Elevated CO₂ levels affect development, motility, and fertility and extend life span in Caenorhabditis elegans

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Hypercapnia (high CO₂ levels) occurs in a number of lung diseases and it is associated with worse outcomes in patients with chronic obstructive lung disease (COPD). However, it is largely unknown how hypercapnia is sensed and responds in nonneural cells. Here, we used C. elegans to study the response to nonanesthetic CO₂ levels and show that levels exceeding 9% induce aberrant motility that is accompanied by age-dependent deterioration of body muscle organization, slowed development, reduced fertility and increased life span. These effects occur independently of the IGF-R, dietary restriction, egg laying or mitochondrial-induced aging pathways. Transcriptional profiling analysis shows specific and dynamic changes in gene expression after 1, 6, or 72 h of exposure to 19% CO₂ including increased transcription of several 7-transmembrane domain and innate immunity genes and a reduction in transcription of many of the MSP genes. Together, these results suggest specific physiological and molecular responses to hypercapnia, which appear to be independent of early heat shock and HIF mediated pathways.

The genetically tractable model organism, C. elegans, is a very powerful system in which to investigate cellular sensing and response to CO₂. Despite being an invertebrate, C. elegans has differentiated tissues including hypodermis, epidermis, muscle, nervous system and others. Also demonstrated is the importance of evolutionary conserved genes in studying diseases and specific biological processes including hypoxia, longevity, and others.
Hypercapnia Extends Life. Growing animals in air containing 19% CO₂ caused an extension in mean life span from 12.5 to 18.0 days at 25 °C and from 19.7 to 24.8 days at 20 °C (Fig. 3A and Table S2). We next tested which of the known aging pathways is involved in response to hypercapnia. The evolutionarily conserved insulin-like IGF-1 signaling pathway is involved in regulating life span of C. elegans (17). In particular, mutations in the daf-2 insulin receptor gene lead to an extension of average life span, and this life span extension phenotype requires the function of the FOXO transcription factor DAF-16 (17). Growing animals with a missense mutation in daf-2, daf-2(e1370), in air containing 19% CO₂ at 20 °C increased their mean life span from 32.6 to 47.5 days (Fig. 3B) while increasing mean life span in the short-lived deletion mutant daf-16(mu86) from 26.0 to 30.0 days (Fig. 3C). Because mean life span was still increased in these mutant strains, the effect of CO₂ is probably independent of the IGF-1 pathway. The eat-2(ad1116) animals have a slower pumping rate and consequently they eat less, thus mimicking the diet restriction longevity pathway (18). Growing eat-2(ad1116) animals in air containing 19% CO₂ at 20 °C increased their mean life span from 23.3 to 29.3 days (Fig. 3D). Likewise, growing clk-1(e2519) animals, which are defective in the ubiquinone

or 13%, respectively (Fig. 1A). Once the embryos were laid, the embryonic lethality was similar to that of nontreated embryos. DIC microscopy and DAPI staining of gonads showed normal size and localization and the presence of sperm cells (Fig. 1B). The only difference was that adults grown in normal air had more embryos (data not shown) suggesting that growing C. elegans in CO₂ atmosphere of 9% and above leads to a significant reduction (P < 0.0005) in brood size without causing apparent defects in gonad morphology. We used CO₂ concentration of 19% for subsequent experiments because of the robust response to this concentration.

We next tested at which developmental stage the egg laying and development were most affected by hypercapnia in animals grown at 20 °C in 19% CO₂, by placing them in the CO₂ chamber or by removing them from the CO₂ chamber (Fig. S1) at different times. Control animals were kept at the same condition in air (Fig. S1C, embryos) or in 19% CO₂ (Fig. S1B, embryos). Subjecting animals to continuous hypercapnia starting at late L4 stage reduced egg laying to 24% compared with egg laying in normal air (n = 20) (Fig. S1B, L4). Interestingly, inserting the animals at the L2 stage reduced egg laying only to 37% compared with egg laying in normal air (Fig. S1B, L2). Transferring L1 stage animals, which were exposed for 18 h in 19% CO₂, to normal air caused only a slight reduction in egg laying (n = 20) (Fig. S1C, L1). Transferring L2-L3 stage animals, which were exposed for 50 h in 19% CO₂, to normal air caused 16% reduction in egg laying (Fig. S1C, L2-L3) suggesting that although exposure to hypercapnia affects development at all embryonic and larval stages (Table S1 and data not shown), egg laying was most affected when CO₂ exposure occurred between the L4 and adult stages.

