REVIEW ARTICLE

The Role of Oxygen in Health and Disease - A Series of Reviews

*This is the fifth and last article in the series of reviews focusing on the role that oxygen plays in health and disease. As a proof of principle, Drs. Zhou, Visk and Haddad have described *Drosophila* genes, beneficial in developing resistance to hypoxia, to provide insight regarding susceptibility and tolerance to low oxygen. This information will pave the way towards developing improved therapies for mammalian species to combat the consequences of low oxygen delivery and low blood oxygen levels.*

Sherin U. Devaskar, M.D.
Editor in Chief

***Drosophila*, a Golden Bug, for the Dissection of the Genetic Basis of Tolerance and Susceptibility to Hypoxia**

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ABSTRACT: We have previously discovered that the adult *Drosophila melanogaster* is tolerant to a low O₂ environment, withstanding hours of total O₂ deprivation without showing any evidence of cell injury. Subsequently, our laboratory embarked on the study of hypoxia tolerance using a mutagenesis and overexpression screens to begin to investigate loss-of-function or gain-of-function phenotypes. Both have given us promising results and, in this article, we detail some of the interesting results. Furthermore, several years ago, we have also started an experimental "Darwinian" selection to generate a fly strain that can perpetuate through all of its life cycle stages in hypoxic environments. Through microarrays and bioinformatic analyses, we have obtained genes (e.g. Notch pathway genes) that play an important role in hypoxia resistance. In addition, we also detail a proof of principle that *Drosophila* genes that are beneficial in fly resistance to hypoxia can also be as well in mammalian cells. We believe that the mechanisms that we are uncovering in *Drosophila* will allow us to gain insight regarding susceptibility and tolerance to low O₂ and will therefore pave the way to develop better therapies for ailments that afflict humans as a consequence of low O₂ delivery or low blood O₂ levels. (*Pediatr Res* 66: 239–247, 2009)

Hypoxia, whether present during physiologic states (e.g. embryogenesis and organ formation) or during pathologic states (e.g. asthma, chronic obstructive pulmonary disease, obstructive sleep apnea, and sickle cell anemia), present

a challenge to the organism. Depending on duration and severity, hypoxia can lead to cell injury and death and consequently organ injury and failure. This is well illustrated in diseases that lead to major morbidity and mortality, such as myocardial infarction, cerebrovascular accidents and brain dysfunction, and placental insufficiency with poor fetal development and potential demise. In fact, the consequences of cerebral and myocardial hypoxia and ischemia are among the most severe in the 21st century. Investigations in the past few decades have started to provide some understanding of the fundamental basis of hypoxia and the mechanisms by which it leads to injury. Furthermore, we and others have also begun to identify the mechanisms that underlie adaptation to and survival in hypoxia such that the molecules that mediate adaptive effects might lead to new therapeutic targets to protect or reverse hypoxia-induced pathology. Such investigations have taken advantage of a variety of models, from invertebrates to vertebrates and to humans.

Many approaches have been used to study questions regarding hypoxic cell injury due to O₂ deprivation. Many investigators, including ourselves, have resorted to *in vitro* techniques and others to more *in vivo* approaches. Some investigators have used acute settings and, mostly, electrophysiologic techniques to examine ionic homeostasis (1), but

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Abbreviations: **AF**, hypoxia-adapted/selected flies; **CH**, constant hypoxia; **dADAR**, pre-mRNA adenosine deaminase; **dMRP4**, *Drosophila* homolog of the human multidrug resistance protein 4; **IH**, intermittent hypoxia; **NF**, naïve control flies

others have relied on morphometric and anatomic approaches. Still others have focused almost exclusively on molecular approaches, especially in settings in which the stress is modest and cells and tissues withstood prolonged periods of hypoxia (2–4). Some of the more recent studies in our laboratory as well as in others, using molecular and genetic approaches, have provided evidence that there are genes that can protect against or predispose to cell injury and death when cells are exposed to O₂ deprivation (see later). Some investigators have studied the effect of hypoxia alone but others have combined it to glucose deprivation as well. There are laboratories that have focused on global ischemia and others have studied focal ischemia. Therefore, it is of no surprise that the results in the literature have been often confusing and sometimes inconsistent. This is compounded also by the fact that experimentally the P_{O₂} in the hypoxia system is not measured and there can be major effects of small changes, especially at the lower levels of O₂. Most importantly, these studies are often biased in that the investigators focus on a pathway or a molecule that they are most familiar with or most interested in, trying to find a role for that particular pathway or molecule in hypoxia or ischemia. Clearly, this is not necessarily faulty but it illustrates the idea that often the work that has been published in the past, until probably a few years ago, was focused on themes that might not have been the most important in understanding normative or pathobiological processes.

