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Aquaporins-2 and -4 regulate glycogen metabolism and survival during hyposmotic-anoxic stress in Caenorhabditis elegans

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Submitted 14 May 2015; accepted in final form 20 May 2015

LaMacchia JC, Roth MB. Aquaporins-2 and -4 regulate glycogen metabolism and survival during hyposmotic-anoxic stress in Caenorhabditis elegans. Am J Physiol Cell Physiol 309: C92–C96, 2015. First published May 27, 2015; doi:10.1152/ajpcell.00131.2015.—Periods of oxygen deprivation can lead to ion and water imbalances in affected tissues that manifest as swelling (edema). Although oxygen deprivation-induced edema is a major contributor to injury in clinical ischemic diseases such as heart attack and stroke, the pathophysiology of this process is incompletely understood. In the present study we investigate the impact of aquaporin-mediated water transport on survival in a Caenorhabditis elegans model of edema formation during complete oxygen deprivation (anoxia). We find that nematodes lacking aquaporin water channels in tissues that interface with the surrounding environment display decreased edema formation and improved survival rates in anoxia. We also find that these animals have significantly reduced demand for glycogen as an energetic substrate during anoxia. Together, our data suggest that reductions in membrane water permeability may be sufficient to induce a hypometabolic state during oxygen deprivation that reduces injury and extends survival limits.

Aquaporins; glycogen; anoxia; osmotic stress; Caenorhabditis elegans

ANIMALS THAT ARE DEPRIVED of oxygen for sufficient lengths of time eventually experience irreversible injury that can lead to death (3, 32). Fundamentally, this is a consequence of physiological ATP demands exceeding the output capacity of anaerobic ATP production, resulting in energetic failure (7, 13, 15). In humans, injuries due to oxygen deprivation are common and are estimated to contribute to >60% of all deaths (34). Oxygen deprivation due to loss of blood flow (ischemia) in metabolically active tissues such as the heart and brain can rapidly cause massive cell death and clinically apparent tissue damage, resulting in heart attack and stroke (8, 19). A hallmark of injury in this context is the collapse of transmembrane ion gradients due to inadequate ion pumping by ATP-dependent proteins such as the Na-K-ATPase (12, 20, 35). This collapse results in cell swelling and membrane rupture due to intracellular water influx. If left unchecked, this eventually results in increased tissue volume (edema), which can independently worsen ischemia by further restricting blood supply (39).

In contrast to mammals, some invertebrate and lower vertebrate species are capable of surviving complete oxygen deprivation (anoxia) without injury for days or even months (3, 4, 36–38). Physiological and metabolic changes in these species during oxygen deprivation that differ substantially from those in anoxia-sensitive species may be indicative of specific mechanisms for survival (4). Previous studies in anoxia-tolerant animals have consistently shown that survival is closely associated with the ability to downregulate overall demand for ATP to compensate for reduced ATP supply (6, 27). Generally referred to as hypometabolism, this adaptation is associated with states of dormancy in which movement and other energetically expensive processes are significantly reduced (3, 14).

Hypometabolism is also associated with coordinate suppression of ATP-dependent processes at the cellular level, as large, reversible decreases in the rates of ion pumping (6), protein synthesis (10, 21), and neuronal firing (22) have been demonstrated in anoxia-tolerant species during oxygen deprivation.

The nematode Caenorhabditis elegans is capable of surviving anoxia for multiple days with no apparent injury (38). During anoxia, C. elegans enters a hypometabolic state called suspended animation in which observable processes such as locomotion, feeding, and cell division cease (29). Entry into suspended animation is required for nematode anoxia survival (28), yet the specific changes to nematode physiology and metabolism during suspended animation remain incompletely understood. We previously developed a C. elegans model of ischemic edema that combines anoxia with a hyposmotic environment to produce visually apparent tissue swelling and rupture (unpublished data). In the present study we investigate the effects of changes in plasma membrane water permeability on nematode survival in this model. We show that genetic removal of aquaporin water channels from major nematode-environment interfaces reduces tissue swelling and increases survival. We also show that these aquaporin mutants possess a decreased rate of glycogen utilization in anoxia, suggesting that decreased water flux across the plasma membrane is a novel means of producing hypometabolism during oxygen deprivation.

