Immotile Sperm and Infertility in Mice Lacking Mitochondrial Voltage-dependent Anion Channel Type 3*

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Received for publication, May 23, 2001, and in revised form, July 20, 2001

Published, JBC Papers in Press, August 15, 2001, DOI 10.1074/jbc.M104724200

Voltage-dependent anion channels (VDACs), also known as mitochondrial porins, are small channel proteins involved in the translocation of metabolites across the mitochondrial outer membrane. A single channel-forming protein is found in yeast, whereas higher eu karyotes express multiple VDACs, with humans and mice each harboring three distinct channels (VDAC1–3) encoded by separate genes. To begin to assess the functions of each of the three isoforms, the VDAC3 gene was inactivated by targeted disruption in embryonic stem cells. Here we show that mice lacking VDAC3 are healthy, but males are infertile. Although there are normal sperm numbers, the sperm exhibit markedly reduced motility. Structural defects were found in two-thirds of epididymal axonemes, with the most common abnormality being loss of a single microtubule doublet at a conserved position within the axoneme. In testicular sperm, the defect was only rarely observed, suggesting that instability of a normally formed axoneme occurs with sperm maturation. In contrast, tracheal epithelial cilia showed no structural abnormalities. In addition, skeletal muscle mitochondria were abnormally shaped, and activities of the respiratory chain complexes were reduced. These results demonstrate that axonemal defects may be caused by associated non-axonemal components such as mitochondrial channels and illustrate that normal mitochondrial function is required for stability of the axoneme.

* This work was supported in part by the Baylor College of Medicine Child Health Research Center and Mental Retardation Research Center, American Heart Association Grant-in-Aid Award 96008130, and National Institutes of Health Grant R01 GM055713–02 (to W. J. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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† The abbreviations used are: VDAC, voltage-dependent anion channel; PCR, polymerase chain reaction.

Voltage-dependent anion channels (VDACs)1 are abundant 30-kDa mitochondrial outer membrane proteins found in all eukaryotes (reviewed by Sorgato and Moran (1)). VDACs are functionally conserved across evolution, with similar electrophysiologic characteristics shared from yeast to humans. VDACs from fungi form large voltage-gated channels that, in the open state, have been shown to preferentially conduct ATP and other anions (2). Mammalian VDACs are reported to associate with the adenine nucleotide translocator at the contact points between the inner and outer mitochondrial membranes (3). VDACs also serve as a binding site for cytosolic hexokinase, providing the enzyme with preferential access to ATP derived from oxidative phosphorylation (4). There is evidence for the involvement of VDAC in the formation of the permeability transition pore (5), and it has been reported recently that VDAC1 constitutes a pathway for the release of cytochrome c during cytochrome c-dependent apoptosis (6, 7), although this conclusion is not universally shared (8, 9). VDACs may participate in the regulation of cellular energy metabolism via the control of mitochondrial outer membrane permeability and compartmentation of high energy metabolites. In growth factor-dependent pro-B lymphocyte cell lines, growth factor withdrawal leads to loss of outer membrane permeability and accumulation of phosphocreatine within the intermembrane space, in association with a defect in ATP/ADP exchange (10). Although it has been reported that interactions between BH4 domain containing Bcl-2 family members mediate VDAC closure (7), direct evidence for this occurring in vivo is lacking.

The mammalian VDACs currently consist of three family members: VDAC1, VDAC2, and VDAC3, encoded by three separate genes (11, 12). VDAC3 undergoes alternative splicing such that a single amino acid is inserted 39 residues into the polypeptide (13, 14). Phylogenetic analysis of the VDAC3 cDNA sequence and complementation studies of a VDAC-deficient yeast strain suggest that it may have a distinct physiologic function (11, 15), and a recent study has demonstrated electrophysiologic differences between the mouse isoforms (16). Here, we describe the generation and initial characterization of a mouse strain lacking VDAC3.