WHY *DROSOPHILA*: A VARIETY OF PHENOTYPES AND DISEASE CONDITIONS

Although flies have been used for over a hundred years, since the days of T. H. Morgan, work on flies focused on being models for human diseases only in the past decade when such work flourished and major discoveries are currently being made. At present, a considerable amount of research in *Drosophila* is tied to the understanding of behavioral, biochemical, or genetic processes at the molecular level and the relation to disease processes in mammals or humans. Examples in point are related, for instance, to the effort that is ongoing at present to solve the molecular underpinnings of aging, tumor formation, alcohol intoxication, neurodegeneration, and memory, to name a few (5–7).

Complex pathways are simpler to elucidate in *Drosophila* compared with those in vertebrates. Single-cell organisms such as yeast and bacteria are useful in determining cell autonomous effects, where the effect is in a single cell, with no interactions between the cells. Higher order metazoans, such as vertebrates, often have redundancies in their genomes with several genes able to compensate for one another. Lower order metazoans, such as *Drosophila*, have far fewer redundancies in their genome, and this represents at least one reason why *Drosophila* is a useful model to study noncell autonomous effects or ones that require a model organism that undergoes development or has variety of organ systems (5). An example of this can be found in studies of heart development. The discovery of the *Drosophila* gene *tinman*, a homeobox transcription factor involved with cardiac specification, and its conservation in vertebrates supplied evidence that conserved pathways controlled cardiac development

in both flies and humans (8,9), driving subsequently the field of cardiac development in humans (10).

Another important example that has illustrated the power of *Drosophila* as a model system is in the area of triplet repeats and neurologic diseases, such as Fragile X syndrome (FXS). In 2002, two groups independently reported that the fly homolog for the protein causing FXS, Fmr1, associates with the RNAi complex in *Drosophila* (11,12) and further research in both *Drosophila* and higher metazoans confirmed the role of FMR1 as an RNA binding protein (13) and showed that Fmr1 mutant flies have impaired long-term memory, analogous to some aspects of mental retardation in humans (13,14). Finally, a *Drosophila* model of FXS used to screen a library of small molecules led to the discovery of nine especially promising compounds that rescued several FXS phenotypes (14).

We have been interested in a variety of questions that span from O₂ sensing to the cellular and molecular responses to hypoxia and to injury from anoxia. More than 12 y ago, we discovered that the adult fruit fly, *Drosophila melanogaster*, is acutely tolerant to a low O₂ environment, withstanding ~3–4 h of total O₂ deprivation (anoxia) without showing any evidence of cell injury (6,15–17). We could not distinguish, using light and electron microscopy, between the cells and cellular organelles in the CNS of flies that were exposed to anoxia for hours from normoxic control flies. This is clearly remarkable, especially, that 3–4 h of the life of a fly is much longer than this specific absolute time if one translates this in terms of “human” time! On the basis of these observations, our laboratory embarked on the study of hypoxia tolerance in this organism for the following reasons: a) *Drosophila melanogaster* is an extremely well-studied organism with thousands of stocked mutants and P-element lines (insertional mutations) that cover the majority of its genome; b) ~70% of all known human disease genes are present in *Drosophila melanogaster*; and c) this organism has had a long history of genetic dissection of normative and disease processes (5–7). This opened major avenues for us because the *Drosophila* has been used so effectively in so many relevant research areas. Indeed, in spite of many advances in monitoring oxygenation, there is still considerable morbidity and mortality arising from conditions with O₂ deprivation leading to hypoxic/ischemic damage, especially, in brain. Part of this failure is related to the complexity of the cascade of events that ensue after hypoxia. Hence, we have used *Drosophila* in our laboratory to solve some of the questions related to tolerance or susceptibility to hypoxia. In this review, the role and importance of genetic models, such as *Drosophila melanogaster*, are discussed using specific examples illustrating the power of *Drosophila* genetics as a tool to understand human disease and in this particular case, hypoxic cell injury. We used three ideas to try to address our questions: 1) mutagenesis and overexpression screens to identify loss-of-function mutants and gain-of-function lines; 2) microarrays on adapted *versus* naïve flies; and 3) studying cell biology and physiology of genes that seem important in flies and mammals. The hope is to learn from these studies about the fundamental basis of tolerance (and susceptibility) to the lack of O₂, and with this knowledge be able to develop better therapies for the future.

