cell incubation for 90 minutes with 100 μM z-LEHD-FMK abolished H_2O_2-induced caspase-9 activation (Bejarano et al., unpublished data). Consistent with the results just presented, incubation with the caspase-9 inhibitor was able to block either H_2O_2- and P-evoked caspase-3 activation (Fig. 2B). These findings strongly suggest that H_2O_2 and the physiological agonist P induce activation of caspase-3 in a manner dependent on caspase-9 and mitochondria.

FIGURE 1

Concentration dependence and time course of H_2O_2- and P-induced activation of caspase-3. Human spermatozoa were stimulated for 30 minutes with increasing concentrations of H_2O_2 (10 μM, 100 μM, and 1 mM; **A**) or for various periods of time (5, 15, 30, 60, and 120 min) with 10 μM H_2O_2 (**B**) or with 20 μM P (PROG; **C**). Caspase-3 activity was estimated as described in Materials and Methods. Values are presented as means ± SEM of 5–10 separate experiments and expressed as percentage above control (untreated samples). *$P<.05$, compared with control values. ■$P<.05$, comparing the two groups indicated by each end of the horizontal line.

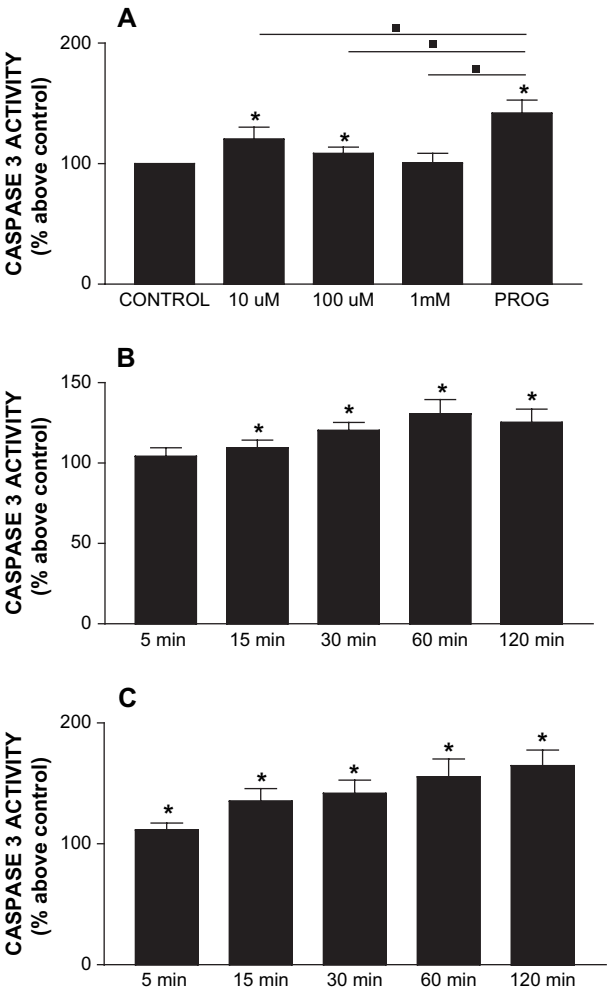

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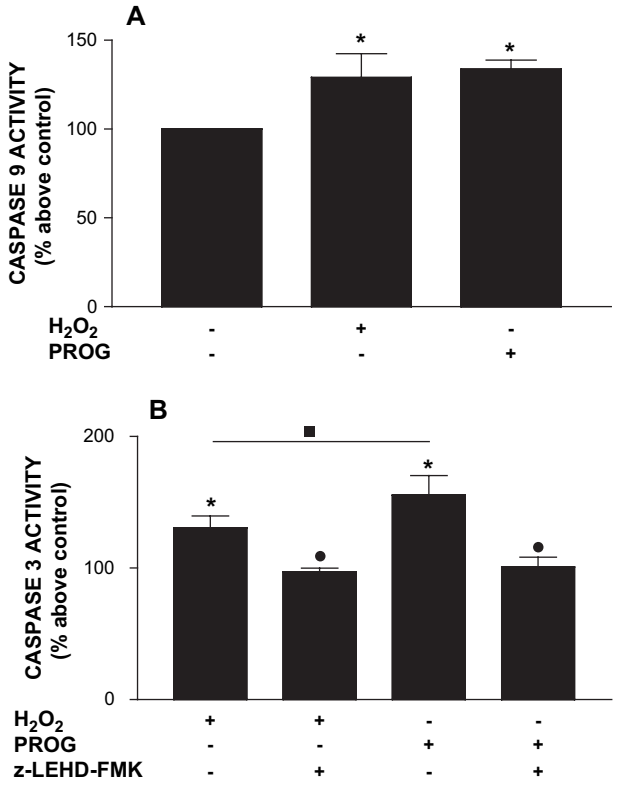
FIGURE 2

