Male Subfertility Induced by Heterozygous Expression of Catalytically Inactive Glutathione Peroxidase 4 Is Rescued in Vivo by Systemic Inactivation of the Alox15 Gene*

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Glutathione peroxidase 4 (GPX4) and arachidonic acid 15-lipoxygenase (ALOX15) are antagonizing enzymes in the metabolism of hydroperoxy lipids. In spermatid cells and/or in the male reproductive system both enzymes are apparently expressed, and GPX4 serves as anti-oxidative enzyme but also as a structural protein. In this study we explored whether germ line inactivation of the Alox15 gene might rescue male subfertility induced by heterozygous expression of catalytically silent GPX4. To address this question we employed Gpx4 knock-in mice expressing the Sec46Ala-Gpx4 mutant, in which the catalytic inactivation of the Alox15 gene might rescue male subfertility induced by heterozygous expression of catalytically silent GPX4. To address this question we employed Gpx4 knock-in mice expressing the Sec46Ala-Gpx4 mutant, in which the catalytic selenocysteine was replaced by a redox inactive alanine. Because homozygous Gpx4 knock-in mice (Sec46Ala-Gpx4+/+) are not viable we created heterozygous animals (Sec46Ala-Gpx4+/−) and crossed them with Alox15 knock-out mice (Alox15−/−). Male Sec46Ala-Gpx4−/− mice, but not their female littermates, were subfertile. Sperm extracted from the epididymal cauda showed strongly impaired motility characteristics and severe structural midpiece alterations (swollen mitochondria, intramitochondrial vacuoles, disordered mitochondrial cap). Despite these structural alterations, they exhibited similar respiration characteristics than wild-type sperm. When Sec46Ala-Gpx4−/− mice were crossed with Alox15-deficient animals, the resulting males (Sec46Ala-Gpx4−/−+Alox15−/−) showed normalized fertility, and sperm motility was re-improved to wild-type levels. Taken together these data suggest that systemic inactivation of the Alox15 gene normalizes the reduced fertility of male Sec46Ala-Gpx4−/− mice by improving the motility of their sperm. If these data can be confirmed in humans, ALOX15 inhibitors might counteract male infertility related to GPX4 deficiency.

The redox equilibrium is an important parameter for the regulation of the cellular phenotype, and pro- and anti-oxidative enzymes have been implicated in maintaining the cellular redox homeostasis (1, 2). A disturbed redox equilibrium has been related to the pathogenesis of metabolic, cardiovascular, and neurodegenerative disorders (3–5) and may also play a role in hyperproliferative diseases (6). The redox homeostasis is balanced among other things by the catalytic activities of pro- and anti-oxidative enzymes. Alox15 (12/15-lipoxygenases) and Gpx4 (glutathione peroxidase 4) have previously been identified as important players in this regulatory network (7, 8). Alox15 is capable of oxygenating polyenoic fatty acids to the corresponding hydroperoxides, and thus, it up-regulates the cellular oxidative potential (9, 10). On the other hand, Gpx4 is the only glutathione peroxidase isoform capable of reducing complex hydroperoxy lipids (7), eliminating these peroxides as sources of free radical-induced secondary reactions. Thus, ALOX15 and GPX4 are functional counter players in metabolism of hydroperoxy lipids (11).

Lipoxygenases (LOX) are expressed in the testis of various mammals (12–14), but systemic inactivation of the genes encoding for Alox15, Alox12, and Alox5 does not induce major phenotypic alterations unless the animals were challenged otherwise (8, 15). However, more detailed studies on the reproduction of Alox15−/− mice indicated that breeding of these animals was hampered since male Alox15−/− mice turned out to be subfertile (16). Although the molecular basis for this subfertility has not been explored in detail, it was suggested that epididymal sperm maturation, in particular the proximal-distal migration of the cytoplasmic droplet, which contains remnants of the spermatogenic cytoplasm as well as Alox15 (17), was impaired in Alox15-deficient sperm (16). Homozygous Gpx4-deficient animals die in utero before mouse embryonic day 7.5 (18, 19). Similarly, homozygous mice expressing catalytically inactive Gpx4 variants (Sec46Ala/Ser mutant) do not survive the seventh day of embryogenesis, and systemic Alox15 deficiency did not rescue the lethal phenotype (20). These data suggest that Gpx4 is essential for normal embryogenesis, but its detailed function remains a matter of discussion. Conditional neuron-specific knock-out of Gpx4 expression in adult mice induces neurodegeneration suggesting that embryonic lethality of Gpx4 deficiency might be related to defective brain development (22). This conclusion was consistent with the previous observation indicating that expression silencing of Gpx4 during in vitro mouse embryogenesis induces developmental defects in the central nervous system (23). More