FORWARD AND REVERSE GENETICS APPROACHES AND RESULTS FROM *DROSOPHILA*

These approaches allow us to use specific techniques and test specific hypotheses aimed at understanding the basis for hypoxia tolerance or susceptibility. Indeed, we have used the following: 1) a classic or forward genetics approach, in which a phenotype is dissected genetically and a gene or multiple genes are identified that could be responsible for the phenotype of interest, *i.e.* tolerance or susceptibility to O₂ deprivation; 2) reverse genetics, in which gene expression is studied and that the differential gene expression obtained can potentially lead to the understanding of the phenotype of interest; and 3) the study of mechanisms of specific genes in flies to understand the cell biology and physiology that is not currently understood in mammals; flies are used here also as a model. In the past several years, we have started to use these approaches in the study of hypoxia in *Drosophila melanogaster*. Both mutagenesis and overexpression screens were begun to investigate loss-of-function or gain-of-function phenotypes and both have given us promising results (18–26). We have taken advantage of both forward and reverse genetic approaches and have successfully used these in the past to address various questions of interest. For example, we have succeeded during the past 5–6 y in generating a fly strain, through experimental selection (see later) that can live and perpetuate through all of its life cycle stages in extremely low O₂ environments. We will detail examples of these approaches below.

Forward Genetic Approaches and Results

Example 1: An adenosine deaminase gene, ADAR. Using the first strategy, or forward genetics, one focuses on defining the phenotype and then isolating the gene(s) responsible for the phenotype after using, for example, a mutagenesis screen. This screen would include a behavioral and/or physiologic assay that is essential to uncover those mutants that have either lost or gained function. After such mutants are isolated, the mutations are mapped and the genes cloned. To prove that such genes are responsible for the phenotype, one would resort to various approaches including the injection of a wild-type cDNA of the gene of interest into a mutant embryo, for example, to rescue the phenotype. This is precisely what we have done and, about a few years ago, we obtained very interesting results pertaining to the gene *hypnos-2^P* or *ADAR* (23). This gene encodes a *Drosophila* premRNA adenosine deaminase (dADAR) and is expressed almost exclusively in the adult CNS. Disruption of the *dADAR* gene results in totally unedited sodium (Para), calcium (Dmca1A), and chloride (DrosGluCl- α) channels, a very prolonged recovery from anoxic stupor, a vulnerability to heat shock and increased O₂ demands, and neuronal degeneration in aged flies. We have also done rescue experiments to ascertain that *hypnos-2^P* or *ADAR* is the gene that induces the sensitivity to anoxia (Fig. 1). These data clearly demonstrate that, through the editing of ion channels as targets, *dADAR*, for which there are mammalian homologues, is essential for adaptation to altered environmental stresses such as O₂ deprivation and for the preven-

tion of premature neuronal degeneration. In addition, these mutant flies recover their evoked potentials after anoxia at a much slower pace than wild-type flies, which is consistent with the behavioral results. Furthermore, in characterizing further the phenotype, we also found that this mutant, although it is anoxia sensitive (loss-of-function mutant for hypoxia resistance), is very resistant to oxidant injury. In addition to phenotypic studies, we have performed studies to determine 1) the expression of *ADAR* (23,27,28) and 2) the targets that are acted upon by *ADAR* (18,24). We found that there are specific regions in the fly CNS where *ADAR* has high expression. Using a variety of molecular and bioinformatic techniques, we have indeed discovered a number of novel targets, which are potentially the target molecules that are important for the phenotype (24).

Example 2: A multidrug resistance protein, dMRP4. Although we have previously shown that *Drosophila melanogaster* is hypoxia-tolerant, how this species senses O₂ deprivation and how it survives oxygen-limiting conditions are as yet poorly understood. We addressed this question here by testing for anoxic responsiveness in *Drosophila* adult flies after an overexpression of existing EP lines. In this screen, we identified *Drosophila CG14709* gene as a homolog of the human multidrug resistance protein 4 (MRP4/ABCC4) that is tightly regulated to oxygen homeostasis. When this gene product is overexpressed, adult flies profoundly delay their recovery time from anoxia. Ubiquitous expression (with daughterless promoter, da) of *dMRP4* (using the EP line 3177, see Fig. 2) in adult flies resulted in increased sensitivity to anoxia as they had longer recovery time from anoxic stupor. When exposed to 4% O₂ chronically (throughout its lifespan), constitutive expression of *dMRP4* in larvae caused larval lethality due to growth arrest (Fig. 1). Mutations of *dMRP4* led to a hypersensitive response to acute anoxia in adult flies but had less impact on larval survival under chronic hypoxia compared with *dMRP4* overexpression. Selective expression of this gene in neurons, but not in glia or muscles, mirrored the same phenotype as the ubiquitous one. Because neuromuscular transmission and muscle evoked responses have been tightly correlated with the recovery behavior of adult flies during and after anoxia (17), it is interesting to know the role of muscle in anoxia tolerance, in addition to neurons. We addressed this question by targeted expression of *dMRP4* specifically in muscles under control of 24B-Gal4. We found that overexpression of *dMRP4* in muscles only did not alter the behavioral response to anoxia in adult flies and had no effect on larval survival when exposed to 4% O₂. Although these results did not completely rule out the potential contribution of muscles to anoxia tolerance, we believe that, based on our results with targeted expression, the *dMRP4* activity in neurons mainly accounts for sensing the oxygen changes by *Drosophila*. In support of this notion, targeted expression of *dMRP4* only in larval imaginal discs did not change adult behavior in response to anoxia. Thus, our data suggest novel roles for MRP *in vivo*: 1) *dMRP4* regulates the sensitivity to acute or chronic O₂ deprivation, and 2) *dMRP* expression in neurons is sufficient to induce the sensitivity to O₂ in the whole organism.

