Drosophila and Antioxidant Therapy Design

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Summary

Antioxidant intervention is a potential therapeutic approach to mitigate the oxidative stress component of various human diseases. Genetic studies in the model organism *Drosophila melanogaster* indicate that the major antioxidant defense systems protect from oxidative stress in a compartment-specific manner. They also suggest that compensation for the loss of an antioxidant enzyme by experimentally inducing expression of a second, distinct defense enzyme is possible in a few cases. However, in other examples, overexpression of an antioxidant enzyme may also exacerbate an oxidant-sensitive phenotype. Thus, a successful antioxidant therapy should target the affected tissues, act in the appropriate sub-cellular compartments and specifically rectify the underlying molecular imbalances.

Introduction

Oxidative stress is characterized by the generation of reactive oxygen species (ROS), primarily including the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical, which damage proteins, lipids and nucleic acids. A variety of human pathologies are associated with oxidative stress, including cancer (Mantovani, *et al.* '02; Lincoln, *et al.* '03), cardiovascular (Levine, *et al.* '96; Meagher and Rader '01), inflammatory (Cuzzocrea, *et al.* '01), viral (Peterhans '97) and neurological (Lodi, *et al.* '01; Pitchumoni and Doraiswamy '98; Halliwell '01) diseases. Nevertheless, despite a variety of antioxidants used for therapeutic purposes, the response of patients has not always been positive

(Ebadi, *et al.* '96; Brown, *et al.* '01). Here we discuss results from *Drosophila*, which underscore the importance of considering ROS compartmentalization and ROS specificity in the design of such treatments.

Results

Many studies have assessed the effects of various antioxidants and of antioxidant enzyme genetic manipulations on flies (for reviews see Le Bourg '01 and Missirlis '03). The recent development of fly disease models permits testing of drugs (Chan and Bonini '00). *Drosophila* contains all major antioxidant defense enzymes that are employed by mammals, with the notable exception of glutathione reductase (Kanzok, *et al.* '01). Two different superoxide dismutase (*Sod*) genes encode cytosolic and mitochondrial proteins (Phillips, *et al.* '89; Kirby, *et al.* '02). A thioredoxin reductase (*TrxR-1*) gene encodes two transcripts that give rise to cytosolic and mitochondrial isoforms (Missirlis, *et al.* '02). Thioredoxin peroxidases (TPxs) are likewise localized in both these compartments (Radyuk, *et al.* '01), whereas a glutathione peroxidase homologue with thioredoxin peroxidase activity (GTPx1) is detected in the secretory pathway (Missirlis, *et al.* '03b).

The compartmentalization of antioxidant defense systems might have a redundant role in oxidative stress physiology, or it might reflect the existence of independent redox environments within the different compartments of the same cell. We investigated the question of independent redox environments by selectively silencing *Sod* in the cytoplasmic or mitochondrial compartments and concurrently assaying the activity of cytosolic and mitochondrial aconitases, as markers of $O_2^{\cdot -}$ reactivity (Missirlis, *et al.* '03a). We showed that diminution of Sod2 results in mitochondrial, but not cytoplasmic aconitase inactivation and conversely diminution of Sod1 inactivates cytoplasmic, but not mitochondrial aconitase. In addition, overexpression of Sod2 could not ameliorate the *Sod1* mutant phenotype, implying that enhancing the mitochondrial $O_2^{\cdot -}$ scavenging potential of cells is not beneficial if excess $O_2^{\cdot -}$ is present in the cytosol. Furthermore, overexpression of Sod2 did not protect from paraquat, a $O_2^{\cdot -}$ generating drug, because paraquat acted in the cytosolic compartment (Missirlis, *et al.* '03a). In contrast, overexpression of Sod1 greatly protects from paraquat toxicity (Parkes, *et al.* '98). These results suggest that $O_2^{\cdot -}$ is confined *in vivo* to the subcellular compartment in which it is formed and antioxidant treatment needs to be targeted to that compartment.

