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Effects of Putative Epididymal Osmolytes on Sperm Volume Regulation of Fertile and Infertile c-*ros* Transgenic Mice

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ABSTRACT: Volume regulation by spermatozoa has been demonstrated to be crucial in both mice and men for transport in the female tract. In order to determine the nature of osmolytes used by spermatozoa, they were released from the cauda epididymis of fertile c-*ros* heterozygous mice into incubation medium of uterine osmolality (representing an osmotic challenge), containing increasing concentrations of compounds that are major epididymal fluid components and known osmolytes in somatic cells. This should nullify the concentration gradients for osmolytes that mediate volume regulation, prevent osmolyte efflux, and lead to swelling. Of the osmolytes tested, K^+ caused the most rapid and extensive volume increases; glutamate, taurine, L-carnitine, and *myo*-inositol also were effective, but glycerophosphocholine was not. Such effects were not observed in cauda sperm from the infertile knockout mice, demonstrating a defect in normal volume regulation. K^+ concentrations in cauda epididymal fluid were 21 mM higher in the knockout than the heterozygous mice, but no differences were found in caudal fluid glutamate, carnitine, or *myo*-inositol. The carnitine content of cauda sperm from knockout males was not different from that of fertile males, but lower amounts of glutamate and inositol were found that could explain the poor volume regulation. In heterozygous mice, cauda but not caput sperm responded to the K^+ channel blocker quinine by swelling, demonstrating development of volume regulation during epididymal transit, whereas knockout cauda sperm showed no response, as with the osmolytes. Major epididymal secretions could serve as osmolytes in murine spermatozoa for volume regulation in response to physiological osmotic challenge in the normal fertile mice; the reduced sperm content of inositol and glutamate in the c-*ros* knockout mice might reflect maturational abnormalities in volume regulation.

Key words: Sperm swelling, regulatory volume decrease, quinine, organic osmolytes, infertility.

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A novel cause of male infertility in 2 transgenic mouse models is the angulated tails of spermatozoa that fail to negotiate the uterotubal junction and hence reach the site of fertilization (Yeung et al, 2000; Sipilä et al, 2002). Tail angulation reflects a volume increase in the spermatozoa (Yeung et al, 2002a,b) caused by an inadequate regulatory volume decrease (RVD), which is normally initiated after osmotic entry of water in hypotonic environments. Although somatic cells rarely experience this phenomenon (O'Neill, 1999), it is a normal occurrence for sperm upon ejaculation when they are rapidly expelled into the uterus (osmotic pressure [OP] around 330 mmol/kg) (Yeung et al, 2000) from the relatively hypertonic environment of the cauda epididymis (OP around 420 mmol/kg) (Yeung et al, 1999). In RVD, the response is to lose cell water in parallel with efflux of osmolytes through pertinent membrane channels. In the early 1970s, studies of bovine ejaculated sperm incubated in medium with osmolality identical to serum showed that Na^+ and K^+ close to serum levels, Ca^{2+}, and serum albumin as well as metabolic factors all contributed to the maintenance of sperm volume stability (Bredderman and Foote, 1971a,b,c). More recently, potassium has been associated with sperm volume regulation in bulls (Kulkarni et al, 1997; Petrunkina et al, 2001), boars (Petrunkina et al, 2001), mice (Yeung et al, 2002a), and humans (Yeung and Cooper, 2001). Besides these reports, little is known about other inorganic or organic osmolytes or osmolyte channels for spermatozoa.

A characteristic of epididymal fluid of all mammalian species is the distally increasing osmolality, which should induce osmotic changes in sperm cells. It has recently been proposed that these conditions, in conjunction with the long time (days) it takes sperm to pass through the epididymis, favor isovolumetric regulation of sperm osmolality (Cooper and Yeung, 2003). In this process in general, major changes in cell volume are avoided during imposition of small incremental changes in extracellular OP that result in influx of osmolytes (Pasantes-Morales et al, 2000; Souza et al, 2000). In the epididymis, the increasing tonicity along the tubule would encourage sim-

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ilar osmolyte uptake by maturing sperm, and it is suggested that these osmolytes, provided by the epididymis, are used by sperm in the female tract in response to its relative hypo-osmolality (Cooper and Yeung, 2003).

