Enhancing our understanding of the molecular responses to hypoxia in mammals using Drosophila melanogaster

GABRIEL G. HADDAD
Departments of Pediatrics, Section of Respiratory Medicine, and Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520

Haddad, Gabriel G. Enhancing our understanding of the molecular responses to hypoxia in mammals using Drosophila melanogaster. J Appl Physiol 88: 1481–1487, 2000.—Drosophila melanogaster has been used as a genetic model, especially in the past decade, to examine normative biological processes and disease conditions very effectively. These span a wide range of major issues such as aging, cancer, embryogenesis, neural development, apoptosis, and alcohol intoxication. Here, we detail how the Drosophila melanogaster can be used as a genetic model to study the molecular and genetic underpinnings of the response to hypoxia. In our study of the basis of anoxia tolerance, one of the potent approaches that we use is a mutagenesis screen to identify loss-of-function mutants that are anoxia sensitive. The major advantage of this approach is that it is not biased for any particular gene or gene product. Although our screen is in progress, we already have evidence that this approach is useful.

central nervous system; differential display; genetics and reverse genet- ics; anoxia; invertebrate models

A simple PubMed database search reveals that, since the mid 1970s, ~1,000 articles are written per year on the subject of hypoxia. Clearly, a variety of subject topics are addressed at a variety of levels, from an integrative to a molecular level. Although this number of publications does not rival the number of articles written, say, for AIDS, many investigators over the past century have been interested in hypoxia.

The reasons for this interest in the subject are varied but can be grouped into two main categories: 1) an interest in how an individual (from yeast to humans) responds to low O2 conditions and how the adaptation to such a stress takes place and 2) interest in mechanisms that lead to cellular and tissue injury and repair. Indeed, in the past two to three decades, a plethora of new ideas has surfaced and this whole field has been exciting, especially because the scientific community involved in this field is multidisciplinary with varied backgrounds.

From all of these studies, therefore, we have learned a great deal. For example, there are many events that follow O2 deprivation, inside and outside cells, that can impact on cell function. Furthermore, there are a number of cellular mechanisms that are activated that allow survival if the stress is not too severe. However, there comes a point in the cascade of events at which irreversible injury sets in, if the stress persists. This injury can also lead to either necrosis or apoptosis. Finally, a large number of molecules are involved in these responses, including, ion channels, neurotransmitters, growth factors, cytoskeletal proteins, lipases and proteases, and transcription factors. Some of these will differ depending on whether the stress is of acute or of chronic onset.

There are still, however, many questions that have no answer. For example, we do not know what is the exact role of each event in the overall scheme of the response to low O2. What is the importance of the downregulation in metabolic rate during hypoxia in some individuals and species and how does one assess its role (10–12, 31)? What is the importance of the increase in intracellular Ca2+ during hypoxia (6)? What is the importance of the opening of K+ channels during hypoxia in nerve cells (5, 7, 8, 14–18, 35)? What is the importance of the activation of a number of intracellular kinases during cellular hypoxia (29)? And what is the role of the increase or decrease in a variety of neurotransmitters and growth factors during lack of O2.
including rodents (neonates and mature) and turtles, to worked with a variety of mammals and organisms, and that are described below took place after we had

elected to delve into a genetic model and try to answer
years ago, while pondering some of these questions, we
discovered that the regions of the central nervous system (CNS), such as the neocortex, the hippocampus, and the brain stem (5)!
And consider the differences even between subregions of the CNS and cell types differences such as in the hippocampus and dentate gyrus (20).

Although some of these questions are readily answerable in mammalian systems, others are not. Several years ago, while pondering some of these questions, we elected to delve into a genetic model and try to answer some of these questions in the Drosophila melanogaster. The experiments that we performed on this model and that are described below took place after we had worked with a variety of mammals and organisms, including rodents (neonates and mature) and turtles, to address some of the questions posed above.