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2 The abbreviations used are: LOX, lipoxygenase; qRT, quantitative real-time; PUFA, polyenoic fatty acid; HETE, hydroxyeicosatetraenoic acid; RP, reverse phase.
recent studies employing conditional knock-out mice, in which expression of the enzyme was selectively inactivated in erythroid precursors, suggest a role of Gpx4 during erythropoiesis (24). These data indicate that embryonic lethality of Gpx4 deficiency in mice might not only be related to developmental defects of the central nervous system. In humans Gpx4 expression is reversibly induced by hepatitis C virus to control lipid peroxidation and to increase virion infectivity (25). Naturally occurring truncation mutants (splicing defects, premature stop codon) in the human Gpx4 gene cause a rare hereditary neonatal lethal disorder called Sedaghatian spondylometaphyseal dysplasia (26). Patients suffering from this disease are characterized by severe metaphyseal chondrodysplasia with limb shortening, cardiac conduction defects, and central nervous system abnormalities (26). These data are consistent with embryonic lethality of Gpx4-deficient knock-out/knock-in mice (18–21).

In adult mammals, testis is the major organ of Gpx4 expression (27–29), and genetic variants of the human GPX4 gene have been related to male infertility (30). In this organ the Gpx4 protein exhibits a moonlighting character as it functions as glutathione peroxidase but also as a structural protein involved in the formation of the sperm specific mitochondrial capsule (31). Male spermatocyte-specific Gpx4 knock-out mice are infertile (32). Their testes display a decreased number of sperm, and isolated epididymal sperm were unable to fertilize oocytes in vitro. The sperm exhibited reduced forward motility and were characterized by structural abnormalities (32). In this model system expression of the Gpx4 protein was completely blocked, and thus, it was impossible to conclude whether the lack of catalytic activity or the absence of Gpx4 as the structural protein was the major reason for the deleterious effects. To address this question, two different strains of knock-in mice were recently created by independent research groups, which express a catalytically inactive Gpx4 variant (Sec46Ala (20) and Sec46Ser (21)), and male heterozygous allele carriers of the Sec46Ser animals were shown to be subfertile (21). Fertility of Sec46Ala males has not been tested, but similar problems were expected. The major reasons why we preferred the Sec-to-Ala strategy are the redox properties of the introduced amino acid. Because of its aliphatic OH group, there is the principle possibility that the Ser site chain might be oxidized by peroxide substrates to an aldehyde or even to a carboxylic acid. In contrast, the Ala side chain is redox-silent and may not be oxidized by peroxides.

Employing Sec46Ala-Gpx4+/− knock-in mice (20), we here confirmed subfertility of male mice, which are deficient in catalytically active Gpx4. Moreover, we found that systemic expression silencing of Alox15 rescued the subfertility of Sec46Ala-Gpx4−/− mice and reimported the motility of their sperm. These changes were paralleled by an enhanced respiratory capacity, suggesting an up-regulated energy metabolism of these sperm.

Results

GPx4 and Alox15 Are Expressed in the Male Reproductive System—Gpx4 is high level-expressed in mammalian sperm (27, 31), but little is known on the expression of LOX-isoforms in the male reproductive system (12). More recent data indicated expression of Alox15 in the plasmatic droplet and suggested a role of this enzyme in sperm maturation (17). To confirm expression of both enzymes in the male reproductive system and to quantify their relative expression levels, we first carried out qRT-PCR with sperm RNA extracts prepared from the epididymal cauda and compared the relative copy numbers of their mRNAs in relation to somatic GAPDH mRNA (Table 1). As expected, mRNAs encoding for the cytosolic, the mitochondrial, and the nuclear Gpx4 isoforms were present in large abundance. In contrast, Alox15 mRNA was present in much lower quantities. Next, we purified epididymal caput sperm by Percoll gradient centrifugation and quantified Alox15 mRNA in these cells. Here we obtained 10.4 ± 0.8 copies of Alox15 mRNA per 104 copies of somatic GAPDH and 71 ± 5.1 copies of Alox15 mRNA per 103 copies of sperm-specific GAPDH. Because possible functional alterations of sperm might also be related Alox15 deficiency in other cells of the male reproductive system, we next explored Alox15 expression in testis and epididymal caput. qRT-PCR of testis indicated 3.3 ± 0.3 copies of Alox15 mRNA per 104 copies of somatic GAPDH. qRT-PCR of RNA extracts from the epididymal caput revealed 6.6 ± 1.1 copies of Alox15 mRNA per 103 copies of somatic GAPDH. These data indicate the presence of Alox15 mRNA in both organs, but they also suggest that Alox15, in contrast to Gpx4, is not expressed at high levels. Thus, Alox15 constitutes a low copy gene in the cells of the male reproductive organs. Furthermore, we carried out immunoblotting of protein extracts prepared from purified sperm, testis, and epididymal caput. qRT-PCR of testis indicated specific Alox15 signals in the appropriate molecular weight range. Finally, we performed immunohistochemistry with our purified sperm preparation employing a homemade anti-Alox15 antibody. Evaluating a large number (>30) of slides, we observed specific Alox15 staining in a few sperm. In these Alox15-positive cells the signal was localized in a microscopic structure, which could be the plasma droplet. However, the majority of the cells were devoid of a specific Alox15 signal. Taken together, these data indicate that Alox15 mRNA is expressed at low levels in testis and epididymis but that the enzyme is only present at low quantities in mature sperm.