A number of low–molecular weight, water-soluble organic components are present in extremely high (mM) concentrations in epididymal fluid (see Cooper, 1998), and several of them (glutamate, taurine, *myo*-inositol, carnitine [a betaine derivative], and glycerophosphocholine) are employed in somatic cells as nonperturbing solutes for volume regulation (Strange et al, 1996; Lang et al, 1998; Furst et al, 2002) and could be relevant for the volume regulatory properties of spermatozoa.

One way to determine the nature of the osmolytes used by spermatozoa in volume regulation is to monitor volume changes in response to inhibitors of channels mediating osmolyte efflux. In this way, evidence for a role of ion channels in sperm volume regulation was provided by the effect of quinine on bovine sperm volume (Kulkarni et al, 1997; Petrunkina et al, 2001). Quinine (a wide-spectrum, though conventional, K^+ channel blocker), $BaCl_2$ (a K^+ channel blocker), and 5-nitro-2-(3-phenypropylamine)-benzoic acid (NPPB; a Cl^- channel blocker) all promote the angulation of murine sperm, reflecting a swollen status (Yeung et al, 1998, 1999, 2002a). This suggests that osmolytes that use these channels are involved in sperm volume regulation.

In this study, the identities of potential osmolytes were elucidated by compromising their concentration gradients across the sperm membrane and examining the effect on RVD. The amounts of these effective osmolytes in caudal epididymal luminal contents were also compared between the fertile heterozygous and infertile homozygous c-*ros* knockout mice because the heterozygous males are identical to the wild type in phenotype, whereas the homozygous mice lack the initial segment of the epididymis that normally differentiates from the proximal caput during puberty (Sonnenberg-Riethmacher et al, 1996). Although sperm production in the testis and deposition in the uterus are normal in the infertile male, spermatozoa recovered from the uterus after mating or released from the cauda epididymis into medium show angulation of the tail, as exhibited by normal sperm swollen by ion channel blockers (Yeung et al, 1999, 2000). Increases in cell volume of these sperm have been confirmed (Yeung et al, 2002a,b). Therefore, these transgenic animals are a useful model for the study of the role of the epididymis in sperm volume regulation.

Materials and Methods

Animals

The c-*ros* transgenic mice generated from C57BL6 and Ola129 parents (Sonnenberg-Riethmacher et al, 1996) were bred and genotyped with standard polymerase chain reaction on tail DNA using the forward primer 5′-GGCTGCGTCTACTTGGAGCA-3′ and reverse primer 5′-GGAAAGTGGGTCTTTGGTCA-3′. Sperm from the heterozygous animals, which are identical in phenotype and fertility to wild-type mice, were used as normal controls for comparison with the knockout mice. The present experiments using these animals were conducted according to the German Federal Law on the Care and Use of Laboratory Animals (license 41/98).

Incubation Media

The basal (control) medium used was BWW (Biggers et al, 1971), containing bovine serum albumin (BSA) at 4 mg/mL, with the osmolality made to 330 mmol/kg with NaCl, which is identical to that of the uterine contents of wild-type mice (Yeung et al, 2000). All chemicals were obtained from Sigma (Taufenkirchen, Germany) unless stated otherwise. Osmolytes tested at various concentrations (see Results section) were first made in individual stock solutions (1.0 M for KCl, 250 mM for the organic osmolytes, including sodium glutamate, taurine, L-carnitine [inner salt] and *myo*-inositol), with pH adjusted to 7.0 when necessary. Cadmium-free glycerophosphocholine (GPC) was dissolved in methanol, and the desired amount was blown dry in glass tubes at 37°C and taken up by BWW medium. The osmolality of all media used for sperm incubation was adjusted to 330 mmol/kg by omission and further adjustment of NaCl content. Quinine was made up in a 100 mM aqueous stock solution and diluted into BWW medium to give 200, 400, or 800 μM just before use.

Sperm Preparation for Cell Volume Estimation

Mice were killed by cervical dislocation after CO_2 asphyxiation. The epididymis was dissected out and cleaned of blood. For the study of mature sperm, the cauda region was decapsulated at around the flexure. From each side, 3 short tubule segments (6 from 1 mouse) were excised and processed in sequence. Each segment was transferred to a spatula into a drop of control or test medium, and more incisions were further made in the tubule to release the sperm. After removing the empty tubule, the released sperm were dispersed in 200 μL of the same prewarmed medium and incubated at 37°C with 5% CO_2 in air. For the comparison of mature and immature sperm, samples were taken from 3 regions of the epididymis: the caput from the lobule just distal to the initial segment (the equivalent gross anatomical site of the epididymal head in the c-*ros* knockout mice, which do not have an initial segment), the corpus region proximal to the narrowest midsegment, and the cauda at the flexure. These sampling sites correspond respectively to region II, proximal region IV, and midregion V described by Abe et al (1983). The order of sampling and the sequence of test media used were alternated between experiments to randomize any possible effect of sperm preparation time.