Hydrogen peroxide and P induce mitochondrial apoptosis in human spermatozoa. Cells were stimulated for 60 minutes with 10 μM H_2O_2 or 20 μM P (PROG). Caspase-9 (**A**) and -3 (**B**) activities were analyzed after the caspase activity assay on the basis of the cleavage of their respective specific fluorogenic substrates, as described in Materials and Methods. (**B**) Human spermatozoa were preincubated at 37°C, either for 90 minutes with 100 μM z-LEHD-FMK (caspase-9 inhibitor) or the vehicle, and then were stimulated with 10 μM H_2O_2 or 20 μM P. Values are presented as means ± SEM of five to eight separate experiments and expressed as percentage above control (untreated samples). *$P<.05$, compared with control values. ●$P<.05$, compared with H_2O_2 or P alone. ■$P<.05$, comparing the two groups indicated by each end of the horizontal line.



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Involvement of Calcium in H_2O_2- and P-Induced Caspase-3 Activation

It has been reported that a prolonged elevation in $[Ca^{2+}]_c$ and alterations in calcium homeostasis initiate the mitochondrial apoptotic pathway (7) and induce endoplasmic reticulum stress that, in turn, leads to apoptosis (25). In the absence of extracellular calcium concentration (calcium-free medium), stimulation of spermatozoa with 10 μM H_2O_2 caused a slow and sustained $[Ca^{2+}]_c$ increase, which reached a stable $[Ca^{2+}]_i$ plateau after 10–15 minutes of stimulation (Fig. 3A), reflecting the release of calcium from intracellular stores.

To determine the role of rises in $[Ca^{2+}]_c$ in caspase-3 activation, we performed a series of experiments in which spermatozoa were loaded with dimethyl BAPTA-AM, an intracellular calcium chelator, by incubating the cells for 30 minutes at 37°C with 10 μM dimethyl BAPTA-AM. As expected, dimethyl BAPTA loading prevented H_2O_2-evoked $[Ca^{2+}]_c$ elevations (Fig. 3A) and significantly reduced H_2O_2-induced caspase-3 activation. As shown in Figure 4A, after stimulation with 10 μM H_2O_2 for 60 minutes, the activity of caspase-3 was 2.3% ± 0.5% above control in dimethyl BAPTA–loaded cells and was 30.6% ± 0.9% above control in the absence of dimethyl BAPTA (Fig. 4A). These findings indicate that changes in $[Ca^{2+}]_c$ are required for H_2O_2-induced caspase-3 activation.

To further investigate the role of $[Ca^{2+}]_c$ in caspase activation, we used P, a calcium-mobilizing classical agonist, to deplete the intracellular calcium stores. Treatment of spermatozoa, in a calcium-free medium, with 20 μM P induced a transitory rise in $[Ca^{2+}]_c$ by depletion of the intracellular calcium stores, which was prevented by dimethyl BAPTA loading (Fig. 3B). In cells loaded with dimethyl BAPTA, the effect on caspase-3 activation of 20 μM P for 60 minutes was smaller than that observed in the cell's respective control (in P-stimulated cells, the activity of caspase-3 was 8.1% ± 0.2% above control in dimethyl BAPTA–loaded cells and was 55.4% ± 1.5% above control in the absence of dimethyl BAPTA; Fig. 4A).

Prevention of H_2O_2- and P-Induced Apoptosis by the Blockade of Calcium Uptake Into Mitochondria