To rule out effects of different pH levels resulting from hypercapnia induced pH reduction, we grew the worms on NGM plates at pH = 5.0, 6.0 or 7.0. We found that within this pH range, there was no effect on brood size (Fig. 1C) or rate of development. We also compared the oxygen exchange rate between animals grown in 19% CO₂ in air and animals grown in normal air by using an oxygen microelectrode to monitor oxygen levels next to the body of the worm and found that the oxygen level curve was similar between air and 19% CO₂, suggesting that oxygen consumption was not significantly affected (average of 6.70 10⁻³ SD 3.16 10⁻³ vs. 2.16 10⁻³ SD 4.80 10⁻³ mMole per organism × min, respectively). In contrast, ATP levels in animals grown in 19% CO₂ were 60% of those in animals grown in air (Fig. 1D). The long living daf-2 strain, which is known to have higher ATP levels (16), served as a control (Fig. 1D).

Hypercapnia Reduces Motility and Causes Abnormal Organization of Muscle Fibers. Animals grown in air containing 15% or 19% CO₂ had slower locomotion. To determine the long-term effects of high CO₂ on movement, we compared the head movement between animals grown in air containing 19% CO₂ and animals grown in normal air. Movement was quantified both on NGM plates and in water, because it tests different subsets of muscles. After 4 days of exposure to 19% CO₂ at 20 °C, after which the animals were transferred to the room atmosphere, the head movement was reduced by 38% in water and by 43% on NGM plates as compared with controls (Fig. 2A), suggesting that chronic exposure to high CO₂ levels causes significant permanent motility defects (P < 0.0005). We next tested the muscle morphology in these animals using thin-section electron microscopy (EM). The EM analysis revealed that the overall body muscle morphology was already affected after 4 days exposure to CO₂. Muscle morphology further deteriorated after 8 or 12 days of growth in air containing 19% CO₂ (Fig. 2B, compare day 12 to day 4).

Fig. 1. Exposure of wild-type C. elegans (N2) to air containing 9%, 15% or 19% CO₂ caused reduced fertility. (A) Wild-type C. elegans (N2) were maintained at 20 °C (Left) or 25 °C (Right) on NGM plates and the number of laid eggs was scored daily. (B) DIC microscopy (Upper) and DAPI staining (Lower) of gonads of animals grown in air (Left) or in air containing 19% CO₂ from the time the eggs were laid until the adult stage (Right). In both cases, the morphology of gonads was normal. (C) Wild-type C. elegans (N2) were maintained on NGM plates at pH = 5.0, 6.0 or 7.0 at 20 °C (19 plates for each pH). The change in pH did not affect the progeny size. (D) ATP was measured using the MBL ApoSENSOR kit (n = 3). *, P < 0.05; ***, P < 0.0005.
Continuous exposure to air containing 19% CO₂ extends average life span. Survival plots of wild-type (N2) animals, or animals mutated in daf-2, daf-16, eat-2, clk-1 or glp-1 genes. Animals were grown at 20 °C in normal air (continuous line in A-E) or in air containing 19% CO₂ (broken lines in A-E), or at 25 °C in normal air (continuous line in F and continuous line with squares in A) or in air containing 19% CO₂ (broken line in F or broken line with squares in A). The average life span is shown in Table S2. There was a significant extension in life span (P < 0.0001) in all animals grown in air containing 19% CO₂.

RNA was isolated and hybridized to GeneArray chips (Affymetrix). The results of transcription profiling analyses appear in Table S3, Table S4, and Table S5. The chip results were verified by RT-qPCR analyses performed on 2 independent RNA isolations. In almost all tested cases (27/33) RT-qPCR and GeneArray results were consistent with each other (Fig. S2). Because it frequently happens in validation experiments, for the few unvalidated genes their Affymetrix probe sets do not correspond to the same sequences detected by RT-qPCR and it is therefore possible that both methods actually represent different transcripts of the same genes due to alternative splicing for example.

After 1 h of exposure to hypercapnia 429 genes were up-regulated and 59 genes were down-regulated at least 2-fold. After 6 h and 72 h of growth in 19% CO₂, there were 374/771 regulated and 59 genes were down-regulated at least 2-fold.

Among the genes that were up-regulated after 1 h there were 8 genes of the 7-transmembrane domain genes, which could be receptors for chemical messengers (21), 37 genes of the nuclear hormone receptor family, which are involved in transcriptional regulation, 3 genes of the E3 ubiquitin ligase family, which are involved in protein degradation and 8 genes that are involved directly or indirectly in innate immune response (Fig. 4 and Table S6).

Although most up-regulated genes regained their normal levels of expression after 6 or 72 h of growth in 19% CO₂, few genes remained up-regulated in all 3 time points including hsp-12.3, far-3, lea-1 and others (Table S6). Interestingly, although hsp-12.3 was up-regulated, most of the heat shock genes were down-regulated or remained unchanged. Among the down-regulated genes after 6 h of exposure to 19% CO₂ were the major...
Discussion

Hypercapnia is independent of these genes.