MATERIALS AND METHODS

Generation of VDAC3-deficient Mice—The organization of the mouse VDAC3 genomic locus has been described (11). The VDAC3 targeting vector is predicted to delete a 4.1-kilobase pair BglII/NoI fragment containing the last four exons of the VDAC3 gene. ES cell culture, electroporation, mini-Southern blot analysis of ES cell colonies, and generation of chimeras and germ line mice was as described (17, 18). PCR genotyping of VDAC3 animals was performed using a three-primer multiplex PCR assay. The PCR primer sets used are as follows (5’–3’): neomycin resistance gene primer (CTCCGAAATCGGGAGCGG- GATACCG), wild type primer (GCGCTCTCAGATCGTCGG), and common primer (CCAAAAGCTCTACTAGAGCAG) (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, 30 s, 40 cycles). The mutant PCR product (neomycin primer + common primer) is 1.1 kilobase pairs, and the wild type PCR product (wild type primer + common primer) is 600 base pairs.

Northern and Western Analyses—Total RNA was extracted from mouse adult tissues, and Northern blotting was carried out as described (5). To prevent probe cross-hybridization with the other VDACs, the membrane was probed with the 3’-untranslated region of the VDAC3 cDNA and then stripped and probed with a cDNA for glyc eraldehyde 3-phosphate dehydrogenase as a control for loading differences and RNA quality. Crude cell fractionation and Western blotting was...
Fig. 1. Gene targeting of the mouse VDAC3 locus. The top panel diagrams the wild type gene structure and the expected gene structure following homologous recombination. Relevant restriction sites are indicated, as are the predicted changes in restriction fragment length. The dark bar represents the DNA probe used to detect homologous recombination, while the middle panel shows the results of Southern blotting ES cell colonies. The lower panel shows the results of a Northern blot of RNA from the indicated tissues of wild type, VDAC3(+/-), and VDAC3(-/-) mice. The probe corresponds to the 3'-untranslated region of VDAC3 (to avoid cross-hybridization with other VDAC mRNAs). Hybridization with a cDNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for mRNA quantity and quality.

performed using VDAC3-specific and VDAC1-specific antibodies, as described (19). In brief, wild type testes were homogenized in RSB (10 mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 7.5) after which 2.5 x MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.5) was immediately added. The homogenate was then centrifuged at 2 °C for 15 min at 17,000 x g to pellet both nuclei and mitochondria, and the cytoplasmic extract (C) was derived from the supernatant. To isolate the mitochondrial fraction (M), the pellet was resuspended in 1 x MS buffer and centrifuged twice at 1200 x g to remove the nuclear fraction. Mitochondria were then pelleted by centrifugation at 2 °C for 15 min at 17,000 x g.

Sperm Characteristics—Sperm counts were obtained in wild type, heterozygous, and VDAC3-deficient male mice at 10 weeks of age. Animals were weighed and sacrificed, and testes weights were recorded. Both of the cauda epididymides were excised and placed in 1 ml of phosphate-buffered saline containing pyruvic acid, lactic acid, and bovine serum albumin. The percentage of mobile sperm was estimated by observation of at least 200 sperm with a light microscope. Motility of the sperm was categorized as actively swimming, twitching, or nonmotile. Sperm from each epididymis from a given male was counted and analyzed, and the two numbers were averaged.

Histochemistry and Electron Microscopy—For histopathological analysis, muscle tissue was dissected and either rapidly frozen to −150 °C in liquid nitrogen-chilled isopentane or fixed in phosphate-buffered 10% formalin. Frozen sections were stained with Gomori’s modified trichrome stain or subjected to histochemical analysis of cytochrome c oxidase activity and NADH dehydrogenase activity, while fixed sections were stained with hematoxylin and eosin.

