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SUPEROXIDE DISMUTASE IS REGULATED BY LAMMER KINASE IN *DROSOPHILA* AND HUMAN CELLS

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Abstract

LAMMER kinases (also know as Cdc-2-like or CLKs) are a family of dual specificity serine/threonine protein kinases that are found in all sequenced eukaryotic genomes. In the fission yeast, *S. pombe*, the LAMMER kinase gene, *Lkh1*, positively regulates the expression of the antioxidant defense genes, superoxide dismutase1 (*sod1+*, CuZn-SOD) and catalase (*ctt1+*, *CAT*). We have shown that mutations in the *Drosophila* LAMMER kinase gene, *Darkener of apricot* (*Doa*), protect against the decrease in life span caused by the reactive oxygen species (ROS) generator paraquat, and at the same time show an increase in cytoplasmic (CuZn-Sod or SOD1) and mitochondrial superoxide dismutase (Mn-Sod or SOD2) protein levels and activity. The siRNA mediated knock down of the human LAMMER kinase gene, CLK-1, in HeLa and MCF-7 human cell lines leads to an increase in both SOD1 activity and mRNA transcript levels. These data suggest that SOD1 is negatively regulated by LAMMER kinases in *Drosophila* and human cell lines and that this regulation may be conserved during evolution.

Keywords

LAMMER kinase; Superoxide dismutase; CDC-Like-kinase; CLK; TG003; Drosophila

INTRODUCTION

The antioxidant defense enzyme, superoxide dismutase (SOD), plays a critical role in the prevention of damage by reactive oxygen species (ROS) and the in vitro activation of SOD can increase differentiation, decrease cell proliferation [1] and in *Drosophila melanogaster* loss of SOD1 causes early mortality [2]. Two SOD genes are found in all organisms, including bacteria [3]. The copper/zinc-containing SOD (SOD1) encoded by the SOD1 gene, is normally

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found in the cytoplasm, nucleus and lysosomes [3], and mutations in the human SOD1 gene have been linked to familial and sporadic forms of amyotrophic lateral sclerosis [4]. Human SOD1 is upregulated in response to phorbol ester treatment, mediated by SP-1, EGR-1 and the EGR-related protein, WT-1 [5]. In rat liver, the *cis*-acting C/EBP element appears to play a major role in regulating the SOD1 gene [6]. The promoter of SOD1 also has a metal response element (MRE) that upregulates the gene in response to heavy metals [7]. The mitochondrial, or iron/manganese-containing form of SOD, (SOD2), which is encoded by the nuclear gene SOD2, is highly inducible by cytokines and oxidants [3,8]. Protein kinase C stimulating agents also induce expression of the human SOD2 gene [9], and increased levels of the transcription factor AP2 decrease its expression[10]. Single amino acid polymorphisms of SOD2 are associated with diabetic nephrology [11], and increased levels of SOD2 have been reported in mesothelioma [12] and colorectal carcinomas [13]. Overexpression of SOD2 in MCF-7 cells abolished tumor necrosis factor-mediated activation of NFκB and suppressed apoptosis [14]. In many eukaryotes there is also an extracellular SOD (EC-SOD, *SOD3)* which, in mammals, is secreted by a limited number of cell types and has been detected in blood plasma, lymph and cerebrospinal fluid [3,15].

In *S. pombe*, the expression of SOD1 is positively regulated by the LAMMER kinase gene *lkh1*$^{+}$, and null alleles of *lkh1* are more sensitive to killing by H_2O_2 then wild type strains [16,17]. LAMMER kinases are members of a family of dual-specificity protein kinases that regulate various aspects of cell growth and differentiation by an as yet unknown mechanism [18]. All LAMMER kinases assayed have been found to phosphorylate serine/arginine-rich splicing factors (SR) and regulate pre-mRNA splicing [19–22]. Human CLK-1 directly and specifically influences the activity of SR factors and may play a role in governing splice site selection [23].