Wild-type animals. These data suggest that the response to hypercapnia was significantly reduced for both strains as compared with controls (Fig. 5A). To test genes that are potentially involved in the transport of CO\textsubscript{2}, we used strains that are each homozygous for a deletion in the aquaporin or rhesus gene. We asked whether under hypercapnia conditions the deletion of the gene further delays development and reduces the number of laid eggs or whether it rescues the hypercapnia-induced phenotypes.

A recent report suggests that the aquaporin 1 gene is involved in transporting CO\textsubscript{2} across membranes (22). AQP-2 is one of the major aquaporins involved in transporting CO\textsubscript{2} in mammalian species (23). AQP-2 in the context of a whole multicellular organism. Our results show that growing C. elegans in CO\textsubscript{2} levels of 9%, which is homozygous for a deletion in the aqp-2 gene failed to lay eggs and developed slower when placed in 19\% CO\textsubscript{2} at 20 °C (Fig. 5A). C. elegans contains 2 rhesus genes: rhr-1 and rhr-2. When grown in 15\% CO\textsubscript{2} (Fig. 5) or at 19\% (data not shown) at 20 °C the rhr-1(ok432) or rhr-2(ok403), the number of eggs laid was significantly reduced for both strains as compared with wild-type animals. These data suggest that the response to hypercapnia is independent of these genes.

Response to High CO\textsubscript{2} Levels Is Independent of Aquaporin and Rhesus Genes. To test genes that are potentially involved in the transport of CO\textsubscript{2}, we used strains that are each homozygous for a deletion in the aquaporin or rhesus gene. We asked whether under hypercapnia conditions the deletion of the gene further delays development and reduces the number of laid eggs or whether it rescues the hypercapnia-induced phenotypes.

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on egg laying occur only at specific time points in development, or both. Supporting the latter possibility is the strong effect of growing L4 animals in air containing 19% CO2.

Chronic exposure to hypercapnia also caused motility defects that were accompanied by deterioration in muscle morphology. Hypercapnia-induced changes in muscle morphology became more severe with aging. Interestingly, muscle weakness is also observed in patients with COPD, suggesting that muscle deterioration in humans could be related to hypercapnia by yet unknown mechanisms.

Another global effect of hypercapnia is the significant increase in longevity of *C. elegans* grown in 19% CO2, which probably occurs independently of the common longevity pathways of the insulin receptor signaling pathway (daf-2 and daf-16), the sterility pathway (glp-1), the diet restriction pathway (eat-2) or the ubiquinone biosynthesis pathway (clk-1). The hypercapnia effect might not be regulated by the diet deprivation-induced life span pathway (26, 27) because it depends on eat-2 and the life span of eat-2 animals was significantly increased in 19% CO2. Finding the aging pathway affected by hypercapnia is a major goal for future studies.

**Exposure of C. elegans to High CO2 Levels Affects Gene Expression.**

Growing *C. elegans* in air containing 19% CO2 caused dynamic changes in transcription profiles. We have identified a large number of genes that already after 1 h of exposure had a change of 2-fold or greater in their level of transcription. Although some genes remained up-regulated or down-regulated, after 6 h most genes went back to their baseline expression levels. It was also interesting to note that after 72 h of exposure to 19% CO2, >6% of the total genes were either up-regulated or down-regulated. The chip microarray results probably represent a true difference in gene activity, because the real time quantitative PCR, which was performed on independent preparations of RNA, gave results that were consistent with the microarray results.

Many of the known genes that were up-regulated after 1 h of exposure to air containing 19% CO2 are probably involved in coordinating the initial response of the animal to hypercapnia. The 7 transmembrane domain genes, which include the G protein coupling chemoreceptor *sre-44*, could aid sensing changes in CO2 levels. The change in many nuclear hormone receptor genes is probably involved in the activation or repression of many genes required to adjust the cellular metabolism.

The change in expression levels of many genes after exposure to air containing 19% CO2 suggests that hypercapnia is a significant stress to the animal. Surprisingly, although most known heat shock genes were either down-regulated or unchanged, only the *hsp-12.3* gene remained up-regulated throughout the entire period of exposure to 19% CO2 and only the *sip-1* gene was up-regulated after exposure to 19% CO2 for 1 or 6 h. In addition, the change in gene expression was completely different from that of exposure to hypoxia (28), suggesting a very different response to elevated levels of CO2. Determining the roles of these genes in sensing and/or response to hypercapnia, alone or in combination is a goal for future studies. A surprise finding was the downregulation of many of the *MSP* genes after 6 h of exposure to 19% CO2. We cannot, however, exclude the possibility of a slight change in the developmental rate, which may account for this difference.