Figure 1. Results of forward genetic screen: dADAR and dMRP4. (A) The prolonged recovery of *hypnos-2^P* (*ADAR^{-/-}* null) flies can be fully rescued by a wild-type dADAR transgene (○, *hypnos-2^P-dADAR*; ●, *w¹¹¹⁸*; □, *hypnos-2^P*; ■, *hypnos-2^P-32B*). (B) The dADAR transcripts are expressed mainly in adult heads, and the deletion in *hypnos-2^P* is an in-frame deletion. (C) The expression of dADAR can be detected by RT-PCR in C-S embryos (6–8 h). No full-length dADAR transcripts can be detected in *hypnos-2^P* embryos and adults. *St*, low mass DNA marker; *Hyp*, *hypnos-2^P*; *E*, embryos (6–8 h); *A*, adults. (D) Localization of dADAR transcripts in adult fly heads (left panel; arrowheads). Sense dADAR RNA probe gave no positive signals in the same tissues (right panel). (E) Misexpression of dMRP4 reduces viability in *Drosophila*. Viability was shown as percentage of first instar larvae surviving to pupae, referring to numbers of *yw* pupae from the normoxic groups as 100%. (□, 21% O₂; ■, 4% O₂). (F) *dMRP4* mRNA expression in pupae. Semi-quantitative RT-PCR was used to determine the expression levels of *dMRP4* mRNA from 24B>EP(3)177 pupae exposed to 4% O₂ for 10 d (line 2) or reared at 21% O₂ (line 3), *yw* flies were used as control (line 1). (Adapted with permission from Ma E *et al.*, J Clin Invest 107:685–693, copyright © 2001, by The American Society for Clinical Investigation, and Huang H and Haddad GG, Physiol Genomics 29:260–266, copyright © 2007 by the American Physiologic Society).

Figure 2. Phenotypic changes in hypoxia-selected *Drosophila melanogaster*. (A) Survival rate of parental *Drosophila melanogaster* population in hypoxic conditions. The parental population survived well at 8% O₂ but their survival rate was reduced at 6%; and 4% O₂ was lethal. (B) No significant differences were found in lifespan between naïve flies (Δ, NF) and hypoxia-adapted/selected flies (□, AF). The mean lifespan for NF is 33.7 ± 6.2 d (*n* = 180), and the mean lifespan for AF is 33.7 ± 3.9 d (*n* = 220). (C) and (D) Body size and weight in AF. The body size and weight of AF were significantly reduced, compared with NF. Data were presented as means ± SD (**p* < 0.05, ***p* < 0.01; *t* test). (Adapted with permission from Zhou D *et al.*, PLoS Genet 4:e1000221, copyright © 2008).

Reverse Genetics

An experimental selection paradigm and a “Darwinian experiment.” Using a reverse genetics approach, we asked whether we can determine which genes are important during

hypoxia. We had two ideas. First, we exposed flies to various short durations of hypoxia (minutes to hours) and determined gene expression; subsequently, we showed that the alterations in gene expression played a role in hypoxia (see below under

acute hypoxia). Second, starting from a relatively large pool of alleles, we exposed flies to hypoxia over generations and determined whether the offspring selected under hypoxic pressure had differential gene expression; we proved that these alterations are important.

Unlike forward genetics, reverse genetics studies the function of a gene starting from the gene itself instead of a phenotype. In reverse genetics, an expression of the gene is altered using various techniques and the effect of such genetic manipulation on the organism is analyzed. In the past several years, we have successfully combined reverse genetics with the strategies of experimental selection and expression profiling in our studies of hypoxia tolerance or injury in *Drosophila*. Our approach was to 1) identify those genes that are differentially expressed in hypoxia, 2) genetically manipulate these genes by various mutagenesis or RNAi strategies, and 3) determine the effect of such manipulations on the response to hypoxia or survival in hypoxia. This strategy has allowed us to study the genetic basis of long-term (over generations) or short-term (hours to days) responses to hypoxia. We summarize here the studies related to the understanding of mechanisms underlying hypoxia tolerance in our newly generated hypoxia-tolerant *Drosophila* strain (25,26).