MATERIALS AND METHODS

Nematode strains and culture. Nematodes were grown at 20°C on nematode growth medium (NGM) agar seeded with Escherichia coli strain OP50 bacteria and manipulated using standard protocols (5) unless noted otherwise. A combination of visual anatomic markers, such as gonad and uterine morphology, and time following the larval stage 4 (L4) molt was used to assess the developmental stage of the animals. Synchronous F1 populations were obtained by treatment of...
gravid adults on the day they laid embryos with a 20% alkaline hypochlorite solution and recovery of their embryos. For all experiments, only animals from populations that had not experienced a starvation event in the previous two generations were used. The following strains were acquired from the Caenorhabditis elegans Genetics Center: N2 (wild-type var. Bristol), RB1715 aqp-2(ok2159), RB1967 aqp-4(ok2587), RB2115 aqp-8(ok2800), RB1914 aqp-9(ok2487), and RB2570 aqp-11(ok3578). The RB1967 aqp-4(ok2587) strain was backcrossed three times into the wild-type N2 strain.

Oxygen deprivation experiments. All oxygen deprivation experiments were performed at an incubation temperature of 20°C unless indicated otherwise. Atmospheres were generated using mass flow controllers and airtight chambers as previously described (26). Immediately before anoxia exposure, L4 nematodes were transferred via wire pick to NGM with live OP50 bacteria or to medium consisting of 2% agar + 5 mM potassium phosphate buffer with or without NaCl as follows: for hyposmotic medium, no NaCl was added; for isosmotic medium, 100 mM NaCl was added; and for hyperosmotic medium, 300 mM NaCl was added (medium was seeded with live OP50 bacteria that had been washed with double-distilled water). After anoxia, nematodes were returned to normoxia and allowed to recover for ≥6 h before being scored for survival. Animals exhibiting obvious lethal trauma (“popped” phenotype) were scored as dead. Animals in which trauma was not apparent were scored as dead if they did not respond to gentle touch with a platinum wire. For glycogen quantification experiments, animals were returned to normoxia and immediately stained with iodine vapor.

Glycogen staining of nematodes with iodine. Whole body glycogen content was visualized and quantitatively assessed using a previously described iodine vapor staining method (11). Animals were first placed on an agar pad and allowed to roam to remove excess OP50 bacteria associated with the animals. Then exposure to iodine vapor was accomplished by inversion of the agar pad for 90 s over an open bottle containing 100 g of iodine chips (Sigma); immediately thereafter, the animals were visualized and photographed using a Nikon SMZ1500 stereomicroscope. For quantification of glycogen content, mean stain intensity and animal area were subsequently calculated from micrographs using the ImageJ program. To account for small variations in stain concentration and animal handling, experimental animals were stained side-by-side with control animals and imaged simultaneously. Known concentrations of glycogen spotted onto NGM plates were used to determine the linear range of the iodine vapor stain through generation of a standard curve: glycogen (mg) = [(area × intensity) + 26]/71. This standard curve was employed in the definition of glycogen content: glycogen content (animal)_{exp} = [(area_{exp} × intensity_{exp} + 26)/(area_{control} × intensity_{control} + 26)].

RNA interference experiments. RNA interference (RNAi) was achieved as previously described (18) by feeding animals E. coli HT115 (DE3) strains expressing double-stranded RNA corresponding to the gene of interest. These strains were generated by the laboratory of J. Ahringer and were obtained as a library from MRC Geneservice. For all experiments, gravid adult animals were placed on agar RNAi feeding plates and allowed to lay embryos for 24 h. Then the adults were removed and the embryos were allowed to hatch and develop to the L4 stage at 20°C. RNAi was confirmed qualitatively by observation of previously described phenotypes of the knockdown or quantitatively by RT-PCR.

Live animal microscopy. L4 C. elegans were transferred to a thin (<1-mm) agarose pad and placed inverted in a glass-bottom atmospheric chamber mounted on a Nikon TE2000 inverted microscope. Anoxic (100% nitrogen) atmospheres were generated using a mass flow controller. Images of whole animals were collected at the designated time points using a Zeiss Axiocam MRc digital camera and analyzed using the ImageJ program. For body volume measurements, animals were treated as cylinders, and volume was calculated as \(V = \pi r^2 l\), where \(r\) is radius and \(l\) is length (24).