Male Sec46Ala-Gpx4+/− Knock-in Mice Are Subfertile, and Their Sperm Exhibit Impaired Motility Characteristics—It has been reported before that mice that heterozygously express a catalytically inactive variant of Gpx4 (Sec46Ser-Gpx4+/− knock-in mice) are subfertile (21), and here we confirmed these data using a similar in vivo knock-in mouse model (20). When we intercrossed Sec46Ala-Gpx4+/− mice, we observed significantly lower litter sizes when compared with intercrossing
wild-type controls. When we crossed male Sec46Ala-Gpx4+/H11001/H11002 knock-in mice with wild-type females, we observed similar alterations (Fig. 1A). In contrast, similar litter sizes were found when female Sec46Ala-Gpx4+/H11001/H11002 knock-in mice were crossed with male wild-type individuals. These data confirm that heterozygous deficiency of catalytically active Gpx4 impairs male fertility.

Next we compared the motility characteristics of sperm extracted from the epididymal cauda of Sec46Ala-Gpx4+/H11001/H11002 and wild-type mice. For this purpose sperm were classified in motile and immotile sperm, and the motile sperm were further sub-classified in sperm with total (any signs of movement) and progressive (rapid and slow forward movement, circular movement) as well as rapid forward movement. The shares of sperm with total, progressive, and rapid motility were significantly lower in Sec46Ala-Gpx4+/− mice when compared with wild-type animals (Fig. 1B).

Sperm of Sec46Ala-Gpx4+/− Mice Express Wild-type and Mutant Alleles in a 1:1 Ratio—Sperm are haploid cells that undergo genome reduction during meiosis. To explore whether sperm carrying the wild-type allele (functional Gpx4) or the mutant allele (dysfunctional Gpx4) are similarly treated during spermatogenesis, we genotyped the sperm isolated from the epididymal cauda of Sec46Ala-Gpx4+/H11001/H11002 mice. As shown in Fig. 1C, we found a 1:1 ratio of the Sec46Ala-Gpx4+/− and the Sec46Ala-Gpx4−/− alleles. These data indicate that sperm carrying the inactive mutant allele are not removed during spermatogenesis.

Deficiency of Catalytically Active Gpx4 Induces Structural Midpiece Alterations—To explore the basis for the impaired motility of Gpx4-deficient sperm, we quantified the structural alterations employing a microscopic scoring system that classifies all sperm in three categories: Grade 1 sperm (normal shape), no midpiece granulation and smooth plasma membranes; Grade 2 (minor alterations), moderate midpiece granulation and rough plasma membranes; Grade 3, severe midpiece granulation and discontinued plasma membrane. When we determined the relative share of grade 1 sperm (normal appearance) in wild-type controls, we found that >90% of the sperm exhibited a “normal” morphological phenotype (Fig.
In contrast, in Sec46Ala-Gpx4+/− mice we did not detect grade 1 sperm at all. In fact, it was possible to conclude the genotype of the animals simply by looking at the morphological characteristics of the sperm. Grade 2 sperm were virtually absent in wild-type controls but together amounted to >90% in Sec46Ala-Gpx4+/− mice.

Next, we explored the midpiece alterations by transmission electron microscopy (Fig. 2B). Here we found that the mitochondria of wild-type sperm are present as condensed organelles, which are well ordered within the mitochondrial capsule (Fig. 2Ba). In contrast, in Sec46Ala-Gpx4+/− sperm the mitochondria are swollen and contain intramitochondrial vacuoles, and the mitochondrial capsule was not well ordered (Fig. 2Bb). Similar alterations have been described for the sperm of spermatocyte-specific Gpx4-knock-out mice (32).

Previous reports have suggested that different Gpx4 isoforms (nuclear, mitochondrial, cytoplasmic) are expressed in different parts of sperm (33). To compare the subcellular distribution of Gpx4 in wild-type and Sec46Ala-Gpx4+/− mice, we carried out immunogold electron microscopy employing a monoclonal anti-Gpx4 antibody, which does not differentiate between the different isoforms (34). Here we found a similar distribution pattern of wild-type Gpx4 and its Sec46Ala-mutant in the head (Fig. 2Ca, and Cb) and the midpiece of cauda sperm (Fig. 2Cc, and Cd). Moreover, Gpx4 protein was also detected in large amounts in the plasma droplet of caput sperm (Fig. 2Ce, and Cf). This is a remarkable finding because the cytoplasmic droplet is the major source of spermatoid Alox15 (16). Although immune electron microscopy is not a strongly quantitative method, staining intensity was not significantly different when we compared sperm of wild-type control animals (Sec46Ala-Gpx4+/−; Fig. 2C, a, c, and e) with those of Sec46Ala-Gpx4+/− mice (Fig. 2, C, b, d, and f).