To identify the mechanisms underlying hypoxia tolerance in hypoxia-tolerant animals and to translate such mechanisms into mammals to protect from hypoxia-induced injury is an attractive way to develop new strategies for therapeutic purposes. To do so, we have generated in the past several years a *Drosophila* strain that can survive perpetually in severe hypoxic environment (*i.e.* equivalent to 4% O₂, an oxygen level at ~4000 m above Mount Everest). In this experimental selection experiment, a balanced pool of wild-type isogenic lines was used as the parental population, which had a significant variability in hypoxia tolerance (1). Hypoxia tolerance of the parental pool was determined at the outset by culturing the F1 embryos of the parental flies under different levels of O₂ (*e.g.* 8, 6, or 4%). This was important because we needed to determine the starting O₂ level for the experimental selection. We found that 6% O₂ significantly reduced the survival rate, and 4% O₂ was lethal. At 8% O₂, however, the majority of embryos (>80%) completed their development and reached the adult stage. Therefore, we initiated the hypoxia selection at 8% O₂. To maintain the selection pressure, O₂ concentration in the selection chambers was gradually decreased by ~1% after a few to several generations. By the 13th generation, we obtained flies that were able to complete development and perpetually live in 5% O₂; and by the 32nd generation, flies could live perpetually under 4% of O₂, a lethal condition for naïve flies. To test whether this hypoxia tolerant trait is heritable, a subset of embryos obtained from the selected flies were collected and cultured under *normoxia* for several consecutive generations. After eight generations in normoxia, their embryos were reintroduced into the lethal hypoxic environment (for naïve flies, 4% O₂), and again, the majority (>80%) of these embryos completed their development and could be maintained in this extreme condition perpetually. This result strongly suggested that the hypoxia-tolerance in the selected flies was indeed heritable. Furthermore, several

remarkable phenotypic changes were observed in the hypoxia-selected flies. For example, the hypoxia-selected flies have a shortened recovery time from anoxia-induced stupor (16), consume more oxygen in hypoxia, and show a significant reduction in cell number and cell size and a reduction in body weight and size (Fig. 2).

To further investigate the genetic basis underlying hypoxia tolerance in this hypoxia-selected *Drosophila* strain, we determined the global gene expression profiles in 3rd instar larvae and adult flies using cDNA microarrays. One reason for performing the microarrays on both larvae and adult flies is to determine how a stress-like hypoxia affects both the developing organism (larvae) and a mature organism (adult). We found 2749 significant alterations (1534 up- and 1215 down-regulated) in the larval stage and 138 significant alterations (95 up- and 43 down-regulated) in the adults ($p < 0.05$ and fold change >1.5). Among them, 51 genes were found to be altered in both larval and adult stages. Interestingly, most of the commonly up-regulated genes encode proteins that are related to stress response and immunity, and the majority of the commonly down-regulated genes encode proteins that are related to metabolism. The significantly altered genes were further categorized based upon their Gene Ontology (GO). We found significant alterations in >30 biologic processes in both larvae and adult. Most of these processes were related to either defense (especially immune responses, $p < 0.05$) or metabolism (especially carbohydrate and peptide metabolism, $p < 0.05$). Interestingly, most down-regulated genes in larvae encoded proteins related to metabolism (1044 genes including 135 genes that are related to carbohydrate metabolism, $p < 0.01$). In contrast, the genes that were up-regulated belonged to multiple components of signal transduction pathways including EGF, insulin, Notch, and Toll/Imd signaling pathways (Table 1).

Previous studies based upon metabolic analyses demonstrated that metabolic suppression plays an important role in hypoxia tolerant animals. For example, Hochachka *et al.* (29–31) have argued that anoxia-tolerant organisms depress their metabolism to minimize the mismatch between supply and demand. Although this idea is intuitively appealing, there was no information about how various metabolic enzymes could be coordinated to survive severe long lasting hypoxia. In the hypoxia-selected flies, we identified significant changes in the family of genes regulating cellular respiration and metabolism, especially in larvae. Indeed, the majority of the genes were down-regulated. For example, besides one pyruvate kinase isoform (*CG12229*), most of the genes encoding glycolytic enzymes were dramatically down-regulated. Similarly, the expression of genes encoding TCA cycle, lipid β -oxidation, and respiratory chain complexes were also significantly down-regulated, especially in larvae (Fig. 3). Because the majority of genes in the TCA cycle were down-regulated, we hypothesized that such down-regulation was coordinated, possibly at a transcriptional level. Therefore, we attempted to identify transcriptional regulators that potentially controlled the coordinated suppression by analyzing the cis-regulatory regions of these down-regulated genes. To do so, the GenomatixSuite (GEMS) software was used to identify transcription factor binding elements in the defined cis-regulatory

Table 1. Changes in signaling pathways in hypoxia-selected *Drosophila melanogaster* at larval and adult stages ($p < 0.05$)