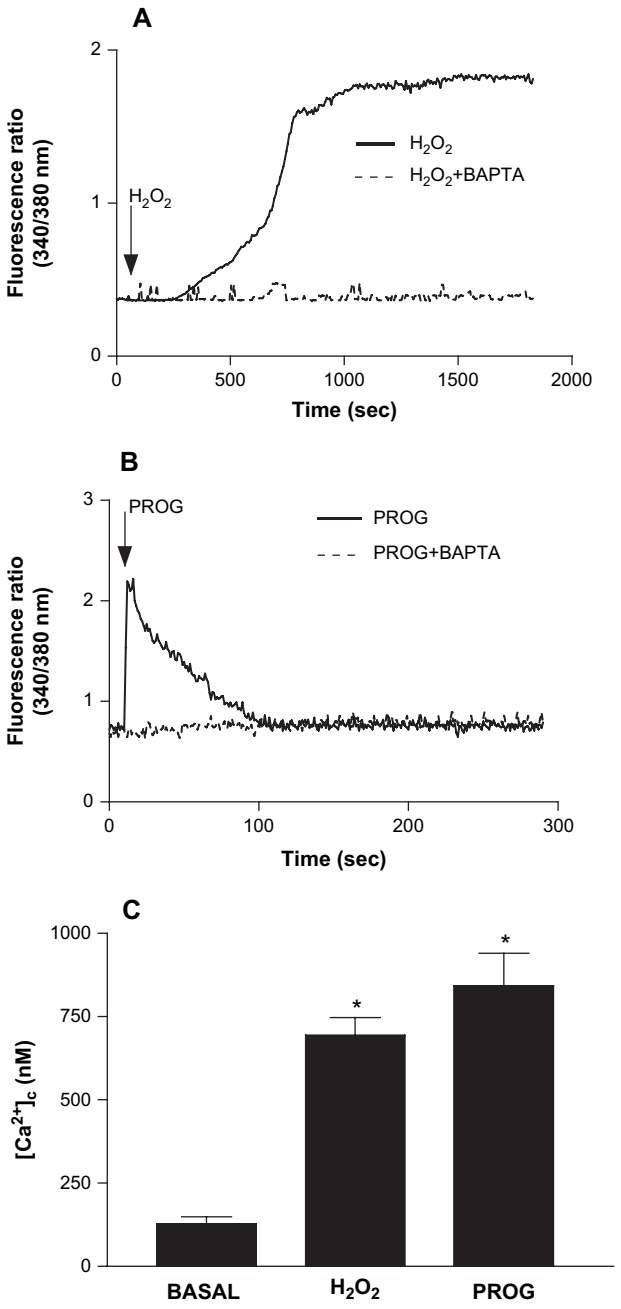
To determine whether H_2O_2-induced apoptotic signals are mediated by the calcium uptake into mitochondria, we investigated the effect of the blocker of calcium entry into mitochondria Ru360 by preincubating the cells for 30 minutes with 10 μM Ru360. As shown in Figure 4A, the blockade of mitochondrial calcium entry significantly ($P<.05$) decreased H_2O_2-induced caspase-3 activation. In Ru360-treated cells, the caspase-3 activity evoked by 10 μM H_2O_2 for 60 minutes was similar in comparison with the control cells (Fig. 4A). Similar results were obtained when apoptosis signals were stimulated with 20 μM P for 60 minutes. In the presence of Ru360, P-evoked caspase-3 activation was significantly ($P<.05$) inhibited (Fig. 4A).

Hydrogen Peroxide and P Induce PS Exposure in Human Spermatozoa

To investigate whether the activation of caspase-3 by H_2O_2 is an apoptotic event, we checked the apoptotic state of spermatozoa by testing PS exposure. As shown in Figure 4B, treatment of spermatozoa with 10 μM H_2O_2 or 20 μM P for 60 minutes induced PS externalization. Spermatozoa loading with dimethyl BAPTA or the blockade of calcium uptake into

FIGURE 3

Mobilization of calcium in response to H_2O_2 and P in human spermatozoa. Cells were stimulated with 10 μM H_2O_2 (**A**) or 20 μM P (PROG; **B**) in calcium-free solution (EGTA, 1 mM), in the presence or absence of 10 μM dimethyl BAPTA. Traces are representative of five to nine independent experiments. (**C**) Histogram of the mean post-stimulus (peak value) $[Ca^{2+}]_c$ under different experimental conditions in five to nine independent experiments. Values are means ± SEM. *$P<.05$.



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the mitochondria by pretreatment with Ru360 (10 μM for 30 min) significantly reduced PS externalization evoked by either H_2O_2 or P, which indicates that $[Ca^{2+}]_c$ is involved in both responses.

We have further investigated whether H_2O_2-mediated PS exposure requires activation of caspase-9 by using a specific caspase-9 inhibitor, z-LEHD-FMK. As shown in Figure 4B, spermatozoa treatment for 90 minutes with 100 μM z-LEHD-FMK abolished H_2O_2-induced PS exposure. Similarly, we also found that P-induced PS externalization was dependent on caspase-9 activation.