**Homozygous Inactivation of the Alox15 Gene Rescues the Subfertile Phenotype of Heterozygous Sec46Ala-Gpx4 Knock-in Mice**—Gpx4 and Alox15 are counter-players in the metabolism of hydroperoxy lipids, and experiments with LOX inhibitors...
AloX15 Knock-out Rescues Gpx4-dependent Subfertility

revealed that functional defects induced by Gpx4 deficiency might be antagonized when Alox15 pathway is inhibited (22, 35). To test whether systemic inactivation of the Alox15 gene is capable of rescuing the subfertile phenotype of male Sec46Ala-Gpx4+/− mice, we crossed Sec46Ala-Gpx4+/− mice with Alox15−/− animals to obtain Sec46Ala-Gpx4+/−+ Alox15−/− individuals. These mice do not express Alox15 any more, as indicated by previous activity assays (20).

Next, male and female Sec46Ala-Gpx4+/−+ Alox15−/− mice were intercrossed (52 matings), and we quantified the percentage of successful mating. Here we found that under our mating conditions 25% of mating resulted in pregnancy (Fig. 3A). In contrast, we only observed 11% pregnancies when male and female Sec46Ala-Gpx4+/−+ Alox15+/+ mice were mated (Fig. 3A). Next, we compared the litter sizes of the resulting pregnancies. As indicated in Fig. 3B the litter sizes of heterozygous Sec46Ala mice with wild-type alleles in the Alox15 locus were significantly lower than those obtained for mice with Alox15 deficiency on Sec46Ala-Gpx4+/− background. Taken together these data indicate that functional inactivation of the Alox15 gene appears to rescue the subfertility of male Sec46Ala-Gpx4+/− mice.

Impact of Alox15 Deficiency on Functional and Structural Characteristics of Sec46Ala-Gpx4+/− Sperm—As indicated in Fig. 2, Sec46Ala-Gpx4−/− cauda sperm exhibit impaired motility characteristics when compared with wild-type controls. In contrast, we did not find significant motility differences when wild-type animals were compared with Alox15 knock-outs (data not shown). However, when the Alox15 knock-out was crossed into the Sec46Ala+/− Gpx4 background, we observed reimprovement of the impaired motility characteristics (Fig. 4). Total motility of Sec46Ala-Gpx4+/− sperm, which was significantly lower than that of wild-type sperm (Fig. 2), was significantly improved by homozygous Alox15-deficiency (Fig. 4A). Similar pictures were observed for progressively (Fig. 4B) and rapidly moving sperm (Fig. 4C). When we compared the motility characteristics of epididymal cauda sperm of Sec46Ala-Gpx4+/−+ Alox15−/− mice with those of animals carrying two wild-type alleles at both gene loci (U46A-Gpx4+/−+ Alox15+/+), we observed a significantly higher total motility of the genetically modified sperm (54% for Sec46Ala-Gpx4+/−+ Alox15−/− mice versus 37% for U46A-Gpx4+/−+ Alox15+/+ mice, p < 0.05). These data suggest that Alox15 knock-out does not only compensate, but over-compensates the defective sperm motility induced by functional Gpx4 deficiency.

To explore whether reimprovement of sperm motility may be paralleled by reversal of the structural alterations induced by Gpx4-deficiency, we quantified the morphological differences of the sperm as described in Fig. 2 for the Sec46Ala-Gpx4+/− mice. Here we found that normalization of the sperm motility
by Alox15-knock-out was not paralleled by complete reversal of structural and ultrastructural alterations induced by Gpx4 deficiency. In fact, for many Sec46Ala-Gpx4+/−/Alox15+/− sperm we observed similar ultrastructural features (similar degree of midpiece granulation, roughening of the plasma membrane, swollen mitochondria, disordered alignment of the mitochondria) as in the Sec46Ala-Gpx4+/− sperm (Fig. 5, A, a–c). These structural alterations are also observed by electron microscopy (Fig. 5B, I and II). On the other hand, we also observed normal-looking sperm produced by Sec46Ala-Gpx4+/−/Alox15+/− animals (Fig. 5BIII). These data indicate a high degree of structural heterogeneity, which is also indicated by interindividual variations in the box plots (Fig. 5A).