Measurement of Sperm Volume by Flow Cytometry

Changes in individual cell volume were estimated by comparing the laser forward and side scatter signals of control and treated sperm samples from the same mouse, using the flow cytometry method established and validated previously (Yeung et al,

2002a). After 1 minute of incubation for dispersion and at 10, 40, and 60 minutes of incubation, a 50 μL aliquot of the incubated sperm suspension was added to 200 μL of the same medium, but without BSA and containing 3 μL of a propidium iodide solution (PI, 500 μg/mL, final concentration 6 μg/mL). The sample was analyzed in a flow cytometer (Coulter Epics XL, version 3.0, Krefeld, Germany) with laser excitation at 488 nm. With cellular debris and aggregates gated out, laser emissions from 10 000 particles were collected. With the use of PI fluorescence signals, sperm were gated as viable (PI-negative) and nonviable (PI-positive), and the forward and side scatter signals from viable sperm were analyzed.

Quantification of Sperm Cytoplasmic Droplets

The possibility that sperm cytoplasmic droplets were associated with the different responses to osmolyte incubation by sperm from the heterozygous and knockout mice was investigated. Sperm from the caudal region released into phosphate-buffered saline (PBS), with osmolality adjusted to 420 mmol/kg (that of cauda epididymal fluid) (Yeung et al, 1999) were immediately fixed with 3% (vol/vol) glutaraldehyde and examined at 200× magnification for the presence of cytoplasmic droplets.

Collection of Cauda Epididymal Fluid and Sperm for Assay of Organic Osmolytes

Mice were killed by cervical dislocation after CO_2 asphyxiation, and the cauda epididymis with the proximal vas deferens were isolated. The vas deferens was cannulated with a drawn-out polyvinyl chloride catheter, and the epididymal luminal contents were flushed out by retrograde perfusion through a cut end of the tubule in the proximal cauda region. The perfusion solution was PBS (Gibco, Berlin, Germany) adjusted to 420 mmol/kg to mimic the osmolality of caudal fluid. The exudate was taken up into a positive displacement pipette, and the collections from both sides of the animal were dispersed in 100 μL of medium. Sperm cells were separated by centrifugation at 2000 × *g* for 2 minutes at 4°C, and the supernatant was centrifuged again at 2000 × *g* for 5 minutes before storing the diluted cauda epididymal fluid at −20°C for use in assays. The sperm pellet was washed twice with the perfusion medium by centrifugation at 600 × *g* for 5 minutes, and the number of spermatozoa collected was estimated by nephelometry (Bone et al, 2000). Sperm pellets were stored at −20°C. To extract sperm for the assay of organic osmolytes, 120 μL of assay buffer was added to each freeze-thawed pellet, and the sample was vortexed and sonicated (1.5-mm tip; Vibra-Cell-Sonicator, Sonics & Materials Inc, Danby, Conn) with 4- by 1-second ultrasound burst. The supernatant was obtained by centrifugation at 20 000 × *g* for 10 minutes at 4°C.

Measurement of Organic Osmolytes in Epididymal Fluid and Sperm

These measurements were taken by fluorometric assays modified for 96-well plate format. L-Glutamate was measured by estimating the H_2O_2 liberated by the action of glutamate oxidase with Amplex Red reagent (Kit A-12216; Molecular Probes, Leiden, The Netherlands; E_x = 530 nm, E_m = 590 nm). L-Carnitine was quantified by measuring, as a fluorescent *N*-[4-(2-benzimidazolyl)phenyl]maleimide (BIPM) adjunct, the free coenzyme A liberated by the action of L-carnitine acetyl transferase (Maehara et al, 1988; E_x = 365 nm, E_m = 460 nm). The *myo*-inositol assay was developed from that of O'Neill et al (1998) in a linked enzyme reaction, in which the $NADH^+$ liberated by the action of inositol dehydrogenase was quantified after conversion by NADH oxidase to H_2O_2 and detection of the latter with Amplex Red (E_x = 530 nm, E_m = 590 nm). To 5-μL (epididymal fluid) or 50-μL (sperm extract) samples was added 140 μL reaction mixture containing (final concentrations) 3.5 mM NAD, 0.18 U/mL inositol dehydrogenase, 0.21 mM flavin adenine dinucleotide, 14.3 mU/mL NADH oxidase, 0.18 μg/mL Amplex Red, and 1.42 U/mL horseradish peroxidase). The samples were incubated for 60 minutes at 30 °C.