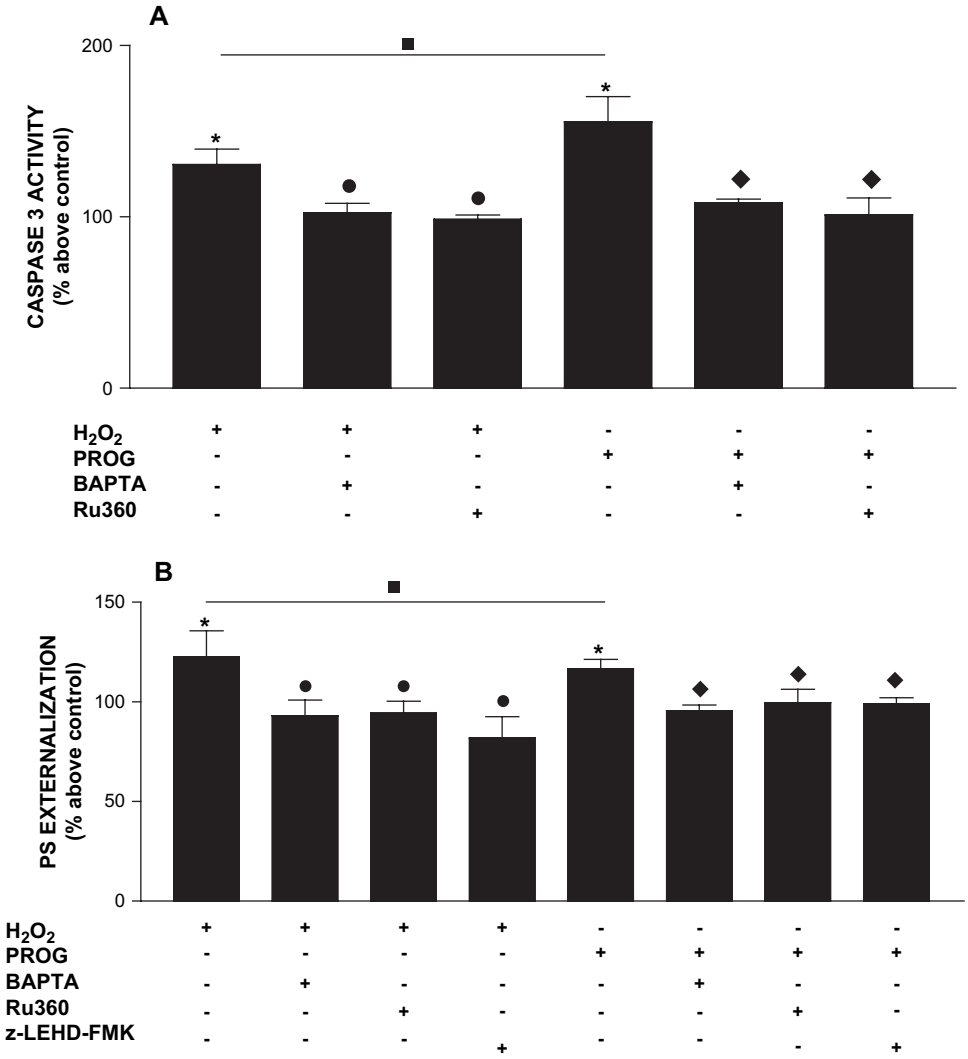
DISCUSSION

This study was designed to determine whether calcium-mediated death signals are involved in ROS-induced cell death, because calcium and ROS have been reported to be critical apoptosis-eliciting mediators. Our studies demonstrate that either H_2O_2 or the calcium-mobilizing agonist P induce apoptotic events in human ejaculated spermatozoa that are mostly of mitochondrial origin, such as caspase-9 activation, and that this apoptosis was mediated by the calcium signaling. In addition, the blockade of mitochondrial calcium entry by pretreatment of the cells with Ru360 showed protection against H_2O_2- and P-induced apoptosis.