**Inactivation of the Alox15 Gene Impacts the Energy Metabolism of Sperm**—The motility of sperm is closely related to the energy metabolism of these cells as ATP is required for movement of the tail. To explore whether the increase in cell motility induced by inactivation of the Alox15 gene (Fig. 4) is related to an altered energy supply, we quantified cellular respiration of sperm isolated from Sec46Ala-Gpx4+/−/Alox15+/− animals and compared it with that of Sec46Ala-Gpx4+/−/Alox15+/+. Here we found that despite the structural alterations induced by heterozygous knock-in of a dysfunctional Gpx4 allele (Fig. 2), the cauda sperm of Sec46Ala-Gpx4+/−/Alox15+/+ mice and of Sec46Ala-Gpx4+/−/Alox15+/− mice show similar basal oxygen consumption rates (Fig. 6A). The addition of inhibitors of the respiratory chain (antimycin as inhibitor of complex 3, rotenone as inhibitor of complex 1) completely abolished the oxygen uptake, indicating the mitochondrial respiratory chain as source for the oxygen uptake measured (Fig. 6A). These data suggest that the observed structural alterations of the mitochondria (Fig. 2) do not significantly impact cellular respiration of the sperm. In contrast, when the Alox15 gene was inactivated on the Gpx4-deficient background, a significantly augmented (almost doubling) basal oxygen consumption rate was measured (Fig. 6, A and B), suggesting an up-regulation of the energy metabolism. Up-regulated cellular respiration indicates a more intense energy metabolism (higher ATP synthesis), providing an explanation for improved sperm motility (Fig. 4).

**Inactivation of the Alox15 Gene Does Not Alter the Degree of Membrane Oxygenation of Sec46Ala-Gpx4+/− Sperm**—Because Alox15 and Gpx4 are antagonizing enzymes in the metabolism of hydroperoxy lipids (11), because Gpx4 functions as endogenous inhibitor of Alox15 (36), and because the expression of both enzymes is inversely regulated (37), we...
Gpx4 is shown in Fig. 7, polyenoic fatty acids (PUFA), which is given in % of the total membrane lipids was defined as relative share of the oxygenated polyunsaturated fatty acids. The oxygenation degree of membrane lipids was determined by HPLC for the content of non-oxygenated and oxygenated PUFAs. After alkaline hydrolysis of the ester lipids under an argon atmosphere, aliquots of the hydrolysates were analyzed by RP-HPLC for the quantification of the oxygenated and non-oxygenated PUFAs. A, representative RP-HPLC indicating the presence of hydroxylated and non-hydroxylated polyenoic fatty acids in the total membrane lipids. The chromatographic scales were calibrated by injecting known amounts of 13-HODE (hydroxy fatty acid), arachidonic acid, and linoleic acid. LA, linoleic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid. B, statistic evaluation of the hydroxy fatty acid/fatty acid ratios of the sperm total lipids. For each genotype, sperm from eight mice were analyzed.

The major finding of this study is that systemic inactivation of the Alox15 gene reversed the defective functional phenotype of Sec46Ala-Gpx4+/− sperm. This functional rescue is somewhat surprising for several reasons. Functional inactivation of the Alox15 gene in mice carrying two wild-type alleles in the Gpx4 locus induced subtle subfertility (16), and we confirmed these findings in our study (data not shown). Although the mechanistic basis for this observation has not been clarified, Alox15-deficient males had significantly more epididymal sperm with retained cytoplasmic droplet (16). Moreover, the cytoplasmic droplets of wild-type sperm had a smooth appearance and contained mostly empty membrane vesicles. In contrast, the cytoplasmic droplet of Alox15-deficient sperm contained functional mitochondria, which are targeted for exocytosis. Moreover, epithelial lesions, phagocytosis-like figures, and missing or aberrant apical blebs were observed in the epididymal caput of Alox15-deficient males (16). From these data, the authors concluded that the process of epididymal sperm maturation and proximal-to-distal migration of the cytoplasmic droplet is altered in Alox15-deficient mice. Thus, Alox15-deficient mice are characterized by normal spermatogenesis but altered epididymal sperm maturation. However, the molecular basis for the developmental role of Alox15 remains elusive.
As an oxidizing enzyme, it might contribute to Gpx4 polymerization during the formation of the mitochondrial capsule, which is an essential step in spermatogenesis. Interestingly, in preliminary experiments we found that sperm of Alox15-deficient mice form significantly less insoluble Gpx4 polymers, as indicated by immunoblotting, which is consistent with a role of Alox15 in Gpx4 polymerization. However, more detailed studies are required to clarify the molecular basis for this observation.