Measurement of K^+ Concentration in Epididymal Fluid Using Ion-Selective Electrodes

The measurement of potential differences in drops of samples against calibration standards with the use of glass capillary microelectrodes was made as previously described for other ions (Xu et al, 2003). Potassium ionophore I cocktail A (Fluka Chemicals, Deisenhofen, Germany) was used to fill the ion-selective electrodes. The perfusion solution for flushing out epididymal luminal contents contained trypan blue (12 mg/mL) to ensure collection of uncontaminated samples.

Statistics

Data were analyzed by SigmaStat software (version 2.03; SPSS Inc, Erkrath, Germany) and presented as mean ± SEM. Differences between the transgenic and control mice within the same epididymal regions and differences between regions within each genotype and over the 60-minute incubation time were tested by 3-way analysis of variance with the Student-Newman-Keuls method for comparison. The effect of different osmolytes and quinine on aliquots of the same source of sperm in each experiment was tested statistically against the controls (expressed as a ratio of control values) by 1-way repeated measures analysis of variance with the Dunnett method. Differences between genotypes in osmolyte contents were tested by the Student's *t* test. Differences were considered statistically significant at $P < .05$.

Results

Effects of Extracellular Putative Osmolytes on Volume of Mature Spermatozoa From the Cauda Epididymis of Fertile Heterozygous Mice

With the method used in this study, it is technically impossible to measure the volume of sperm in the epididymis at the time of release because they need to be dispersed. The basal medium was a physiological solution with osmolality mimicking uterine fluid, which is about 90 mmol/kg lower than that of epididymal fluid. It took about 1–2 minutes to obtain the earliest flow cytometric measurement after dispersion, by which time the sperm could have started swelling, and this measurement was used as a reference value for each experiment to eliminate

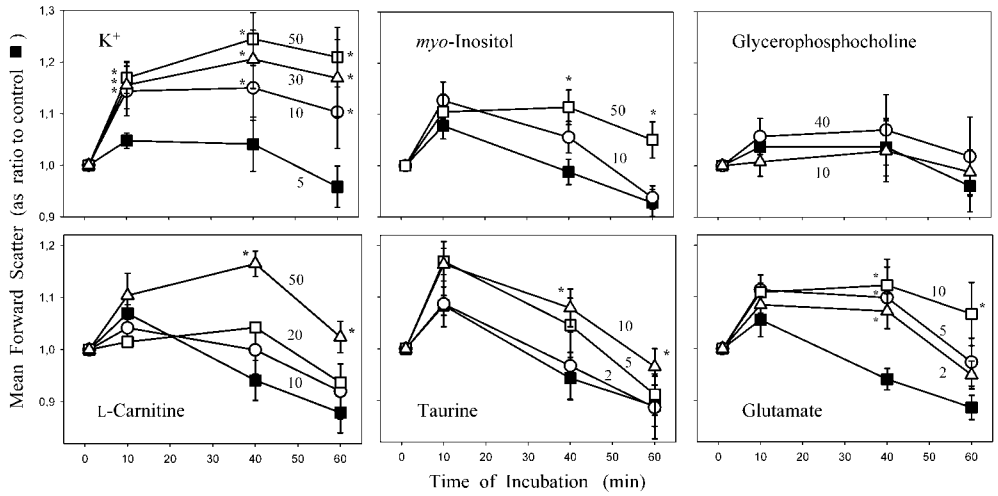

Figure 1. Effects of extracellular putative osmolytes on the volume of normal cauda epididymal sperm measured as laser forward scatter signals by flow cytometry. Changes over 60 minutes of incubation in various concentrations of osmolytes (mM) are expressed as ratios (treated/basal value obtained in each experiment at the beginning of the incubation, with the control medium containing 5 mM KCl and none of the other tested substances). Values are mean ± SEM (n = 5–8). An asterisk indicates a significant ($P < .05$) difference from the basal (control) value (■) obtained at the same incubation time point.

between-animal variability. The volume of mature sperm in the basal (control) medium showed a tendency to increase in the first 10 minutes and gradually decrease on further incubation up to 60 minutes, as reflected by laser forward scatter (Figure 1).