Immunohistochemistry with a VDAC3-specific antibody was carried out as described (20). The tissues are fixed in 20% formalin for 12 h, dehydrated, impregnated with paraffin, and cut at 5 µm. Sections were placed on “plus” slides. Antigen retrieval was performed by placing the slides in citric acid, pH 6.0 (0.01 M), followed by three 5-min bursts of high microwave heat. Chicken anti-VDAC3 antibody was used at a dilution of 1:20 and placed on the tissues for 48 h at 4 °C. The secondary antibodies were applied using the ABC Elite kit from Vector Labs. DAB was used as the chromogen.

For transmission electron microscopy, skeletal muscle samples were placed into 3% glutaraldehyde, prepared as described (21), and examined in a Phillips electron microscope EM 300. Sperm and testes electron microscopy was carried out in a similar fashion, with the testis and cauda epididymis being placed immediately in fixative.

Respiratory Chain Activity Assays—Mitochondrial enzyme activity and electron transport complex activity rates were measured in 600 x g supernatants made from snap frozen skeletal muscle homogenates. NADH dehydrogenase, NADH oxidase, ubiquinol-cytochrome c reductase, succinate-cytochrome c reductase, cytochrome c oxidase, and citrate synthase activities were measured as previously described (22).
VDAC-deficient Male Mice Are Infertile

When VDAC3-deficient male mice were mated with female mice they demonstrated normal copulatory behavior, as evidenced by the presence of vaginal plugs in their mates, but no pregnancies were observed in over 100 matings. When homozygous null VDAC3 females were mated with wild type or VDAC3 heterozygous males, normal sized litters were produced. VDAC3(--/--) males showed normal numbers of sperm per epididymis when compared with wild type and VDAC3(+/--) males (Table I). In addition, no significant differences were found in the size, weight, or histologic features of testes between the three groups. To determine whether the absence of VDAC3 leads to enhanced apoptosis, the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay was carried out on testicular histologic sections. The level of apoptosis was comparable between wild type and VDAC3(--/--) animals (data not shown).

There was no distinguishable difference in the motility of wild type and VDAC3(+/--) sperm. In these groups, ~70% of the sperm were categorized as motile, with half of those actively swimming and the remainder twitching. 17% of VDAC3(--/--) sperm were categorized as motile (Table I; p < 0.001 versus wild type), with only 3% of the sperm actively swimming. Sperm motility was also examined over a 1-h time frame (25), and no appreciable change in activity over time was observed. Incubation with a variety of energy substrates such as lactate, pyruvate, glutamate, succinate, or malate failed to improve motility. Capacitation was not examined, nor was response to glucose in the incubation media.

Structural Abnormalities in Sperm—When viewed by electron microscopy, 68% of VDAC3(--/--) epididymal sperm axonemes (247/362) in cross-section demonstrated some structural aberration, most commonly loss of one outer doublet from the microtubule doublet arrangement. This compared with structural abnormalities in 9% of wild type axonemes (37/423). Axonemes can be oriented by bisecting the axoneme frame (25), and no appreciable change in activity over time was observed. Incubation with a variety of energy substrates such as lactate, pyruvate, glutamate, succinate, or malate failed to improve motility. Capacitation was not examined, nor was response to glucose in the incubation media.

Structural Abnormalities in Sperm

VDAC-deficient Male Mice Are Infertile

Table I

Comparison of VDAC3(+/+), VDAC3(+/-), and VDAC3(--/--) sperm counts and percentage absolute motility

| Genotype   | n    | Wt/Wb | Sperm count (×10⁶) | Sperm motility |
|------------|------|-------|-------------------|----------------|
| VDAC3(+/+) | 5    | 0.153 ± 0.052 | 20.5 ± 6.8 | 63.9 ± 5.3 |
| VDAC3(+/-) | 5    | 0.162 ± 0.038 | 16.7 ± 5.8 | 66.0 ± 7.1 |
| VDAC3(--/-) | 8    | 0.142 ± 0.045 | 21.3 ± 9.9 | 17.2 ± 4.4 |

Males were sacrificed at 10 weeks, and testes weight (Wt) was compared with total body (Wb), n = number of animals.