On heterozygous Sec46Ala<sup>+/−</sup> background, systemic inactivation of the Alox15 gene induced re-improvement of the impaired fertility, which was induced by functional Gpx4 deficiency. The morphological alterations induced by heterozygous Gpx4 deficiency (modified midpiece structure, swollen mitochondria, disordered mitochondrial capsule) were detected in both epididymal caput and epididymal cauda sperm (data not shown). In fact, there was no significant difference in the degree of morphological alterations between these two sperm maturation stages. On the other hand, the functional defects (lower sperm motility) were only observed in the cauda sperm (data not shown). Thus, there is no 1:1 translation of the morphological alterations induced by Gpx4 deficiency into the functional characteristics of the sperm. Although the detailed molecular mechanisms for the maturation-dependent sperm dysfunction remains to be investigated, the most probable explanation is that the morphological defects, which are already visible at earlier stages of spermatogenesis, are not sufficiently severe to cause functional alterations at early developmental stages. However, when sperm mature, the morphological changes might become functionally more relevant as the alterations exceed the compensation capacity of the sperm.

As pro- and anti-oxidative enzymes Alox15 and Gpx4 have been implicated as counter-players in the regulation of the cellular redox homeostasis (1, 41, 42) and a number of studies suggested a functional interplay in vivo, and in vitro studies suggested a functional interplay between the two enzymes (22, 35, 43, 44). In some of these studies inhibitors of Alox15 were used to silence the catalytic activity of the enzyme. Unfortunately, the use of such compounds is problematic for the reasons below.

Lacking Isoform Specificity—In humans six different LOX isoforms including ALOX15 have been identified, but for most commercially available LOX inhibitors the isoform specificity has not been tested in detail. Recently, a systematic study was carried out in which all rat 12-lipoxygenating LOX isoforms were overexpressed in HEK cells, and an array of commercially available LOX inhibitors was tested (45). The data obtained indicate that frequently employed LOX inhibitors (nordihydroguaiaretic acid, propyl gallate, baicalin, and cinnamyl-3,4-dihydroxy-a-cyanocinnamate) are catechols and, thus, may function as anti-oxidants. Thus, in addition of being LOX inhibitors they impact cellular redox homeostasis by distinct mechanisms. For in vivo studies it is difficult to discriminate which of the two functions (LOX inhibition versus antioxidant function) is the major reason for an observed biological effect. Similar effects may be discussed for non-catecholic LOX inhibitors, which may be biotransformed to catechols. To avoid misinterpretations and to improve reliability inhibitor studies should always be confirmed by supplementing loss-of-function strategies.

 Naturally occurring mutations in the human GPX4 gene have been related to the pathogenesis of oligoasthenozoospermia (30) and to Sedaghatian-type spondylometaphyseal dysplasia (26). Here we report in a murine system that defects in the Gpx4 gene can be compensated by inactivation of Alox15 expression. If this is also the case in humans, ALOX15 inhibitors might constitute potential drugs for such diseases. Unfortunately, for the time being no ALOX15 inhibitor has been approved for clinical use, and thus, it is impossible to test this therapeutic concept.

Materials and Methods

Chemicals—The chemicals were from the following sources. HPLC standards of 5(±)-HETE, 12(±)-HETE, and 15(±)-HETE were from Cayman Chemical (distributed by Spi Bio, Montigny le Bretonneux, France), sodium borohydride was from Life Technologies, HPLC solvents were from J. T. Baker or Montigny le Bretonneux, France), according to the vendor’s instructions. The chemicals were purchased from Invitrogen.

Animals and Breeding—All the mice were bred and maintained in a specific pathogen free animal facility according to the FELASA recommendation with food and water ad libitum. All animal experiments were performed in compliance with the German animal welfare law and have been approved by the institutional committee on animal experimentation. Alox15 knock-out mice (strain name: B6.129S2-Alox15<sup>tm1Fun/J</sup>) were obtained from The Jackson Laboratory (Bar Harbor, ME) (48) and backcrossed for seven generations into black six background.

PCR Genotyping of the Gpx4 and Alox15 Locus—For routinely genotyping, mouse-tail biopsies were used. To genotype the Gpx4 and the Alox15 loci, genomic PCR was carried out employing allele-specific primer combinations. PCR was performed with the MyTaq<sup>TM</sup>Red Mix (Bioline, Luckenwalde, Germany) according to the vendor’s instructions. The primers used to detect the Gpx4 wild type or Gpx4 Sec46Ala mutant allele were as follows: forward, 5′-GACAGATGCTCTCTG-GACCTGGGTG-3′; reverse, 5′-TAATCTGGCGTGTTA-GGGGCCAGAC-3′. The wild-type band is 412 bp long, and the Sec46Ala mutant band is 587 bp long, which included a residual LoxP/FRT sequence after neo deletion. Heterozygous allele carriers have two bands, whereas homozygous allele carriers would
either have the lower (homozygous wild type) or the higher (homozygous mutant) band. For genotyping the Alox15 locus, the primer sequences were recommended by The Jackson Laboratory: oMR9535-mutant reverse (5′-GGG AGG ATT GGG AAG ACA AT′-3′), oMR9711-common (5′-GCC TGC CTG AAG AGG TAG AGC-3′), and oMR9712 wild-type reverse (5′-CTA TAG ACG AGA CCA GCA CA-3′). The wild-type band is 200 bp, and the knock-out band is 417 bp. Homozygous allele carriers only show the upper band. The same method was employed to genotype the sperm extracted from the epididymis.