**WHY GENETIC MODELS AND WHY DROSOPHILA?**

Because we were convinced that the differences detailed above could be explained, at least in part by inheritance, we considered a number of potential genetic models for use. In addition, there were two major questions that we were very interested in at that particular juncture: 1) What is the genetic basis for the wide heterogeneity in the response to lack of O2? 2) Can we use anoxia-tolerant organisms to understand the basis of O2 responsiveness? It was also clear to us at that time that the freshwater turtle that was considered the par excellence model for an anoxia-tolerant organism was not an optimal organism, since they sense the lack of O2, a situation that is similar to mammals, since they sense the lack of O2 and respond to it like mammals (13, 19).

Furthermore, there is a complete recovery of muscle-evoked potentials with reoxygenation, after a latency that is proportional to the anoxic period, a physiological response that is again similar to the behavioral one. This stereotypical response in flies is very different from that in turtles. Turtles do not seem to lose neuronal activity, even after very prolonged anoxia (10, 34), extending to hours. Hence, flies, unlike turtles, seem to have different strategies for survival under very low-O2 conditions. From the point of view of sensing, flies seem to behave phenotypically in a manner that is similar to mammals, since they sense the low-O2 conditions and respond to it like mammals (13, 19).

However, flies seemingly do recover from prolonged anoxia, but this is not the case in mammalian organisms and tissues. Hence, the question is how can flies (and other organisms capable of tolerating anoxia) recover from anoxia and survive the severe stress?

Although some of the genetic models, including Drosophila, have been in use for many decades, the conservation of complements of genes with evolution, from prokaryotes to eukaryotes and from yeast, Drosophila, Caenorhabditis elegans, zebrafish to humans, has become more appreciated in the past decade. With this important discovery of gene conservation, these models have become even more timely and useful in trying to solve problems relevant to human physiology, biology, and disease. Genes responsible for functions as varied as circadian rhythms, aging, alcohol intoxication, and development of tracheal buds, heart chambers, and CNS have all been cloned first in model systems (1, 2, 21, 26, 28, 30, 32, 33) and then studied in mammals as well as humans. Genes responsible for programmed cell death were first cloned in C. elegans (27), and their homologues were found afterward in mammals and humans.

Very recently, Wingrove and O’Farrell (34) have also shown that the nitric oxide pathway is conserved in flies and humans in O2 sensing.
There are a number of reasons for the success of these genetic models in understanding normal biology. One major reason is that they enjoy important advantages, some conceptual and others technical. Although the space allocated for this mini-review does not allow detailing these advantages, I will briefly mention a few that have been particularly helpful in work done in Drosophila. 1) The Drosophila has a number of useful characteristics of a genetic model: a small number of chromosomes, a generation time of 10 days at 25°C, and a generation size of more than 200–300 per female. 2) There is an enormous number of mutant lines (deficiencies, inversions, P elements, and so forth) and chromosomal markers available for use. 3) Molecular tools such as libraries are available. 4) There are tools available for the study of cell or organ physiology in Drosophila (see below). Finally, 5) P elements, which are transposable DNA elements with known sequences, have been very useful in Drosophila for cloning and mapping purposes.

SPECIFIC QUESTIONS AND APPROACHES IN DROSOPHILA

Our long-standing interest has been in the area of cell and tissue hypoxia. More specifically, we have been very interested in the response of cells, in particular nerve cells, to O₂ deprivation and in nerve cell injury. If this is our interest, how does a genetic model such as Drosophila help us? Because Drosophila survives long periods of anoxia, we took advantage of this situation and specifically asked what is the Drosophila endowed with genetically to be able to resist anoxia for many hours?

To answer this question, a number of approaches can be used. We have used three of these possible approaches in parallel and have obtained very interesting results. Below is a brief outline of each approach and a summary of the results obtained.