All of the osmolytes tested, except glycerophosphocholine, caused an increase in the laser forward scatter (Figure 1) during the 60-minute incubation. Dose responses were demonstrated, particularly with K^+, taurine, and glutamate, although statistically significant effects by carnitine and *myo*-inositol were obtained only at the highest dose tested (50 mM). Among these osmolytes, K^+ induced the most immediate and the largest effect and was effective already at 10 mM, which was double the concentration in the control medium mimicking serum K^+ concentration.

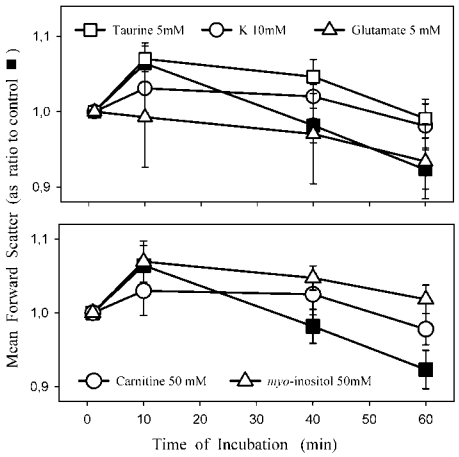

Figure 2. Lack of effects of extracellular putative osmolytes on the volume of cauda epididymal sperm from c-*ros* knockout mice measured as laser forward scatter signals by flow cytometry. Measurements obtained over 60 minutes of incubation in each osmolyte tested are expressed as ratios to the basal value obtained in the control medium in each experiment at the beginning of the incubation. Values are mean ± SEM (n = 7).

*Lack of Effect of Extracellular Putative Osmolytes on Volume of Sperm From c-*ros *Knockout Mice*

At concentrations causing volume increases in sperm from the c-*ros* heterozygous fertile mice, none of the osmolytes affected the volume of sperm released from the cauda epididymis of the infertile c-*ros* knockout mice (Figure 2).

This lack of response to the osmolyte incubation by knockout mouse sperm was not due to the absence of cytoplasmic droplets, where the bulk of sperm cytoplasm is located, because there was no difference from the heterozygous mice in the percentage of sperm bearing cytoplasmic droplets (83% ± 2% vs 77% ± 2%; n = 7).

Contents of Osmolytes in Luminal Fluid and Sperm From the Cauda Epididymidis of Heterozygous and Knockout Mice

The concentrations of K^+ in cauda epididymal fluid measured by ion-selective microelectrodes were significantly higher in the knockout than the heterozygous mice. The content of organic osmolytes measured by spectrofluorometric methods showed no difference between the 2 genotypes in the luminal fluid, but glutamate and inositol contents were lower in the sperm recovered from the knockout compared with the heterozygous mice as normal controls (Table).

*Concentrations of organic osmolytes in cauda epididymal luminal fluid and spermatozoa from heterozygous and knockout c-*ros *transgenic mice; values are means ± SEM of (n) samples standardized against protein content of the fluid or the number of spermatozoa*

	Osmolyte Concentration	
	Heterozygous	Knockout
Epididymal fluid		
Potassium (mM)	30.5 ± 5.3 (11)	52.0 ± 5.1* (10)
Glutamate (nmol/mg)	4.74 ± 0.62 (13)	3.91 ± 0.54 (16)
Carnitine (nmol/mg)	484 ± 38 (16)	470 ± 26 (16)
Myo-inositol (nmol/mg)	205 ± 27 (17)	220 ± 26 (16)
Spermatozoa		
Glutamate (pmol/10^6 cells)	9.26 ± 0.92 (6)	5.12 ± 1.09* (7)
Carnitine (nmol/10^6 cells)	1.83 ± 0.22 (8)	1.49 ± 0.15 (8)
Myo-inositol (nmol/10^6 cells)	2.15 ± 0.17 (7)	1.65 ± 0.13* (7)

* Significantly different from heterozygous, $P < .05$.