\textit{Cross-breeding Studies—}Three- to six-month-old mice, wild-type mice ((Sec46Ala-Gpx4/−/−+Alox15+/+), heterozygous Sec46Ala knock-in mice (Sec46Ala-Gpx4+/−+/+), and heterozygous Sec46Ala knock-in mice with Alox15-deficient background (Sec46Ala-Gpx4+/−+/+Alox15−/−)) were mated as described previously (20). All mice were genotyped by genomic PCR employing allele-specific primer combinations.

\textit{DNA Preparation—}Genomic DNA was prepared from mouse-tail biopsies or cauda sperm using the Invisorb Spin Tissue Mini kit (Berlin, Germany) extraction kit and employed for genomic PCR-based genotyping.

\textit{Sperm Extraction and Morphological Analysis—}After euthanasia, testes and epididymides were isolated and kept at 4 °C. Both testes and epididymides were freed from surrounding tissue and blood vessels. Each testis was dissected from the epididymis and weighed. One testis per male was fixed in Bouin’s solution, and histological sections were prepared and stained by conventional hematoxylin-eosin staining.

For sperm extraction epididymal caput and epididymal cauda were minced in M199-k medium of M199 (Sigma M7528) supplemented with 1 mM sodium pyruvate, 14 mM sodium lactate, and 0.4% (w/v) BSA (Mercat) at room temperature (20–23 °C). After 5 min of incubation, the suspension was filtered through 30-μm nylon mesh (Partec, Goerlitz, Germany). For morphological analysis, droplets of a 5-μl sperm suspension were spread on a slide, fixed, and stained with the Spermac® stain kit (Minitüb, Tiefenbach, Germany). After Spermac® staining acrosomes and midpieces appeared dark green, nuclei appeared red and the equatorial region was colored light green.

\textit{Percoll Gradient Sperm Purification—}Freshly prepared sperm from the epididymal caput were centrifuged at 500 × g for 5 min and resuspended in 1 ml of PBS. This suspension was then placed on top of a 30–45% discontinuous Percoll gradient. The sample was centrifuged for 30 min at 1500 × g, and the resulting upper layer, which contained contaminating cell debris and somatic cells, was removed. The purified sperm were isolated from the lower layer and washed by centrifugation.

\textit{RNA Extraction and qRT-PCR of Gpx4 and Alox15 mRNA—}Total RNA was extracted from the sperm using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). Synthesis of the cDNAs was performed with 1–3 μg of the total RNA preparations using RevertAid™ Premium First Strand cDNA Synthesis kit (Schwerte, Germany). Quantitative real-time PCR was carried out with a Rotor Gene 3000 (Corbett Research, Mortlake, Australia) using the SensiMix™SYBR PCR Kit (Bio-line). GAPDH, Alox15, and Gpx4 isoform-specific amplification employed here are described in Refs. 20 and 23. The primers used to detected sperm-specific GAPDH were as follows: forward (5′-AGCTAGAGACGAGTGAGGTAT-3′) and reverse (5′-CCACCACTAGTTGCTGCTATTCTATGGT-3′).

The experiment raw data were analyzed with the Rotor-Gene Monitor software (version 4.6). To generate standard curves for quantification of gene expression levels, specific amplicons were used as external standards for each target cDNA. All RNA preparations were analyzed at least in triplicate, and the means ± S.D. are given.

\textit{Sperm Motility Assays—}To evaluate the sperm motility, the filtered sperm suspension was diluted with prewarmed (38 °C) M199-k medium to a density of 10–50 × 10⁶ cells/ml, and 5 μl of the suspension was added in a prewarmed Makler chamber (Sefi-Medical Instruments, Haifa, Israel). Sperm motility was analyzed by the computer-assisted sperm analysis system AndroVision (Minitüb, Tiefenbach, Germany) under an AXIO Scope A1 microscope (Zeiss, Oberkochen, Germany) equipped with negative phase contrast optics and a video camera Basler avA1000–100gc (Basler AG, Germany). For each field, 100 pictures were recorded at 100 Hz, and 8 fields with 20–50 spermatozoa per field were evaluated per sample. The percentage of motile sperm was evaluated. Motile sperm showed local and progressive movement. Progressive sperm represent cells with any kind of circular or forward movement. Rapid sperm move faster than 80 μm/s.