Quantitative real-time PCR. Synchronized L4 C. elegans were grown on isosmotic or hyposmotic agar medium for 24 h, washed three times with water, and frozen in liquid nitrogen. TRIzol reagent (Life Technologies; 1 ml/g worms) was added, and RNA was extracted according to the manufacturer’s instructions. Total RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific), and cDNA was synthesized using a SuperScript III kit (Life Technologies) according to the manufacturer’s instructions. Quantitative PCR was performed on an ABI 7900HT real-time PCR system using iTaq SYBR Green (Bio-Rad Laboratories) according to the manufacturer’s instructions. Standard curves were generated using cDNA, and expression levels were determined using the Pfaffl analysis method (30). Levels of aqp-4 cDNA were normalized to the geometric mean of the cDNA levels of the housekeeping genes act-1 and gpd-2. The size of all PCR products was verified to be correct by gel electrophoresis.

Adaptation to altered culture medium osmolarities. Synchronous populations of nematodes were raised from embryo to day 1 of adulthood at 20°C on hyposmotic (10 mosM) or isosmotic (200 mosM) medium that had been seeded with live OP50 E. coli. Animals were then transferred by a platinum wire to fresh medium and assayed for 24-h survival in a 100% nitrogen atmosphere as described above.

Statistical analysis. All statistical analyses were carried out in Microsoft Excel or Prism 6 (GraphPad Software).

RESULTS

We hypothesized that reductions in membrane water permeability would blunt nematode body swelling and increase survival in the nematode during hypoxic anoxia. To test this, we obtained C. elegans genetic loss-of-function mutants for four nematode aquaporin water channel homologs: aqp-2, aqp-4, aqp-8, and aqp-11. Aquaporins represent a functionally conserved class of membrane-bound proteins that facilitate rapid, bidirectional water flux driven by the osmotic gradient (1). Experiments carried out in the Xenopus oocyte model have demonstrated that genetic removal of aquaporin channels decreases membrane water permeability by as much as 10-fold (17, 31). We found significant increases in survival rates of the aqp-2(ok2159) and aqp-4(ok2587), but not the aqp-8(ok2800) and aqp-11(ok3578), mutants relative to wild-type animals following 24 h of hypoxic anoxia (Figure 1A). To rule out the possibility that the enhanced survival phenotypes of the ok2159 and RB1967 mutants were due to linked, epistatic mutations elsewhere in the genome, we treated wild-type (N2) animals with RNAi targeting aqp-2 or aqp-4. We found that both of these knockdowns resulted in significantly increased survival rates following hypoxic anoxia, confirming that expression of aqp-2 and aqp-4 impedes survival in this environment (Fig. 1B).

We chose to further investigate the mechanisms responsible for the high level of resistance to hypoxic anoxia in the aqp-4(RB1967) mutant. To determine if RB1967 animals are generally anoxia-resistant, we exposed them to long-term (48 h) anoxia while on isosmotic media, thereby uncoupling anoxic stress from osmotic stress. We found that RB1967 animals possess levels of resistance in this assay that are similar to those seen in hypoxic anoxia (Fig. 1C), suggesting that loss of a functional aqp-4 gene confers resistance to anoxia that is not solely dependent on the external osmotic milieu. To determine if nematode aqp-4 expression is regulated by hypoxic environments, we raised wild-type animals from embryo to adulthood on hypoxic media and then measured aqp-4 mRNA levels by quantitative PCR. We found that aqp-4
Fig. 1. Removal of aquaporin water channels promotes nematode survival in hyposmotic anoxia, as well as long-term hypoxia. A: percent survival of aquaporin (aqp) loss-of-function mutants following 24 h of exposure to hyposmotic anoxia. B: percent survival of control(RNAi), aqp-2(RNAi), and aqp-4(RNAi) larval stage 4 (L4) animals following 24 h of exposure to hyposmotic anoxia. C: percent survival of wild-type (N2) and aqp-4(ok2587) animals following 48 h of anoxia on conventional nematode growth medium (long-term anoxia). Values are means ± SE of ≥5 trials, each with ≥20 animals. *P < 0.05, **P < 0.01 (by 2-tailed t-test).

mRNA expression is reduced in this environment to about one-third of that in controls (Fig. 2), suggesting that a component of the nematode adaptive response to cell swelling during hyposmotic stress involves reducing membrane water permeability. Consistent with this, we found that aqp-4(RB1967) animals are deficient in their ability to undergo hyposmotic preconditioning prior to hyposmotic anoxia (Fig. 3).