The single LAMMER kinase gene in *Drosophila* is encoded by the *Darkener of apricot* (*Doa*) locus. Like other LAMMER kinase family members, the *Doa* gene encodes a dual specificity serine/threonine kinase capable of phosphorylating SR proteins and posses the highly conserved EHLAMMERILG motif [19,24,25]. Mutations in *Doa* were initially identified as dominant second site suppressors of mutations induced by *copia* retrotransposon insertions [24,25]. Most *Doa* alleles are recessive lethal; one exception is the hypomorphic allele *Doa*^{Dem} [17,25]. The amino terminal non-catalytic domain of LAMMER kinases are not well conserved across species, but the catalytic kinase domain of LAMMER kinases is well conserved across orthologues, and of the six *Doa* splice forms identified in Drosophila all share a common catalytic domain [26]. Based on the catalytic domains, DOA is most similar to that of the human LAMMER kinase CLK-2 with 75% identity, but is also shares a significant similarity to catalytic domain of human CLK-1 with 62% identity [17].

We observed in *Drosophila melanogaster* that mutations in *Doa*, protected flies against acute lethality caused by feeding the ROS generating agent paraquat. We focused our studies on the regulation of SOD since the over-expression of SOD1 in both *S. cereviseae* and *Drosophila* has been reported to reduce oxidative damage and extend life span [27]. We found that flies with mutant *Doa* have increased expression of SOD1 and smaller increases in SOD2. We extended the studies to human cancer cells that have 4 LAMMER kinase genes and found that inhibition of CLK-1 alone increased expression of SOD1 but not SOD2. Thus, an increase in SOD1 by inhibition of CLK-1 appears to be a conserved mechanism that provides a possible explanation for the increased paraquat resistance caused by D*oa* mutation in *Drosophila*. In addition, we suggest that this mechanism may provide protection against ROS-induced carcinogenesis in human cancer cells.

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MATERIALS AND METHODS

Drosophila stocks and genetics

Stocks of Drosophila melanogaster were maintained on standard cornmeal-molasses medium and all crosses were carried out at 25° C unless otherwise noted. Stocks of w^a; *Doa*$^{\gamma 3B}$/TM6B, eTb, yw^a; Pr Doa^{HD}/TM6B, e Tb and w^a; *Doa*^{Dem}/TM6B, eTb were a generous gift from L. Rabinow (Univ. Paris). All other stocks were obtained from the Bloomington Drosophila Stock Center.

Paraquat resistance test

To examine the effects of genetic background on resistance to the O_2.- generator paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) (Sigma Chemical, St. Louis, MO) a modification of the method of Seong et al [28] was used. Animals eclosing within a 2-day period were pooled, starved on 1.5% agar for 6 h and transferred (20 flies/vial) to vials containing GF/A filter disks (Whatman) soaked in 10 mM paraquat in 5% sucrose. Control flies received 5% sucrose without paraquat. At least 100 flies were used for each genetic background, and approximately equal numbers of males and females were tested. Dead flies were counted twice daily for 5 days, and the time of 50% mortality was determined from a second order polynomial regression curve fitted to the data using Microsoft Excel.

Cell culture

HeLa and MCF-7 cells were maintained in Dulbecco's modified eagles medium supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT). The LAMMER kinase inhibitor TG003 (EMD, San Diego, CA) [29], was dissolved in DMSO as a 20 mM stock solution and added to log-phase cultures at a final concentration of 10 μM. Control cells received DMSO without inhibitor. Following incubation at 37° C for the times indicated cells were harvested for SOD assays, and Western blot analyses, as described below. To assess the effect of TG003 on cell growth, AlamarBlue was added to a final concentration of 10%, and its reduction was monitored at 570 nm and 600 nm [30].