Measurements of $[Ca^{2+}]_c$ showed that H_2O_2 increased $[Ca^{2+}]_c$ in the absence of extracellular calcium, indicating that H_2O_2 mobilizes calcium from intracellular stores in human spermatozoa. Similar results have been obtained by us in other cellular types, such as mouse pancreatic acinar cells (26, 27), human platelets (28, 29) and neutrophils (30), and rat hippocampal astrocytes (31). Sustained $[Ca^{2+}]_c$ elevation has been recognized as a trigger of apoptosis cell death (7, 32). In addition, calcium transient evoked by calcium-mobilizing classical agonists, such as thrombin in human platelets or cholecystokinin in pancreatic acinar cells, also can induce apoptotic events (18, 21). In addition, calcium overloading in mitochondria can induce apoptosis by stimulating the release of apoptosis-promoting factors such as cytochrome *c* and by generating ROS as a result of respiratory chain damage (4, 12, 13, 15). Our study demonstrated that H_2O_2 and P induce activation of caspase-3 and -9 and PS externalization, an early detectable event on membranes during apoptosis. There is some evidence suggesting the involvement of caspase-3 activation in H_2O_2-induced apoptosis in HL60 cells (33), cultured astrocytes (34), activated human peripheral blood lymphocytes (35), hepatoblastoma HepG2 cells (36), and human neuroblastoma cells (37). The effects of H_2O_2 on caspase-3 activation and PS exposure, observed by us, are dependent on elevations in $[Ca^{2+}]_c$, as we demonstrated by loading the spermatozoa with the intracellular calcium chelator dimethyl BAPTA. To further investigate the role of calcium on apoptosis, we used P in calcium-free medium, which fully depletes the calcium intracellular stores. The cellular effect of P can be summarized as being calcium store depletion and elevation of $[Ca^{2+}]_c$. As observed

FIGURE 4

Effect of intracellular calcium on H_2O_2- and P-induced caspase-3 activation and PS exposure in human spermatozoa. Control or dimethyl BAPTA– (10 μM for 30 min), Ru360- (10 μM for 30 min), or z-LEHD-FMK– (caspase-9 inhibitor; 100 μM for 90 min) pretreated cells were stimulated for 60 minutes with 10 μM H_2O_2 or 20 μM P (PROG), and then caspase-3 activity (**A**) and PS exposure (**B**) were determined as described in Materials and Methods. Values are presented as means ± SEM of five to eight separate experiments and expressed as percentage above control (untreated samples). *$P<.05$ compared with control values. ●$P<.05$ compared with H_2O_2 alone. ◆$P<.05$ compared with P alone. ■$P<.05$, comparing the two groups indicated by each end of the horizontal line.



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with H_2O_2, in dimethyl BAPTA–loaded cells, treatment with P demonstrated that rises in $[Ca^{2+}]_c$ are required for either caspase-3 activation and PS externalization. Both H_2O_2- and P-evoked apoptotic events are mostly of mitochondrial origin, as clearly demonstrated by the activation of caspase-9, an initiator caspase that is involved in the initial steps of mitochondrial apoptosis (5), and by the inhibitory effect of z-LEHD-FMK, a specific caspase-9 inhibitor, on either caspase-3 activation and PS exposure induced by H_2O_2 and P.

However, our results also demonstrated that the effect of H_2O_2 on caspase-3 activation did not show a clear relation to dose response. In fact, the highest concentration of H_2O_2 (i.e., 1 mM) did not activate caspase-3, whereas the lower concentration (i.e., 10 μM) significantly increased caspase-3 activity. In this regard, it has been postulated that low ROS concentrations (in the range of micromoles) could play a physiological role in mammalian sperm, such as sperm capacitation, whereas higher concentrations (in the range of millimoles) are harmful (38, 39).

Translocation of PS from the inner to the outer leaflet of the plasma membrane is one of the earliest signs of apoptosis. In human sperm, it has been shown that PS externalization induced by the calcium ionophore A23187 is an event linked to the acrosome reaction rather than to apoptosis (40). However, other investigators have shown evidence that exposure of PS in human sperm is characteristic of apoptosis (41). Our results showed that H_2O_2 and P induced PS externalization, as well as caspase-3 and -9 activation, which are the typical signs of apoptosis. We cannot reject the possibility that PS exposure is involved in the capacitation of human spermatozoa and plays a physiological role in sperm, being necessary for its acquisition of fertilizing ability.

Finally, oxidative stress enhancement and calcium accumulation in mitochondria have been reported to play roles in cell death (42). In our experiments, we provide compelling evidence supporting that mitochondrial calcium uptake is involved in caspase activation. Our results show that the blockade of calcium uptake into mitochondria with Ru360 was able to decrease apoptosis mediated by H_2O_2 and P, which were able to release calcium from intracellular stores.

In summary, our findings show that H_2O_2 and P stimulate caspase-3 and -9 activation and PS exposure. Our results also suggest that H_2O_2- and P-induced apoptosis is basically of mitochondrial origin, which requires rises in $[Ca^{2+}]_c$ and calcium entry into mitochondria.

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