A genetic approach. Assuming that there are genes in the Drosophila that protect cells and tissues from anoxic injury, the main idea here is to mutagenize the fly genome and develop a mutagenesis screen to identify flies that have lost these genes, i.e., flies that have loss-of-function mutations. Then, these mutations are mapped in detail using marker chromosomes and small deficiencies and then cloned. The genes responsible for the abnormal phenotype are then ascertained in various ways, including the use of transgenic techniques and rescue experiments. This approach has two main advantages: 1) we start with a phenotype that is useful and that is relevant to the question asked, and 2) there is no bias in terms of the genes and molecules found; whatever genes are found in the mutagenesis screen are studied.

Although we had no a priori reason to suspect that the genes of interest are exclusively located on the X chromosome, we focused our screen for mutations on that chromosome for two reasons. The first is to limit our initial task, but the main reason is strategic. An advantage of focusing on the X chromosome is that, using a specific cross, the screening for mutations on the X chromosome for a specific phenotype can be observed in the immediate next generation without the need for subsequent single-pair matings. Thus we will be able to screen for recessive mutations in the immediate next generation, since the investigation is done in males.

There are at least three ways for mutagenizing the genome in the Drosophila: 1) X-rays, 2) ethylmethane sulfonate, and 3) P element insertion mutagenesis. There are advantages and disadvantages for each of these methods, but space in this mini-review does not allow us to detail these. We have started with X-ray mutagenesis and P elements, but most of our results so far are obtained from X-ray mutagenesis. We therefore mutagenized (X-ray, 4,000 rad) C-S or wild-type males and crossed them to attached X females [c(1)yw]. By doing this, irradiated males (and hence carrying mutations on all three chromosomes) will transmit their mutated X chromosome to the male offspring (which is different from the usual situation in which the male offspring inherits the X chromosome from the female parent). Therefore, by testing the first generation male progeny, we could test for mutations, irrespective of whether the mutations are recessive or dominant, since, like humans and mammals, male flies have one X chromosome. In a specialized apparatus, more than 22,000 flies, carrying mutagenized chromosomes, have been screened so far in our laboratory (13). Because we had already studied in detail the wild-type responses and developed distribution profiles (histograms) about the recovery from anoxia (13), a threshold (close to the 96th percentile of the wild-type distribution) was used to identify and isolate mutants. To date, we have identified 10 mutants that have loss-of-function mutations and 8 complementation groups (2 mutations have 2 weaker alleles isolated as well), and the mutants have profoundly altered distribution of recovery times after reoxygenation. The marked delay in recovery after anoxia displayed by these mutant flies suggested to us that they were much more sensitive to lack of O₂ (13, 19) (Fig. 1).

The behavioral testing, which showed delayed recovery from anoxia in the mutants, led us to believe that these mutations affected the CNS (13, 19). To further our understanding of these mutations, we directly examined their effect on CNS function. I identified neurons that can be studied electrophysiologically in Drosophila are those of the giant fiber system (13, 19). During reoxygenation, the wild-type flies started to respond by firing evoked potentials (neurons in CNS are stimulated and muscle action potentials are recorded across several synapses) after 2 min into recovery. However, flies with mutations had a much longer latency time to firing of the first evoked potential, with some mutant flies requiring up to 25–30 min for the first evoked response (13, 19) (Fig. 2).

Mapping of the induced mutations was performed with X-chromosomal markers and complementation tests (13). Several markers were used, including y, cv, v, f, car, and su(f). Complementation testing was done on several X-linked recessive mutations obtained. A
Fig. 1. Distribution and cumulative frequency of recovery times for wild-type (C-S; A) and mutant flies (B–F). Mutants are abbreviated by N, P, L, or I on the plots. Y is used to indicate Y chromosome in males. These represent 3 complementation groups as examples. Note the major difference in the distribution of wild-type vs. mutants with almost complete lack of overlap between wild-type and some mutant populations. Female flies heterozygous for mutations P, L, and I have a phenotype similar to that of wild-type flies (D–F). In F, the phenotype of N heterozygotes is also shown. +, Wild-type chromosome.