SOD assays

To measure the effect of Doa alleles on SOD activity, female flies heterozygous for the desired allele were crossed with Ore R males, and male progeny were collected 1 day after eclosion. Groups of 10 males were homogenized on ice in pre-chilled homogenization buffer (20 mM HEPES, pH 7.2, 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) at a ratio of 1 fly per 40 μl, using a PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA). Tumor cells were rinsed briefly in PBS, and homogenized in homogenization buffer (20 mM Hepes, pH 7.2, 1mM EGTA, 210 mM mannitol, and 70 mM sucrose). Homogenates were centrifuged at 3000 × g for 10 min at 4°C, and the supernatants were serially diluted with 50 mM Tris-HCl, pH 8.0, to dilutions ranging from 1:40 to 1:640. Quadruplicate 10 μl aliquots of each dilution were assayed for SOD activity using an SOD Assay kit (Cayman Chemical Co, Ann Arbor, MI). SOD2 activity was determined in the presence of 5 mM NaCN. SOD1 activity was estimated by subtracting the SOD2 activity from the total activity. Protein concentrations in the homogenates were measured with a Coomassie Plus Protein Assay Reagent (Pierce Chemical, Rockford, IL). Data are presented as means ± SEM, and the significant variation was determined using a 2-tailed t-test.

siRNA treatment, mRNA Measurement and RT-PCR

Total RNA was isolated from MCF-7 and HeLa cells using the PARIS kit (Ambion, Applied Biosystems) according to the manufacturer's protocol. TaqMan quantitative reverse transcription-PCR was done on the ABI 7300 system using the TaqMan One-Step reverse

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transcription-PCR Master Mix kit and predesigned primer/probe pairs for LAMMER kinases and β2-microglobulin (Applied Biosystems). Normalization and analyses were carried out with β2-microglobulin as the internal reference by the -CT method [31] using the Applied Biosystems GeneAmp 5700 SDS software. HeLa and MCF-7 cells were transfected with siRNA in 6-well plates with Dharmafect 2 (Dharmacon, Chicago, IL) according to the manufacture's protocol. SMARTpool siCLK1, siCLK2, siCLK3, siCLK4 and non-targeting siSCR RNAs were obtained from Dharmacon (Lafayette, CO). One microgram of total RNA was reverse-transcribed and amplified with the following primers: SOD1 Exon 1 forward CCTCGGAACCAGGACCTC, SOD1 Exon 3 reverse GTGAGGACCTGCACTGGTA, SOD1 Exon 3 forward GGTGGGCCAAAGGATGAAGAG, and SOD1 Exon 5 reverse TTCACAGGCTTGAATGACAAAC.

Electrophoresis and Western blots

Adult flies, 1–3 days post-eclosion, were homogenized in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.5% Triton-X100, 1% deoxycholate, 2 mM EDTA and protease inhibitor cocktail (Sigma). Human tumor cells grown in 6-well plates were lysed in 250 μl of the same lysis buffer and briefly sonicated. Homogenates were centrifuged at 16,000 ×g for 5 min. Supernatants (25 μg/lane) were fractionated on 10% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to PVDF membranes. Blots were probed with rabbit anti-human SOD1 antiserum (Upstate) diluted 1:300 (Drosophila samples) or 1:1,000 (human samples) or with rabbit anti-human SOD2 antiserum (Upstate) diluted 1:1000 (Drosophila samples) or 1:5,000 (human samples), followed by anti-rabbit IgG-horseradish peroxidase conjugate diluted 1:2500 (Invitrogen). Bands were visualized with Western Lightning Plus chemiluminescent reagent (Perkin Elmer). Subsequently, blots were stripped in 0.2 N NaOH for 5 min at room temperature, then reprobed with mouse anti-α-actin diluted 1:20,000 (Chemicon) and anti-mouse IgG conjugated to horseradish peroxidase diluted 1:20,000 (Invitrogen).