*Comparison of Laser Scatter by Cauda and Caput Spermatozoa From c-*ros *Heterozygous and Knockout Mice in Basal Medium*

Mature sperm from the cauda epididymis of the fertile c-*ros* heterozygous mice showed a tendency toward volume decrease after the initial increase, unlike immature sperm from the caput region, which showed a continuous volume increase for up to 40 minutes of incubation in basal medium (Figure 3). By comparison, cauda sperm from the knockout mice exhibited larger volumes, especially during the first 10 minutes of incubation, but also showed a decline with time, whereas caput sperm were smaller than those from the heterozygous mice but swelled to the same extent with time. Sperm from the corpus epididymis from both genotypes responded identically, exhibiting larger forward scatter than the caput and cauda sperm initially, with a slight tendency of decrease over time (Figure 3).

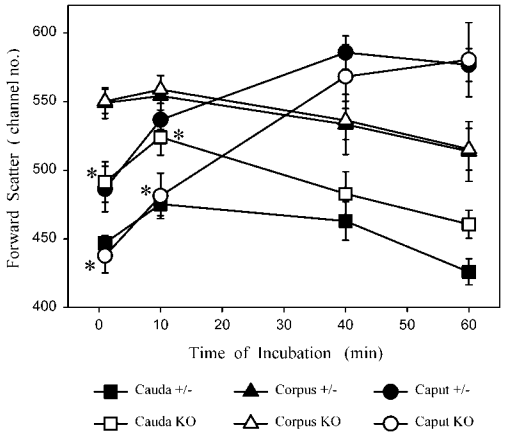

Figure 3. Changes in cell volume as measured by laser forward scatter of sperm collected from various regions (caput, corpus, and cauda) of the epididymis of c-*ros* heterozygous (±, closed symbols) and knockout (KO, open symbols) mice monitored over 60 minutes of incubation. Values are mean ± SEM (n = 8). An asterisk indicates a significant difference ($P < .05$) between genotypes for the same region at the same incubation time point.

Differences in Response to Quinine by Cauda and Caput Spermatozoa From Heterozygous and Knockout Mice

In the presence of the ion channel blocker quinine, mature (cauda) sperm from the c-*ros* heterozygous mice manifested marked, immediate, and persistent dose-dependent increases in laser forward scatter (Figure 4), whereas cauda sperm from the knockout mice failed to show any significant response. On the other hand, immature (caput) sperm from the fertile genotype failed to respond to quinine with volume increases characteristic of mature sperm. Surprisingly, the knockout caput sperm responded with a transient increase at 10 minutes of incubation (Figure 5).

Discussion

Although sperm volume regulation and its association with fertility has been highlighted in the infertile c-*ros* knockout mice, it can be envisaged that multiple factors

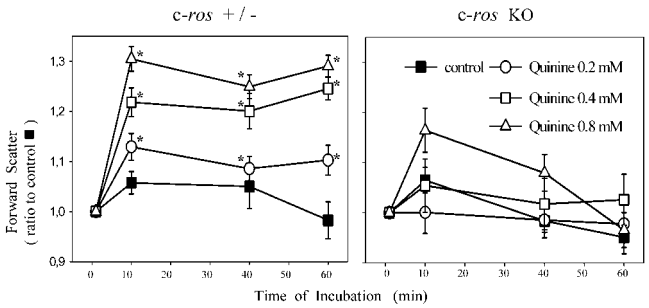

Figure 4. Dose-dependent effects of quinine on sperm volume, reflected by laser forward scatter and expressed as ratios to the basal value obtained in the control medium in each experiment at the beginning of the incubation of sperm collected from the cauda epididymis of c-*ros* heterozygous mice (left panels, n = 6), but not those from the knockout mice (right panels, n = 5–7). Values are mean ± SEM. An asterisk indicates a significant difference ($P < .05$) from the control measured at the same incubation time point.