Fig. 2. Muscle evoked potential (EP) response during and following anoxia. A: Drosophila dorsal longitudinal muscle (DLM) EP response to stimulation in normoxia in central nervous system (CNS). B: response of DLM to anoxia. Note that, in 16 s, anoxia eliminated any EP. N2 was then replaced with room air, and DLM was stimulated via the CNS to determine the minimum time for recovery of EP. C: response of DLM to 30 min of anoxia and during recovery phase. Note that it took ~14 min to start seeing an EP response to muscle stimulation. D: response of DLM to 2.5 h of anoxia and during recovery. After 22 min of reoxygenation, a muscle EP response starts to be seen. All times indicate time into the recovery period.
number of these mutations were mapped, and they are spread in a number of locations on the X chromosome. For example, one of them is on the right side of f or forked and another is between y and cv. One of the mutations has been refined in terms of the mapping, and we have localized it to a rather narrow region, in between y and cv, using a number of deficiencies. Cloning of the first mutation is underway at present, taking advantage of the possibility that X-ray-induced mutations are usually sizeable. Because this region has been totally sequenced by the University of California-Berkeley/European Drosophila Genome Project, we have taken a two-step approach to clone this first mutation. We will first narrow the region of interest in which the mutation lies using Southern analysis and genomic DNA from the mutant and wild-type flies. Subsequently, we will design flanking primers (based on the sequenced DNA) and PCR and clone the open reading frames present in that sequence in both wild-type and mutant Drosophila. In addition to this approach, we are using P-element jumping to clone this mutation.

The current genetic screen has not been saturated. However, we had to screen more than 22,000 mutagenized flies to obtain the mutations obtained. This suggested that a limited number of genes could be mutated in Drosophila to produce similar phenotypes. Because these mutations profoundly disrupted the recovery from anoxia, we believe that this approach can be used effectively to dissect the genetic basis of resistance to anoxia and can help delineate the genes responsible for protection against low O2.

Differential display and reverse genetics. The idea behind this approach is to identify those genes that are differentially expressed during a condition or a stress. In our case, we use very low levels of O2 and assess differentially expressed during a condition or a stress. The approach and the first one, it is clear that the main difference is that this approach starts with a gene (reverse genetics), whereas the first starts with a phenotype (genetics). Hence, in this second approach, the proof that a particular gene is relevant to the phenomenon of interest would have to wait additional investigations beyond the differential display such as transgenic studies as we have done (see below).

The data from our differential display clearly showed that certain genes were upregulated, whereas others were downregulated, during anoxia (23, 24). From the PCR reactions, we found that the expression level of several transcripts was visibly affected by anoxia. We have selected one transcript, which was markedly upregulated, to focus on and study. We termed this transcript fau (fly, anoxia, upregulated) (24). The fau cDNA and its deduced protein sequence have several interesting characteristics. For example, 1) several ATTT motifs were found in its 3'-UTR. These motifs, reportedly, play a role in the stability of transient mRNAs (24) and may therefore play a similar role in hypoxia-induced mRNAs and could regulate their expression (24). In addition, there are two (TA)9–10 stretches in the 3'-UTR of this cDNA. Although their function is not clear, together, these unique sequences in the 3'-UTR with high GC content in the fau cDNA open reading frame may define a functional anatomy important in the transient or stress-induced mRNAs. 2) The deduced protein sequence of fau cDNA also has a high number of phosphorylation sites, which makes it an appropriate substrate for phosphorylation. It is possible then that the fau protein can participate in transient pathways that depend on phosphorylation or dephosphorylation. Because our computerized search did not reveal a significant homology with published sequences, the deduced fau protein is most likely a novel phosphorylated one (Fig. 3).

In addition to the cloning of this gene, we have performed chromosomal in situ hybridization (24). Interestingly, this gene is present on the X chromosome (7C-D). This localization is generally helpful for developing mutants with P elements. Furthermore, we have also localized the mRNA of this newly cloned gene in the CNS of the fly as well as by Northern analysis.