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bulles (doublets 3 and 8), with three doublets on one side and four on the other side (26). In the majority of abnormal VDAC3(+/−) axonemes, the missing doublet corresponds to the last of the four doublets (doublet 7) (see Fig. 4A, top panels, and Fig. 5, A–F), reflecting a single recurring defect in the axonemal structure. Images through the midpiece of epididymal sperm demonstrate the same defect in doublet 7 (Fig. 5), while some images appear to represent intermediate steps in doublet loss (e.g. Fig. 5, A, D, and F), with the missing doublet losing the normal positional relationship to other doublets and the outer dense fibers. The associated outer dense fiber of a missing doublet was often also found to be missing. Electron microscopy of spermatids in the testes revealed enlarged and abnormally shaped mitochondria along the midpiece (Fig. 4A, lower panels). Cross-sections through the distal principal piece of testicular sperm rarely revealed structural abnormalities (Fig. 5C), suggesting that the structural abnormality found in epididymal sperm represents instability of the axoneme and not necessarily a defect in assembly. Other less frequently observed structural defects in the axoneme include ectopically placed doublets (Fig. 5A), partial axonemal duplications (Fig. 5D), and the absence of half of the doublets (Fig. 5D).

In contrast to the ultrastructural abnormalities observed in sperm axonemes, cilia from tracheal epithelia have a normal 9 + 2 structural arrangement (Fig. 4B) and normal ciliary movement when examined by dark field microscopy. However, when sections were dissected 2 mm below the vocal cords and examined by scanning electron microscopy, there consistently appeared to be reduced numbers of ciliated cells (Fig. 4B), although no increase in respiratory pathology has been seen in the mice.

Abnormal Respiratory Chain Activity in Skeletal Muscle—Since VDAC3 undergoes alternative RNA splicing in brain and muscle (13, 14), skeletal muscle was examined histologically and biochemically for abnormalities. By hematoxylin/eosin staining of muscle cross-sections, no abnormalities in fiber structure or size were seen. Histochemical analysis of frozen sections does not reveal any significant changes in NADH dehydrogenase or cytochrome c oxidase activities, and no increase in ragged red fibers was observed (data not shown). Although no mitochondrial proliferation was seen and citrate synthase activity was not increased, electron microscopy did show unusually shaped mitochondria in skeletal muscle, with both variably enlarged size and extended intermyofibrillar growth (Fig. 6A). Respiratory chain activity in skeletal muscle mitochondrial preparations revealed significant reductions in the activity of succinate-cytochrome c reductase (complex II/III), ubiquinol-cytochrome c reductase (complex III) and NADH oxidase (complex I/IV) (Fig. 6B), while cytochrome c oxidase (complex IV) was reduced but not to a level that reached sta-
VDAC3-deficient Male Mice Are Infertile

**DISCUSSION**

The mitochondrial outer membrane has historically been viewed as a constitutively permeable membrane, with the inner membrane being the site of regulated transport of metabolites involved in coupled respiration. It is becoming increasingly clear that the outer membrane potentially constitutes an additional site of regulation via control of metabolite flux. The outer membrane also functions as a docking site for cellular kinases, along with being an important site of apoptotic signaling. A key aspect in this latter process may be the closure of VDACs in response to as yet unknown signals. While VDACs can adopt multiple conductance states *in vitro*, it has not yet been established that VDACs can change conductance states *in vivo*. Although VDACs are highly conserved across species, the specific function of each isoform remains unknown. It was recently reported that, when singularly expressed in yeast lacking the endogenous VDAC (*por1*), each isoform confers different permeability properties to the outer mitochondrial membrane. When incorporated into artificial bilayers, each isoform also exhibits electrophysiological differences, with VDAC3 showing the least voltage dependence and the lowest conductance (16).