RESULTS

Doa regulates resistance to oxidative stress in *Drosophila*

In order to determine whether *Drosophila* heterozygous for mutations in *Doa* showed sensitivity to oxidative stress, we fed *Doa* mutants the $O_2^{\cdot -}$ generator paraquat [32]. To reduce genetic background and balancer chromosome effects, virgin wild type (*Ore R*) females were mated with males that were either $Doa^{\gamma 3B}$ or Doa^{HD}, and the newly-eclosed F1 progeny of the genotypes $Doa^{\gamma 3B}$/+ and Doa^{HD}/+ were fed 10mM paraquat. The Doa^{HD} allele is due to a transposable element insertion and the Doa_{3B} allele is caused by a chromosomal breakpoint [17]. Approximately equal numbers of males and females were assayed, and age-matched *Ore R* animals were used as controls. The results are shown in **(**Figure 1). During the first 24 hours few animals of any genotype died. By 74 hours exposure, approximately half the *Ore R* animals had died and by 112 hours all were dead. In contrast, the $Doa^{\gamma 3B}$/+ and the Doa^{HD}/+ animals survived considerably longer than the controls. The estimated times of 50% mortality (TM_{50}) were 74.3 hours for *Ore R*, 109.2 hours for $Doa^{\gamma\ 3B}$/+ and 116.2 hours for Doa^{HD}/+. Less than 1% of the control animals maintained in the absence of paraquat died during the experimental period (data not shown). The results suggest that decreased levels of *Doa* in adult flies significantly increases their resistance to oxidative stress.

Doa alleles affect SOD expression in *Drosophila*

To determine whether the increased resistance of the *Doa* mutant flies to paraquat was due, at least in part, to increased SOD activity in the *Doa* mutants, homogenates of young adult *Ore R*, $Doa^{\gamma 3B}$/+ and Doa^{HD}/+ flies were assayed for total SOD activity and SOD2 activity. Because the only SOD genes in the *Drosophila* genome are *Sod1* and *Sod2* [33], the SOD1

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activity could then be estimated by subtracting SOD2 activity from total SOD activity. The $Doa^{\gamma 3B}$/+ and Doa^{HD}/+ animals exhibited approximately 2-fold increases in both SOD1 and SOD2 activity over those measured in wild type (*Ore R*) controls (Figure 2A). Western blots revealed that SOD1 and SOD2 protein levels corresponded with activity (Figure 2B). Animals heterozygous for the apparent null allele, $Doa^{\gamma\ 3B}$, appeared to express more SOD2 than either the wild-type controls or those carrying the hypomorphic allele, Doa^{HD}. These results suggest that *Doa* regulates the levels of SOD2 and SOD1 expression in *Drosophila*.

Because both the $Doa^{\gamma 3B}$ and the Doa^{HD} alleles are homozygous lethal, the SOD studies described above were carried out using animals heterozygous for *Doa*. In crosses of Doa^{HD} and the hypomorphic allele Doa^{Dem} a small percentage of the progeny that eclose are of the *trans*-heterozygous genotype, Doa^{Dem}/Doa^{HD}. To examine SOD activity in the presence of *Doa* levels lower than those found in either $Doa^{\gamma 3B}$/+ or Doa^{HD}/+ flies, we collected F1 male progeny *trans*-heterozygous $yw^{a}/Y;\ Doa^{Dem}/Pr\ Doa^{HD}$, along with their $yw^{a}/Y;Pr\ Doa^{HD}/TM6B$, and $yw^{a}/Y;\ Doa^{Dem}/TM6B$ siblings, and determined their levels of SOD **(**Figure 2C). Age-matched yw^{a}/Y males were assayed as wild-type controls. In these experiments, the SOD2 activity in Doa^{Dem}/Doa^{HD} *trans*-heterozygotes was 3.2-times that in wild-type controls. The activity in $Doa^{Dem}/TM6B$ and $Doa^{HD}/TM6B$ animals was increased by 2.0-fold and 1.6-fold, respectively. In contrast, SOD1 activity in Doa^{Dem}/Doa^{HD} animals was increased only 1.4-fold compared to controls, and the SOD1 activity in $Doa^{Dem}/TM6B$ and $Doa^{HD}/TM6B$ remained close to control levels. The activities of both SOD1 and SOD2 in *TM3/TM6B* males were comparable to those in wild-type controls. Taken together, these data suggest that, in *Drosophila*, the LAMMER kinase gene *Doa* regulates the expression of SOD2 and SOD1.