Symbol	Gene name	Fold change	
		Larvae	Adult
EGF receptor signaling pathway			
E(spl)	Enhancer of split	1.81	NS
ed	Echinoid	1.85	NS
Egfr	Epidermal growth factor receptor	1.52	NS
Nrg	Neuroglian	1.68	NS
spi	Spitz	1.51	NS
Insulin receptor signaling pathway			
Akt1	Akt1	2.20	NS
CG10702	CG10702	1.51	NS
CG11910	CG11910	1.83	NS
chico	Chico	2.10	NS
Pi3K21B	Pi3K21B	1.59	NS
Pk61C	Protein kinase 61C	1.51	NS
Pten	Pten	1.68	NS
JNK cascade			
Aplip1	APP-like protein interacting protein 1	−1.57	NS
bsk	Basket	1.99	NS
Cdc42	Cdc42	1.79	NS
Crk	Crk	1.83	NS
R	Roughened	1.82	NS
Rho1	Rho1	1.60	NS
Sac1	Sac1	−2.18	NS
Notch signaling pathway			
aph1	Anterior pharynx defective 1	1.63	NS
Brd	Bearded	1.65	NS
E(spl)	Enhancer of split	1.81	NS
fng	Fringe	1.55	NS
HLHmβ	E(spl) region transcript mβ	1.64	1.60
HLHmδ	E(spl) region transcript mδ	2.20	NS
HLHmγ	E(spl) region transcript mγ	1.92	NS
mα	E(spl) region transcript mα	2.27	NS
m4	E(spl) region transcript m4	2.21	NS
nct	Nicastrin	1.72	NS
O-fut1	O-fucosyltransferase 1	2.74	NS
SP1070	SP1070	2.00	NS
Su(dx)	Suppressor of deltex	−1.60	NS
Toll signaling pathway			
ECSIT	ECSIT	−1.79	NS
ird5	Immune response deficient 5	1.78	NS
key	Kenny	1.81	NS
nec	Necrotic	−1.73	NS
PGRP-SA	Peptidoglycan recognition protein SA	2.51	NS
PGRP-SB1	PGRP-SB1	7.56	2.03
Pli	Pellino	1.72	NS
snk	Snake	−2.06	NS
Spn27A	Serpin-27A	1.87	NS
spz	Spatzle	1.55	−1.71

NS, not significant.

regions of genes encoding the TCA cycle. We divided the TCA cycle related genes into two groups, one containing the significantly down-regulated genes (as the experimental group, 16 genes) and the other containing the insignificantly altered or up-regulated genes (as the reference group, eight genes). Of major interest was that the binding elements of the *Drosophila* transcriptional suppressor, *hairy*, were overrepresented in the regulatory regions of the down-regulated genes (15 of 16) but not in the reference group (one of seven) ($p <$

0.0001, χ^2 -Test). Furthermore, the expression level of *hairy* was also found to be significantly up-regulated in the hypoxia-selected flies. These results suggested that *hairy*, a key transcriptional suppressor reduced the expression of the TCA cycle genes in hypoxia. Physical binding of *hairy* to the *cis*-regulatory regions of the candidate TCA cycle genes were confirmed by chromatin immunoprecipitation-PCR assay (ChIP-PCR) in *Drosophila* Kc cells that were treated with hypoxia. Furthermore, the hypoxia-induced suppression of TCA cycle genes was abolished in the *hairy* loss-of-function mutants, *h*¹ or *h*^{1j3}. Following these studies, two *hairy* loss-of-function mutants were used to determine their survival rates in a relatively mild hypoxic condition (*i.e.* 6% of O₂). We found that both *hairy* loss-of-function mutants exhibited much lower survival rate ($p < 0.0001$, χ^2 test) compared with controls, proving the role of *hairy* in hypoxia tolerance in flies (Fig. 4).

Acute hypoxia and survival. This previous experiments focused on a long-term type of hypoxia, occurring over many generations. However, from a clinical point of view, acute hypoxia (lasting min or hours) is also pertinent. In addition, because we have evidence that intermittent hypoxia is rather different from constant, we also made provisions to investigate both constant and acute stresses. Both of these frequently occur in disease states. For example, intermittent hypoxia (IH) is associated with obstructive sleep apnea, central hypoventilation syndrome, and intermittent vascular occlusion in sickle cell anemia. Constant hypoxia (CH) is associated with pulmonary disease such as asthma, congenital heart disease with left to right shunt. Hypoxia can occur even under normal condition such as high altitude. Various studies, using rodents as animal models, have examined experimentally the effects of chronic CH and IH on specific tissues such as heart, brain, and kidneys (32–36) CH and IH are very different in their effect on growth, proliferation, generation of reactive O₂ species, and neuronal injury (33,37–40). Furthermore, *in vivo* studies have shown organ-specific phenotypic differences to low O₂ such as hypertrophy in heart or decrease in myelination and *N*-acetyl aspartate/creatine ratios in brain (36–38). In this study, we focus on gene expression changes associated with severe short-term constant (CH) or intermittent hypoxia (IH) in adult flies. Our hypothesis was that CH induces different gene expression profile than IH in *Drosophila*, and that these expression changes are important for inducing tolerance to short-term hypoxia. Indeed, our microarray analysis has identified multiple gene families that are up- or down-regulated in response to acute CH or IH. Furthermore, we observed distinct responses to IH and CH in terms of gene expression that varied not only in the number of genes but also type of gene families. During CH, stress response genes (heat shock) were the most overrepresented family whereas multidrug resistance genes were predominantly up-regulated during IH. With the use of P-elements and EP lines, we have also shown that these differentially expressed genes are important for the phenotype of survival. These data provide further clues about the mechanisms by which IH or CH lead to cell injury and morbidity or adaptation and survival.