Fig. 3. In situ analysis of gene fau, pulled from the differential display technique. A: localization of fau mRNA (byagenes) using the "squash technique" and 3rd instar larvae salivary gland chromosomes. These chromosomes were probed with a labeled fau cDNA. Signal was detected where the arrow indicates (region 7C-D). B: localization of fau mRNA in the fruit fly CNS using 10-µm sections and a cRNA-labeled probe. A high level is seen in the lamina (arrows) and cortex (arrowheads) (B1); fau sense probe gave little or no signal (B2).
Does the fau protein play a role in anoxia tolerance? Despite the fact that we have no direct evidence that this gene is involved in anoxic survival in Drosophila melanogaster, we believe that fau plays an important role during anoxic stress. The expression of this gene was upregulated when the overall reduction of protein synthesis in general occurs during the lack of O2. Overexpression of fau in transgenic flies prolonged their recovery from anoxia. Furthermore, the putative protein encoded by this gene is probably highly regulated by phosphorylation, suggesting that it is active during O2 deprivation. Finally, the richness of the AUUU motif in 3′-UTR of this mRNA could make it a transient one, with relevance during O2 deprivation.

Known gene products in mammalian cells and tissues. Another approach is to clone from Drosophila the genes that seem to be important in mammals. This would allow the study of gene networks and therefore the study of the genes that modify the expression or the function of particular genes of interest. For example, we know now that the hypoxia-inducible factor 1 (HIF-1) plays an important role in mammals in hypoxia and development. To study the genetic network that HIF-1 is involved in mammals is rather difficult, especially when one considers in vivo experiments. However, this is possible in Drosophila. Therefore, this is first studied in the fly (22, 23), and then the genes discovered in the fly are looked for in mammals. The use of Drosophila would likely be useful, since it would be much easier to investigate modifier genes and their pathways in a well-studied genetic model rather than in mammals. We have also adopted such a method and have done some work in this area, as illustrated in our previous publications (22, 23).

FUTURE DIRECTIONS: IS DROSOPHILA RESEARCH A "DETOUR" OR A "SHORTCUT"?

From our work to date on Drosophila, we conclude that there are specific individual genes in the Drosophila genome that are protective against hypoxic injury and that their mutations lead to cellular injury. Furthermore, these studies have taught us that it is possible to start to dissect the genetic nature of anoxia tolerance in tolerant organisms, a phenomenon that has so far been elusive at best. We also conclude that future experiments in our mutagenesis work should include 1) refining the mutational maps of the loci of interest, 2) building further alleles of the mutations that we have already isolated, 3) characterizing further the phenotype of our loss-of-function mutants and extending the mutagenesis screen to the analysis of the autosomes, 4) expanding the mutagenesis screen to include insertional mutagenesis with P elements, 5) cloning the various mutations and studying the epistatic relationships between them, and, finally, 6) studying the cell biology and physiology of these genes. In addition, we conclude that reverse genetics work should include 1) performing additional differential display reactions to examine all or near all of the fly genome, 2) studying their tissue expression as well as their chromosomal localizations, 3) constructing transgenic flies carrying the mutant gene or an overexpressed gene to study its impact on whole animal function, and 4) finding homologues of the Drosophila genes in mammals.

So far, when the work done on Drosophila and other genetic models, including the C. elegans and the zebrafish, over the past two decades is critically evaluated, it becomes clear that these models have enhanced our understanding of normal biological processes and disease conditions not only in these model systems but also in mammals and in humans. Furthermore, with the completion of the genome project in yeast and C. elegans and soon with the completion of the genome project of the fly and shortly in humans, the pace at which we will uncover molecular mechanisms and how genes function will increase, and the best is yet to come! The current consensus is that the work in Drosophila is a shortcut that will enhance, at a faster pace, our understanding of the molecular basis of biology and disease states in humans.

Address for reprint requests and other correspondence: G. G. Haddad, Dept. of Pediatrics, Section of Respiratory Medicine, 333 Cedar St., FMP 506, New Haven, CT 06520 (E-mail: gabriel.haddad@yale.edu).

REFERENCES