\textit{Electron Microscopy and Immunogold Staining—}For electron microscopy, sperm were fixed in 3% (w/v) glutaraldehyde, washed in PBS (pH 7.2), and fixed again in 2% osmium tetroxide. Dehydration of the fixed preparations was performed in ethanol, and samples were embedded in EPON 812 before preparation of ultrathin sections and staining with uranyl acetate and lead citrate. For immune electron microscopy, sperm were fixed in Karnovsky’s solution (Serva, Heidelberg, Germany) washed in PBS, dehydrated in ethanol, and embedded in LR white resin. Ultrathin sections were incubated at room temperature with a 1:2000 dilution of our monoclonal antihuman Gpx4 antibody (34). After 1 h of incubation, sections were washed with a few drops of PBS and then treated with a commercial goat anti-mouse IgG antibody (1:40 dilution), which was labeled with 12 nm of colloidal gold. After 45 min at room temperature the sections were washed with PBS and water. Electron microscopy was carried out with FEI TecnaiSpiritBT device (120 kV; FEI Deutschland GmbH).

\textit{Measurements of Sperm Respiration—}For quantification of sperm respiration, the epididymal cauda was minced in Heps-buffered modified Tyrode’s medium (mT-H: 131.89 mM NaCl, 2.68 mM KCl, 0.49 mM MgCl₂·6H₂O, 0.36 mM Na₂HPO₄·2H₂O, 20 mM Hepes, 5.56 mM glucose, 1.80 mM CaCl₂) that was supplemented with 4 mg/ml fatty acid-free bovine serum albumin at room temperature (52). After 5 min at 37 °C the suspension was filtered through a 30-μm nylon mesh (Partec). The sperm suspension was centrifuged for 2 min at 500 × g, the supernatant was removed, and sperm were resuspended in 1 ml mT-H. Sperm were counted with a Neubauer chamber. Four to five individuals of the different mouse strains were employed to quantify the respiration characteristics. For respiration measurements, 2.5 × 10⁷ sperm were seeded on an XFp cell culture miniplate that had been coated with concanavalin A to ensure proper sperm adhesion. Two wells were left without cells to perform background correction. After 1 min, the plate
was centrifuged for 2 min at 1200 × g. This centrifugation was repeated, changing the plate orientation to ensure an even adhesion of cells to the bottom of the well. The supernatant was removed, 175 μl of M199-k medium were added, and sperm adhesion was checked by microscope. Then the plate was placed in the instrument. An XFp extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA) was used. Oxygen uptake measurements were carried out at 37 °C for ~16 min. Then rotenone (1 μM final concentration) and antimycin A (1 μM final concentration) were added to block mitochondrial respiration. The oxygen uptake was normalized to the sperm number present in the incubation sample. Basal mitochondrial respiration was calculated by subtracting the oxygen uptake measured after the addition of rotenone and antimycin A from the overall oxygen uptake determined during the first 16 min of the measuring period.

**Degree of Oxygenation of Membrane Lipids—** Sperm were prepared as described above with the exception that pre-warmed PBS was used instead of M199-k medium. Cells were washed, and the total sperm lipids were extracted according to the Bligh/Dyer method (53). The solvent was evaporated, the remaining lipids were reconstituted in 0.35 ml of anaerobic methanol, and 0.075 ml of anaerobic 40% KOH was added. The ester lipids were then hydrolyzed under an argon atmosphere (20 min at 60 °C), and the samples were cooled on ice and neutralized by the addition of 0.075 ml of acetic acid. Precipitate was spun down, and aliquots of the clear supernatant were injected to RP-HPLC. HPLC was performed on a Shimadzu LC-20 instrument recording simultaneously light absorbance at 235 nm (detection of hydroxy PUFAs) and 210 nm (detection of PUFAs). A Nucleodur C18 Gravity column (Macherey-Nagel; 250 × 4 mm, 5 μm particle size) was used, and fatty acid derivatives were eluted with a solvent system consisting of methanol/water/acidic acid (85/15/0.05 by volume) at a flow rate of 1 ml/min. The chromatographic scale was calibrated by injecting known amounts (6-point calibrations for each compound) of 13-HODE, linoleic acid, and arachidonic acid.

**Statistics—** Statistical analyses were carried out with SPSS 23 (IBM New York) software package, and the results are presented as medians. Non-parametric tests, most frequently the Mann-Whitney U test, was carried out. Two-tailed significance was accepted at p < 0.05. Within the box plots the black vertical lines indicate the median. The 25th and the 75th percentile are visualized as upper and lower box limits.

**Author Contributions—** S. H. B. carried out most of the experiments and contributed to writing the paper. M. R. carried out lipid analysis and contributed to writing the paper. S. R. R. performed genotyping of animals and sperms and contributed to writing the paper. K. M., S. E., D. V., and C. F. contributed to characterization of sperm motility and performed electron microscopy analysis. H. K. contributed to study design, drafted the manuscript, supervised the study, and contributed to sperm lipid analysis. A. B. contributed to study design and writing the paper and performed the respiration measurements of the sperm.

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