Figure 3. Suppression of genes encoding glycolytic, TCA cycle enzymes, and components of the respiratory chain complexes. (A) Schematic illustration of the alterations in genes encoding glycolytic and TCA cycle enzymes (Green, down-regulated genes; Black, not significantly changed genes; Red, up-regulated genes). (B) Cluster map of changes in genes encoding glycolytic enzymes in NF and AF larvae. (C) Cluster map of changes in genes encoding TCA cycle enzymes in NF and AF larvae. (D) Cluster map of changes in gene expression encoding components of respiratory chain complexes. Each cluster contained nine hybridizations of NF and eight hybridizations of AF samples. (E) Summary of alterations in each respiratory chain complex measured in larvae. (F) Comparison of gene expression changes in each respiratory chain complex between larvae and adult. (Adapted with permission from Zhou D *et al.*, PLoS Genet 4:e1000221, copyright © 2008).

Figure 4. Effect of the transcriptional suppressor, hairy, on TCA cycle gene expression and hypoxia tolerance. (A) Genomic localization of hairy binding elements in the *cis*-regulatory region of genes encoding TCA cycle enzymes (Arrowheads, consensus hairy binding sites; Arrows, transcriptional start sites; Bold, down-regulated genes; Regular, not significantly changed or up-regulated genes). (B) Significant up-regulation of hairy expression in hypoxia-selected AF populations (□, NF; ■, AF) (***p* < 0.01). (C) Abolished suppression of genes encoding TCA cycle enzymes in hairy loss-of-function mutants (■, AF; □, h¹ mutant; ▤, h^{1/3} mutant). (D) Significant reduction in hypoxia survival in hairy loss-of-function mutants (open bar: normoxia; gray bar: 6% O₂) (***p* < 0.01). (Adapted with permission from Zhou D *et al.*, PLoS Genet 4:e1000221, copyright © 2008).

Although it is not clear at this stage from the results above on the acute or long-term effect of hypoxia in flies whether the transcriptional changes are in fact a result of HIF-1 α activation or other signaling pathways, our belief at present is that there is evidence that there are a number of switch mechanisms during hypoxia and these may vary from cell-to-cell, tissue-to-tissue, and species-to-species, just to name a few variables. Consider for example the metabolic switch that we

have recently described, such as *hairy*, a target of Notch. Another example is the *ADAR* gene that we obtained from the forward genetic approach that we did several years ago and its ability to activate a switch mechanism that affects double-stranded premRNAs. Our prediction is that a number of such switch mechanisms will be discovered soon with all the advanced technology that is and will be at our disposal in the next few years.

WHY DOES IT MATTER IF FLIES ARE RESISTANT TO LOW O₂? WHAT ABOUT A PROOF OF CONCEPT?

Like with any microarray analysis, the major question that emanates at the end of such an analysis is whether the genes that have altered expression have something to do with the phenotype of interest. Although hypoxia tolerance is likely to be a complex trait which involves coordinated action of many genes, individual gene expression profiling can provide clues about genes that control multigenic traits. However, it is likely that only some of the differentially expressed individual genes are directly responsible for hypoxia tolerance in the selected flies. To further identify the genes that functionally contributed to the tolerance of the lethal level of hypoxia (5% O₂), we adopted a strategy that uses P-elements. These were first used for genes that were down-regulated in the microarray experiments (single P-element insertion *Drosophila melanogaster* lines). In addition, to further confirm that survival in these severe O₂ conditions was related to the P-element insertion in these particular genes, we excised the P-element alleles of one gene, namely *sec6*, and found that the precise excision lines had less than 10% of eclosion in 5% O₂, a level that is similar to naive controls. Therefore, the precise excision of these P-elements reversed the hypoxia tolerance phenotype. The *sec6* gene encodes a protein that is homologous to a mammalian *sec6* protein, and it is predicted to be involved in synaptic vesicle recycling (25). This presents the first evidence that this gene is involved in conferring hypoxia tolerance in *Drosophila*, possibly through regulation of neurotransmitter release.