It has been demonstrated previously that nematode aqp-4 is expressed at the apical membrane of the intestine, a major interface between the nematode and its external environment (17). Changes in aqp-4 expression could, therefore, significantly alter the rate at which water moves into or out of the animal. To determine the effect of aqp-4 loss on cell/tissue swelling, we measured body volumes of RB1967 animals at several time points during hyposmotic anoxia. We found significantly smaller increases in body volume in RB1967 than wild-type animals during hyposmotic anoxia (Fig. 4A). During hyposmotic stress, cells rely on ATP-consuming (active) mechanisms of ion transport to limit swelling. We considered a model for aqp-4-mediated anoxia survival in which loss of functional aqp-4 results in decreased rates of active transport due to reduced membrane water permeability. While previous studies have associated suppression of active transport with reduced energetic demand (hypometabolism) during anoxia (6, 9), it is unclear if this suppression is sufficient to produce hypometabolism. We therefore investigated the effects of aqp-4 loss on nematode energy consumption during hyposmotic anoxia. To accomplish this, we quantified animal glyogen content at different time points during hyposmotic anoxia. Glycogen is the primary energy source utilized during anoxia and is not regenerated in C. elegans until normoxia is restored (11). Therefore, the rate of decrease in glycogen content during anoxia is proportional to the rate of energy consumption. We found that this rate in RB1967 animals during hyposmotic anoxia was less than half that in wild-type (N2) animals (Fig. 4C). This indicates that the presence of a functional aqp-4 channel in wild-type animals increases energetic demand during hyposmotic anoxia. Interestingly, decreases in glycogen

Fig. 2. Expression of aqp-4 is downregulated in hyposmotic environments. Levels of aqp-4 mRNA were quantitated by real-time RT-PCR in wild-type (N2) L4 animals following 24 h on isosmotic (Iso, 200 mosM) or hyposmotic (Hypo, 10 mosM) medium in normoxia. cDNA of aqp-4 is shown as average increase relative to geometric mean of cDNA levels of the housekeeping genes act-1 and spd-2. Values are means ± SE. *P < 0.05 (by 2-tailed t-test).

Fig. 3. Hyposmotic preconditioning to hyposmotic anoxia requires functional aquaporin 4. N2 and aqp-4(ok2587) L4 animals were placed on isosmotic or hyposmotic medium for 24 h and then exposed to hyposmotic anoxia for 24 h. Percent survival increase was calculated as percent difference in survival following hyposmotic anoxia of animals preconditioned on hyposmotic medium relative to those preconditioned on isosmotic medium. Values are means ± SE of ≥4 independent trials, each with ≥20 animals. *P < 0.05 (by 2-tailed t-test).
content during hyposmotic anoxia coincided temporally with decreases in survival for both N2 and RB1967 animals, suggesting that the probability of surviving anoxia is related to body glycogen content at that point in time.

**DISCUSSION**

In this study we present results supporting the hypothesis that membrane water permeability can influence animal survival limits during oxygen deprivation. We demonstrate in the nematode *C. elegans* that loss of the aquaporin water channel gene homologs *aqp-2* and *aqp-4* results in an up to 10-fold increase in nematode survival in a model of anoxic edema (hyposmotic anoxia). Both *aqp-2* and *aqp-4* are known to be expressed at major nematode-environment interfaces (the apical membrane of the intestine and the excretory cell, respectively), suggesting that the protective effects of aquaporin loss in this context are associated with the blockade of water influx into the animal from its surroundings. We go on to show that hyposmotic preconditioning of wild-type (N2) nematodes leads to significant *aqp-4* downregulation and that a functional *aqp-4* gene is required for efficient preconditioning, suggesting that the wild-type adaptive response to this environment involves active reduction of membrane water permeability. Because we previously observed a high correlation of wild-type lethality in hyposmotic anoxia with tissue swelling and rupture, we measured nematode body volume over the course of hyposmotic anoxia. While wild-type (N2) animals display a ~50% increase in body volume after 16 h in this environment, this increase is only ~25% in *aqp-4* mutants. This suggests that aquaporin loss leads to decreased membrane water permeability, which in turn results in a decreased rate of water movement into tissues during hyposmotic anoxia.