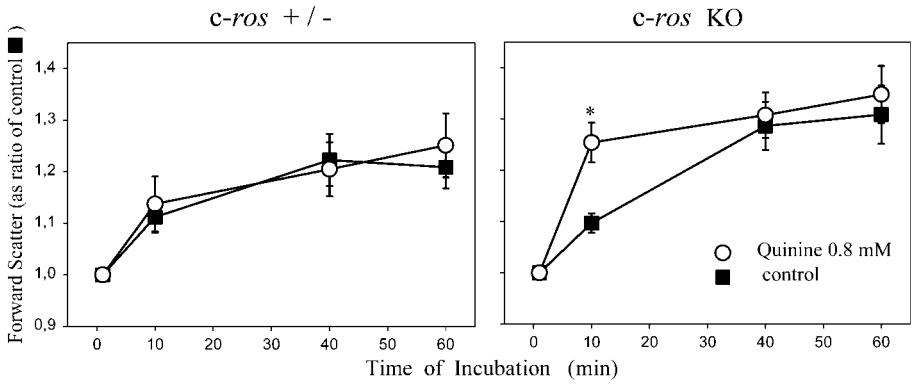

Figure 5. Effects of quinine on volume of caput sperm from the c-*ros* heterozygous (±, n = 6) and knockout (KO; n = 7) mice, measured as laser forward scatter and expressed as ratios to the basal value obtained in the control medium in each experiment at the beginning of the incubation. Values are mean ± SEM. An asterisk indicates a significant difference ($P < .05$) from the control measured at the same incubation time point.

should be involved because the lack of a differentiated epididymal initial segment would render the luminal milieu abnormal and, hence, affect various aspects of maturational changes in sperm. This study concentrated on sperm volume regulation. The working hypothesis was that sperm migrating through the epididymis are gradually confronted with increases in osmolality and consequently take up osmolytes that subsequently would be lost in RVD in the female tract (see the Introduction). These osmolytes should be present at high concentrations in the epididymal lumen, and their identities would be revealed if spermatozoa were to swell in media containing these molecules at concentrations approaching intrasperm levels that prevent their diffusional efflux. In the c-*ros* knockout males, sperm osmolytes are anticipated to be limiting.

When subjected to the osmolality faced in the uterus, caudal sperm from heterozygous males initially swelled and then reduced their volume, as previously demonstrated (Yeung et al, 2002b). That caudal sperm from knockout mice were larger in initial volume but also reduced their volume with time is consistent with the view that they contain a reduced complement of osmolytes available for volume regulation, although defective ion channels in an abnormal plasma membrane cannot be ruled out. Corpus sperm from both genotypes behaved similarly; namely, they were unable to reduce their larger volume over 1 hour, but their volumes did not increase with time, indicating a minor ability to regulate volume. The larger volume might reflect a higher intracellular osmolality compared with cauda sperm. Caput sperm from both genotypes swelled continuously during incubation—even faster for the knockout sperm, which were initially smaller—demonstrating a complete lack of ability to regulate volume as they entered the epididymis from the testis.

The amino acid content of whole epididymal tissue from the mouse is known to be high, with taurine and glutamate among the highest in caput tissue, with glutamate content decreasing and taurine increasing toward the cauda (Kochakian, 1975). Little is known of the nature of luminal osmotic components in the murine epididymis, and most knowledge has come from the rat (see Cooper, 1998), in which L-carnitine (60 mM), *myo*-inositol (50 mM), GPC (40 mM), and taurine (3 mM) are major osmolytes in caudal fluid: corpus fluid contains 20 mM glutamate and 6 mM taurine, and caput fluid contains 50 mM glutamate and 2 mM taurine. Each of these components has a distinct profile, such that sperm entering the epididymis are bathed consecutively in high concentrations of GPC followed by glutamate and taurine, K^+, carnitine, and then *myo*-inositol (Hinton and Palladino, 1995; Cooper and Yeung, 2003). Even less is known of intrasperm concentrations of epididymal osmolytes: the carnitine content of sperm from many species increases distally (Cooper, 1986), whereas intracellular potassium in the mouse is reported to be 90–120 mM in mature sperm (Babcock, 1983; Chou et al, 1989; Zeng et al, 1995).

From the data presented here, assuming an approximate dilution of 50- to 100-fold when luminal contents were flushed out and dispersed in 100 μL of medium, the corresponding neat concentrations would be 35–70 mM for *myo*-inositol, 60–120 mM for carnitine, and 0.23–0.45 mM for glutamate, which are values similar to those measured in rats. A difference in provision of osmolytes in this fluid in the infertile c-*ros* knockout males was not evident because no differences between genotypes were detected for the organic osmolytes expressed per unit protein of fluid. No differences in taurine content of epididymal fluid between genotypes were previously reported by Xu et al (2003), and neither is there any detectable differences in the expression of the epithelial carnitine transporter genes *OCT1*, *OCT2*, *OCT3*, and *OCTN2* (Cooper et al, 2003). By contrast, higher K^+ concentrations in cauda epididymal fluid were detected in the infertile knockout mice. Thus, the only detectable change in caudal fluid from the mutant males with compromised fertil-

ity was an increase, rather than decrease, in a potential osmolyte, which nevertheless indicates abnormal epithelial function in the c-*ros* knockout male. It is tempting to speculate that the increased extracellular K^+ concentration leads to cellular K^+ uptake and swelling, as shown in other cells (Lang et al, 1998). This in situ swelling might then inhibit the cellular accumulation of organic osmolytes, such as glutamate and *myo*-inositol (see the Table). It could be the lack of these osmolytes that leads to deranged cell volume regulation and function of sperm from the knockout mice.