In this report, we show that the absence of VDAC3 in the mouse leads to at least two abnormalities: defective axonemal structures in sperm and perturbed oxidative phosphorylation in skeletal muscle. Defects in the respiratory chain are consistent with our previous results in VDAC3-deficient cultured cells, in which oxygen consumption in both coupled and uncoupled mitochondria was reduced (27). We speculate that abnormal respiratory chain activity in part reflects disturbance of metabolite flux, but other possibilities, such as altered kinase binding or perturbations in mitochondrial-cytoskeletal interactions, require investigation. In this regard, mitochondrially bound hexokinase type 1 activity in brain extracts, which is typically 65–70% of total hexokinase activity, is unchanged from control values in the absence of VDAC3 (data not shown), but the subcellular localization of other kinases known to bind mitochondria has not yet been addressed. The appearance of mitochondria with abnormal size and shape suggests altered cytoarchitecture, and it has been previously reported that VDACs interact with the microtubule-associated protein MAP2 (28, 29). The respiratory chain assays were performed on previously frozen samples; hence, the observed defects should not simply reflect substrate flux at the time of assay, since the outer membrane is disrupted by freezing. Thus, less dynamic and more long lasting alterations in the respiratory chain must occur to account for these results. The respiratory complex defects are reminiscent of the loss of cytochrome c oxidase activity in yeast lacking *por1* (30). Use of fresh skinned muscle fibers should allow for the direct measurement of outer membrane permeability in intact preparations.

VDAC3 is also necessary for the maintenance of ciliary structures. Interestingly, this role appears limited to axonemes of sperm and not those of tracheal airway cilia, while cilia of the inner ear have not yet been examined. This differential effect on sperm and airway cilia has previously been recognized clinically in infertile men (31). Infertility affects as many as 5–10% of men (32), making it one of the most common disorders in humans. Sperm motility is one of the major determinants of fertility in men, and poor sperm motility is a major cause of male infertility (33). In the case of VDAC3-deficient mice, the normal structures found in spermatids within the testes suggest that the defect develops with maturation of sperm in the transition from the testes to the epididymis. Each microtubule doublet has a corresponding outer dense fiber, all of which are morphologically distinguishable. Two of the outer dense fibers that are associated with microtubules 3 and 8 terminate within the principal piece and form the longitudinal columns of the fibrous sheath that partition the axoneme into two unequal compartments. It has been proposed that loss of doublets 4–7 represent sliding of microtubules during attempted motility, with extrusion of half of the axoneme (35). Although, based on the results presented here, expression of VDAC3 protein appears ubiquitous, VDAC1 and VDAC2 reportedly are expressed in a more limited fashion, with VDAC1 restricted to Sertoli cells and VDAC2 found in secondary spermatocytes and in round and elongated spermatids (36). These differences in cell...
type expression imply distinct isoform-specific functions for the VDACs in gonadal cell lineages.

Thus, VDAC3-deficient male mice represent a novel model of sperm immotility due to a mitochondrial defect and demonstrate that functional mitochondria are required for the structural maintenance of the axoneme. The defect in skeletal muscle respiratory chain complex activity suggests the possibility of a similar deficiency in sperm mitochondria, but how this would relate to axonemal stability is not clear.

Recently, Jonas et al. (34) have reported that patch clamp recordings from presynaptic mitochondria detect a rapid increase in large conductances (0.5–2.5 nanosiemens) following a series of action potentials. This conductance depended on the presence of extracellular calcium, and they speculated that conductance reflected activation of mitochondrial outer membrane VDACs. Since the change in conductance outlasted the input stimulus in a time frame very similar to that seen in post-tetanic long term potentiation, the authors further proposed that mitochondrial conductance contributes to synaptic plasticity. Studies are currently under way to address this issue.

Acknowledgments—We thank Bobbie Antalffy and Jim Barrish for technical assistance.

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