Although the phenotype of hypoxia survival is, in all likelihood, complex and a number of genes may be acting in a coordinated way to affect hypoxia survival, what is surprising in our studies is that single-gene alteration was able to affect the survival of flies to very low O₂ environment! Clearly, this does not indicate that multiple genes are not involved and that an even better survival might be achievable if a number of

genes were altered at the same time. Our data indicate, however, that even single genes can be important in modulating this impressive phenotype and that a strategy like ours, which involved single P-element fly studies, has a chance in dissecting the importance of single genes in this phenotype of hypoxic survival.

That flies can become more tolerant or are naturally more tolerant to low O₂ than mammals may not be surprising or, to some investigators, not relevant to human health. However, we believe that this can be a narrow view and that work in *Drosophila* can enhance our ability to understand biologic normative processes and most probably human diseases. We will illustrate here with an example as a proof of concept.

One of the important questions that we asked was whether trehalose, which is a glucose dimer, can play an important role in protecting flies against anoxic stress. We had discovered in nuclear magnetic resonance (NMR) experiments that trehalose was present in abundance in *Drosophila* heads. We first cloned the gene for trehalose-6-phosphate synthase (*tps1*), which synthesizes trehalose, and examined the effect of *tps1* overexpression or mutation on the resistance of *Drosophila* to anoxia (20,21). Upon induction of *tps1*, trehalose increased, and this was associated with increased tolerance to anoxia. A transposable genetic element (p-element) inserted into an intron of the *tps1* gene resulted in an embryonic lethal fly (19). To determine whether trehalose could protect against anoxic injury in mammalian cells, we transfected the *Drosophilatps1* gene (*dtps1*) into human embryonic kidney cells (18). Glucose starvation in culture showed that HEK 293 cells transfected with pcDNA3.1 (–) *dtps1* (HEK-*dtps1*) did not metabolize intracellular trehalose and, interestingly, these cells accumulated intracellular trehalose during hypoxic exposure. In contrast to HEK 293 cells transfected with pcDNA3.1 (–) (HEK-v), cells with trehalose (HEK-*dtps1*) were more resistant to low oxygen stress (1% O₂; Fig. 5). Insoluble proteins were three times more abundant in HEK-v than in HEK-*dtps1* after

Figure 5. Left upper panel: Cell viability assay after exposure to 1% O₂ for 4 d. There were four times more dead cells in HEK-v than in HEK-*dtps1*. Right upper panel: Apoptosis assay of the cells after hypoxic treatment for 2 d. Annexin V-FITC and propidium iodide stained on average $13.4 \pm 3.2\%$ and $6.4 \pm 0.3\%$ of the HEK-v cells but a much lower proportion of HEK-*dtps1* cells ($1.4 \pm 0.4\%$, $p < 0.001$ and $0.3 \pm 0.2\%$, $p < 0.0001$). Left lower panel: (A) Western signals of Na⁺-K⁺ ATPase and β-actin. (B) The Na⁺-K⁺ ATPase amount present in the insoluble proteins fraction increases dramatically in HEK-v cells after 2 and 3 d of exposure (■, HEK-v; □, HEK-*dtps1*) (* $p < 0.05$). Right lower panel: Intracellular trehalose reduces ubiquitinated proteins during hypoxia (■, HEK-v; □, HEK-*dtps1*) (* $p < 0.05$). (Adapted with permission from Chen Q *et al.*, J Biol Chem 278:49113–49118, copyright © 2003 by The American Society for Biochemistry and Molecular Biology, Inc.).

3 d of exposure to low O₂. The amount of Na⁺–K⁺ ATPase present in the insoluble proteins dramatically increased in HEK-v cells after 2 and 3 d of exposure, whereas there was no significant change in HEK-*dtps1* cells. Ubiquitinated proteins increased dramatically in HEK-v cells but not in HEK-*dtps1* cells over the same period. Our results indicate that increased trehalose in mammalian cells after transfection by the *Drosophila tps1* gene protects mammalian cells from hypoxic injury!

HYPOXIA AND FUTURE RESEARCH

With the advances in technology, molecular biology, and genomics and with the use of model systems, whether it is yeast, *C elegans*, *Drosophila*, or mice, research focusing on hypoxia has taken many twists and turns over the past 1–2 decades, as it always is, in basic and clinical research. One of the major questions that many investigators have tried to tackle in the past is that of O₂ sensing. Extensive efforts have provided some answers but, by and large, many tissues and cell types seem to have their own specific important pathways and generalizations may not be very helpful (41).

Two other major areas have seen enormous growth in hypoxia research. One is related to cancer biology and the other to injury and repair mechanisms in various tissues, especially in brain, heart, and kidneys. It is very clear now that hypoxia is part of the overall picture of tumor growth, metastasis, and treatment, and many laboratories have focused their efforts on understanding the basis of the hypoxia modulation of cancer and on potential clinical trials. Injury and repair are also a major area now, and the use of animal models, as we show in this review, has been an important addition to our armamentarium and our flexibility to impact on the field and be able to re-engineer susceptible cells into more resistant ones and help in either prevention or repair of tissues.

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