In somatic cells, the predominant osmolyte and the mechanism of volume regulation can vary under different conditions and is dependent on cell type. In cardiomyocytes, RVD induced by drastic hypo-osmotic change is achieved mainly by taurine efflux, whereas in isovolumetric regulation (IVR) with gradual osmolality decrease, K^+ loss is predominant (Souza et al, 2000). However, in hippocampal tissue, IVR does not involve K^+ but mainly taurine efflux, whereas RVD is associated with loss of glutamate, taurine, and K^+ (Franco et al, 2000). The nature of osmolytes used by sperm is unknown, but hyperosmotic stress in chimpanzee sperm can be alleviated by 2 mM taurine (Ozasa and Gould, 1982), suggesting that it might be a physiological osmolyte taken up by sperm during the sojourn of increasing osmolalities in the epididymis.

In this study, when cauda sperm were subjected to a physiological 90 mmol/kg decrease in extracellular osmolality, K^+ was the most effective extracellular osmolyte tested that caused swelling of murine mature sperm and induced the fastest response. *Myo*-inositol and L-carnitine at assumed physiological concentrations were able to sustain high cell volumes over 40 minutes, whereas cell volumes began to decline in the presence of supraphysiological concentrations of glutamate and taurine, suggesting that other osmolytes were operating to maintain volume. GPC had no effect on sperm volume, probably because it is impermeant, as it is for renal cells (Zablocki et al, 1991). These positive responses in the induction of swelling from almost all the osmolytes tested suggest that murine sperm can use a number of different molecules for volume regulation.

The failure of knockout cauda sperm to respond to the osmolytes tested could be because the sperm are already swollen (Yeung et al, 2002a). The same argument would explain the resistance of c-*ros* knockout sperm to swelling induced by quinine. Normal immature sperm from the caput swelled in the basal medium and did not respond to quinine because the volume regulation mechanism is largely undeveloped. Although quinine enhanced the swelling of caput sperm from the knockout mice at 10 minutes, this effect was not sustained at later time points. This could mean that because the knockout sperm had been in the caput environment longer than the normal sperm because of the missing initial segment, they might have started the early stages of the development of volume regulation mechanism but failed to complete it normally because of epididymal malfunction. The swollen status of the knockout cauda sperm in the basal medium could be a consequence of abnormal osmolyte uptake in the mutant epididymis, and indeed, glutamate and *myo*-inositol were decreased in sperm from the knockout males, although the carnitine and taurine levels (Xu et al, 2003) within spermatozoa were not different between genotypes.

This study demonstrates that major epididymal secretions could serve as osmolytes in murine spermatozoa for volume regulation in response to physiological osmotic challenge. This capacity of volume regulation is developed during the sojourn in the epididymis and is important for normal sperm function in the female tract. The infertile c-*ros* knockout mouse sperm were found to have less sperm glutamate and *myo*-inositol, despite normal concentrations in epididymal fluid. Insofar as this animal model can be useful for investigating the relationship between epithelial and sperm function, for purposes of developing a contraceptive for males, these observations suggest that attacking the sperm channels to limit the uptake of epididymal osmolytes might be more effective than targeting the epithelial transporters in order to limit the provision of luminal secretions to the sperm. This reiterates findings in rats and hamsters that reducing epididymal carnitine by increasing excretion of pivalolyl carnitine does not lead to infertility or reduce sperm motility because sperm carnitine was unaltered (Cooper et al, 1997; Lewin et al, 1997).

Because K^+ and quinine consistently provided the most rapid and extensive swelling responses, K^+ could be a major regulator of sperm volume. Again, the nature of the channels used by sperm in mediating osmolyte influx and efflux in the male and female tracts requires investigation and could be controlled by secretions of the initial segment. Such elucidation of sperm volume regulation mechanisms contributes to the understanding of infertility and the development of new male contraceptives because volume regulation has been demonstrated in human sperm and swollen sperm have altered motility pattern that hinders mucus penetration (Yeung and Cooper, 2001; Yeung et al, 2003).

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