Mutant in the rhesus and aquaporin genes show a stronger effect of CO2 than does wild type (N2), suggesting that these genes may play roles in resisting to hypercapnia. This is in line with previous reports in *C. elegans* or in other organisms (7, 29) and suggests that their role in allowing CO2 to enter cells is evolutionarily conserved.

**Materials and Methods**

**Maintenance of Strains and Growth in CO2 Chamber.** *C. elegans* strains were handled as described in ref. 15. N2, *aqp-2(ok2159), rhr-1(ok432), rhr-2(ok403), daf-2(e1370), daf-16(mu86), glp-1(oi178), eat-2(ok1116), clk-1(e2519)* were obtained from the *C. elegans* Genetic Center. These mutants are out-crossed to the N2 animals. DYNAMENT CO2 controller with a mini infrared sensor (0–20% CO2) was connected to a sealed Perspex incubator. CO2 was flowed to the incubator via the controller until reaching the desired level and was balanced using air.

**Progeny Number, Life Span, and Locomotion.** Synchronized embryos were transferred to air containing 19% CO2 and allowed to reach adulthood. Each experiment contained 30 plates and each plate contained 1 animal. Aging experiments were performed as described in ref. 30, gravid worms were allowed to lay eggs for 6–24 h at 20 °C in normal air conditions and embryos were transferred to 19% CO2 (day 1). The aging experiments with the *glp-1* T5 mutant were at 25 °C; the *glp-1* worms were allowed to lay eggs for 6 h at 20 °C and then were transferred to either air or 19% CO2 at 25 °C.

Animals were considered dead when they did not respond to prodding with a platinum wire. *P* values were calculated using the log-rank method. For the motility measurements, individuals L4 animals were transferred to NGM plates seeded with OP50 or to a drop of M9 and filmed with a webcam. Body bends were counted every time the part of the worm just behind the pharynx reached a maximum bend in the opposite direction from the bend last counted.

**ATP Measurements.** Young adult worms from five 90-mm NGM plates were collected and aliquots of 100 μL were prepared and stored at −70 °C until used. For in vitro ATP measurement 100 μL of worms in 1 mL of DDW were transferred to boiling water for 15′ and then centrifuged for 5′ at 14000 rpm. Serial dilutions were used to measure ATP content with luciferase-based kit according to manufacturer instructions (MBL; ApoSENSOR kit). Results were normalized to protein content.

**Fig. 5.** *Aqp-2, rhr-1* and *rhr-2* affect the hypercapnia-induced egg laying. (A) Animals homozygous for a deletion in a specific gene were maintained in air containing 15% or 19% CO2 and the number of laid eggs was scored and divided by the number of eggs laid by animals of the same genetic background grown in normal air. *, *P* < 0.05; ***, *P* < 0.0005. (B) The effect of mutations in *aqp-2, rhr-1*, and *rhr-2* on the rate of development in normal air and in air containing 19% CO2.
Transmission electron microscopy (TEM) analysis of *C. elegans* was done as described in ref. 31. DIC and immunofluorescence images were taken with an Axiocam CCD camera mounted on a Zeiss Axioplan II microscope equipped for fluorescence and DIC, or with an MRC-1024 BioRad confocal scanhead coupled to a Zeiss Axiovert 135M inverted microscope equipped with a 63× NA = 1.3 oil-immersion objective.

**RNA Isolation, Microarray Analysis, and Quantitative RT-PCR.** Wild-type (N2) *C. elegans* were grown for 1, 6 or 72 h in 19% CO2 at 20 °C. All microarray experiments were performed in a pairwise manner. For the 1 and 6 h of exposure, animals were grown at normal conditions until L4 and only then were transferred to 19% CO2 for either 1 or 6 h, whereas control animals kept growing for either 1 or 6 h in air. For the 72 h of exposure, animals were grown from embryos until they reached the young adult stage, which takes between 72 and 80 h under 19% CO2 and the developmental stage was determined using DIC microscopy. The control animals for that experiment were collected at the same developmental stage. RNA preparations were isolated using TRIzol and were used to synthesis first strand cDNA according to manufacturer instructions, or to perform RT-qPCR. The RNA preparations were hybridized to *C. elegans* affymetrix gene array according to manufacturer’s instructions. Real-time RT-PCR was performed on 2 independent isolations of RNA from each time point, which were different from the RNA isolation used for the microarray analysis. A Corbett ROTOR-GENE 6000 instrument using an AGene absolute qPCR SYBR green kit were used according to the manufacturer’s instructions. act-1 was used as the gene of reference.

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