Although our work is the first association of aquaporins with survival of oxygen deprivation in *C. elegans*, prior studies in mammalian systems have implicated these water channels in the pathophysiology of ischemic disease. Knockout of glially expressed *AQP-4* in mice, for instance, results in reduced cerebral edema following stroke, as well as significant improvements in animal survival and neurological score (2, 23). Similarly, myocardium from *AQP-4* knockout mice displays decreased infarct size relative to wild-type animals in models of heart attack and global ischemia-reperfusion injury (33). The parallels between these prior studies and the results we present here strongly support the argument that hyposmotic anoxia is a physiologically relevant nematode model for anoxia-dependent edema. Subsequent studies that combine hyposmotic anoxia with genome-wide screening techniques are likely to provide insight into the genetic regulation of anoxia-dependent edema, a pathophysiological process that is currently poorly understood.

Two decades ago, the channel arrest hypothesis was proposed to explain how anoxia-tolerant animals are able to maintain homeostasis and cell volume while simultaneously suppressing mechanisms of active ion transport during oxygen deprivation. Because ion transport accounts for a large percentage of total cellular ATP demand, even modest reductions in demand for this process are likely to lead to large decreases in overall energetic demand. Measurements in anoxia-tolerant turtle hepatocytes demonstrated a 90% reduction in Na-K-ATPase activity during anoxia, with no concurrent changes in plasma membrane ion potential. According to channel arrest, these data were indicative of decreased transmembrane ion permeability in compensation for decreased transmembrane ion transport. Subsequent studies of channel arrest intuitively focused on identifying specific ion channels as effectors of membrane permeability changes and did not consider whether decreased transmembrane water flux could also result in measurable hypometabolism. We provide data clearly linking aquaporin downregulation with reduced energetic demand. Glycogen is the primary energetic substrate utilized by the nematode during anoxia, and we show that *aqp-4* mutant animals have a significantly reduced rate of glycogen utilization in hyposmotic anoxia. While cell swelling can be triggered by changes in intracellular and/or extracellular osmolarities, it is ultimately a direct result of water influx (16, 25). Therefore, we propose a model for aquaporin-mediated survival in which decreased water flux due to *aqp* loss places less demand on mechanisms of cell volume homeostasis, such as ion transport. This in turn results in reduced energetic demand during anoxia, as measured by glycogen consumption rate. Further studies are necessary to determine whether changes in aquaporin expression influence the activity of ca-

**Fig. 4.** Rates of body swelling and glycogen consumption in hyposmotic anoxia are reduced in *aqp-4(ok2587)* mutant animals and are correlated temporally with animal survival. A: body swelling during hyposmotic anoxia is blunted in *aqp-4(ok2587)* mutant animals. Percent increase in whole animal body volume is shown for N2 (solid line) and *aqp-4(ok2587)* (dashed line) L4 animals. Values (means ± SE of ≥5 animals) were normalized to volume at time 0. *P < 0.05* (by 2-tailed *t*-test). B: representative micrographs of N2 L4 animals stained for glycogen following anoxia or normoxia. C: quantification of glycogen staining [arbitrary units (AU), solid lines] in N2 (●) and *aqp-4(ok2587)* (●) animals at 0–24 h of hyposmotic anoxia. Dashed lines represent percent survival for respective strains at 0–24 h. Values (means ± SE of ≥5 animals) were normalized to normoxic control. *P < 0.05*, *aqp-4(ok2587)* vs. N2 (by 2-tailed *t*-test).
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nonical ion transporters such as the Na-K-ATPase or whether other, less-studied transporters are involved.

ACKNOWLEDGMENTS

We thank all members of the Roth Lab for valuable insight and critique. We thank the Caenorhabditis elegans Genetics Stock Center for strains.

GRANTS

This work was funded by Defense Advanced Research Projects Agency Project 214759 and the Achievement Rewards for College Scientists Foundation (Seattle Chapter).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.C.L. and M.B.R. developed the concept and designed the research; J.C.L. performed the experiments; J.C.L. analyzed the data; J.C.L. interpreted the results of the experiments; J.C.L. prepared the figures; J.C.L. drafted the manuscript; J.C.L. and M.B.R. edited and revised the manuscript; J.C.L. and M.B.R. approved the final version of the manuscript.

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