We have also generated transcript-specific mutants in the *TrxR-1* gene, impairing the thioredoxin-dependent defense system in mitochondria or cytosol, respectively (Missirlis, *et al.* '02). Loss of either activity resulted in lethality, suggesting that both isoenzymes are required for

ROS detoxification. Transgene-dependent rescue experiments indicated that cytosolic TrxR-1 can compensate only for the lack of cytosolic TrxR-1 activity (as expected), but cannot substitute for the lack of mitochondrial TrxR-1 (although the two TrxR-1 isoforms have very similar biochemical properties) and *vice versa*. We conclude that separate compartmentalized ROS defense systems operate in the cytoplasm and mitochondria to sustain cell viability and propose that compromise of either the Sod-dependent or the thioredoxin-dependent antioxidant defenses cannot be compensated by isoforms that reside in the wrong subcellular compartment.

ROS specificity is a second determinant for the appropriate selection of antioxidants used in different pathological conditions. We have previously assessed the impact of Sod1 and Cat overexpression in the mutant for cytosolic TrxR-1 (Missirlis, *et al*. '01). The results were surprisingly contrasting, as Cat offered a substantial rescue of viability and lifespan, whereas Sod1 impacted further the *TrxR-1* phenotype (Missirlis, *et al*. '01). We also investigated whether overexpression of GTPx1 is beneficial in flies lacking Sod1 or Cat. Biochemical characterization of GTPx1 shows that this enzyme is highly active with H_2O_2 as a substrate, *in vitro* (Missirlis, *et al*. '03b). We generated transgenic *Drosophila* that overexpress *GTPx1*, either from its endogenous or from an inducible promoter. Transgenic flies were resistant to paraquat toxicity, implying an important role for GTPx1 in antioxidant defense. However, *GTPx1* overexpression did not enhance the viability of the *Cat* mutant (Missirlis, *et al*. '03b). A similar function between two enzymes *in vitro* does not necessarily reflect the actual situation *in vivo*.

We then generated flies heterozygous for the genomic *GTPx1*$^+$ transgene on their second chromosome and heterozygous for two different *Sod1* null-activity alleles on the third. Only 5-8% of *Sod1* homozygous flies successfully eclose from their pupal case as adults and *GTPx1*$^+$ segregates at the expected Mendelian ratio in wild type flies. We performed sibling analysis and counted how many of the viable *Sod1* homozygous mutants contained zero, one or two copies of the *GTPx1*$^+$ transgene. Table 1 shows that a *GTPx1*$^+$-heterozygote, *Sod1* mutant fly is much less likely to eclose than a sibling *Sod1* mutant with wild type second chromosomes (d"50%; expected ratio is 1:2:1). Two extra copies of *GTPx1* are almost incompatible with viable *Sod1* mutants. This interaction is specific to *Sod1*; *Cat* mutants are not affected by the presence or absence of the transgene (see also Missirlis, *et al*. '03b). The finding that *GTPx1* overexpression negatively impacts *Sod1* mutants is even more surprising when one considers that the same genetic manipulation confers resistance to the $O_2^{\cdot -}$ generating herbicide, paraquat. Although paraquat administration is often considered a pharmacological parallel to the genetic ablation of *Sod1* (Missirlis, *et al*. '03a), its mechanism of action also implicates $O_2^{\cdot -}$ independent pathways (Berisha, *et al*. '94).

Table 1. Genetic interaction between GTPx1 *and* Sod1*: Overexpression of* GTPx1 *enhances the* Sod1 *mutant phenotype, contrary to the prediction that increasing an antioxidant enzyme would be beneficial to an oxidatively-stressed individual.*

	wild-type	*GTPx1*$^{+}$ heterozygote	*GTPx1*$^{+}$ homozygote
Sod1^{x39}	78	57	6
Sod1^{n108}	12	13	0
Cat^{n1}	131	264	90

Further experiments are required to explain these observations, however they underscore the importance of understanding the substrate specificity, subcellular location and cofactor requirements for each of the proteins in question.

Conclusions

By studying the antioxidant defense systems of *Drosophila*, we have shown that oxidative stress in one subcellular compartment is fairly insensitive to the redox status of adjacent compartments. We have also observed that, although antioxidant defense systems cooperate to protect from ROS, we cannot assume that enhancing one system will always be protective, especially if different antioxidant systems are failing. Thus, before designing an antioxidant therapy, the source (tissue, intracellular location) of oxidative stress and the type of oxidative insult should be clarified. The need for development of antioxidant drugs that target different aspects of the machinery in different compartments is crucial. Complementing current efforts of generating drugs that have multi-enzyme properties or therapies that use antioxidant cocktails, we should also invest on issues of specificity.

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