CLK-1 negatively regulates SOD activity in mammalian cells

In order to test whether SOD is regulated by LAMMER kinases in human cells, we treated human adenocarcinoma (HeLa) cells with siRNA against the human LAMMER kinase genes CLK-1, CLK-2, CLK-3 or CLK-4 and assayed total SOD activity 72 hours later. We found that knockdown of CLK-1 led to a 1.5 fold increase in total SOD activity ($P < .05$), while individual knockdown of CLK-2, CLK-3 and CLK-4 had no significant effect on total SOD activity (Figure 3A).

When CLK-1 was knocked down there was a 1.6 fold increase in SOD1 activity in HeLa cells, and a 2.5 fold increase in SOD1 activity in breast carcinoma (MCF-7) cells (Figure 3B). However, in neither cell line did knockdown of CLK-1 cause a significant change in SOD2 activity (Figure 3C). This suggests that CLK-1 is sufficient for regulation of SOD1 in human cells. However, CLK-1 either does not regulate SOD2, or CLK regulation of SOD2 is at least partially redundant, in human cells. This effect on SOD1 activity in human cells lacking CLK-1 is similar to what is seen in *Drosophila Doa* mutants (Figure 2).

SOD1 protein and transcript are regulated by CLK-1 in mammalian cells

LAMMER kinases are known to regulate pre-mRNA splicing [19–22]. We therefore asked if knockdown of CLK-1 would affect SOD protein and transcript levels in HeLa and MCF7 cells. We used a 72-hour timepoint after treatment with siRNA against CLK-1. We found that HeLa and MCF-7 cells showed significantly increased SOD1 protein and transcript after treatment. SOD1 protein increased approximately 2.5 fold in HeLa cells and 1.6 fold in MCF-7 cells. SOD2 protein did not change in HeLa or MCF-7 cells (Figure 4A). RT-PCR analysis showed that the SOD1 transcript increases 2.1 fold in MCF-7 and (Figure 4B). 2.2 fold in HeLa cells (Figure 4C). There was no significant difference in the SOD2 transcript.

To determine if the change seen in SOD1 transcript levels were due to changes in splicing we performed RT-PCR on total RNA from HeLa and MCF-7 cells treated with siRNA against

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CLK-1. RT-PCR amplification of SOD1 did not show any evidence of alternate splicing of the SOD1 transcript (Figure 5).

The LAMMER kinase inhibitor TG003 increases SOD expression in mammalian cells

To determine whether pharmacological inhibition of LAMMER kinase would increase SOD expression in human cells, we used the LAMMER kinase inhibitor TG003. TG003 acts as a competitive inhibitor of ATP binding to the kinase domain. It has a K_i of 10 nM for CLK-1/Sty in HeLa cells, and exhibits IC_{50}s of 20 nM, 200 nM, >10 μM and 10 nM for mouse CLK-1, CLK-2, CLK-3 and CLK-4, respectively [29].

As reported previously for HeLa cells [29], treatment of cells with TG003 had no significant effect on the growth of HeLa or MCF-7 cells (data not shown). Western blot analysis of lysates from HeLa and MCF-7 cells grown for 48 hours in 10 μM TG003 showed an approximate 2 fold increase in SOD1 protein (Figure 4D). The response of SOD1 protein levels to TG003 was similar to what we observed in CLK-1 siRNA treated cells **(**Figure 4A) suggesting that this response is due to CLK-1 inhibition by TG003. SOD2 protein levels did not change in MCF-7 cells, but did increase approximately 3 fold in HeLa cells (Figure 4D).

The effect of treating MCF-7 cells with TG003 on SOD2 activity (Figure 4D) is different from what is seen when CLK-1 alone is knocked down (Figure 4A), but is similar to what is seen in *Drosophila Doa* mutants (Figure 2C). One explanation for the difference between the siRNA and TG003 findings is that in MCF-7 cells SOD2 regulation requires simultaneous inhibition of CLK-1, CLK-2 and CLK-4 activity which is only seen in the case of TG003 treated cells.

DISCUSSION

Our studies have shown that mutations in *Drosophila Doa*, cause increased levels of both SOD1 and SOD2 protein and activity, providing a possible explanation for the antioxidant mechanism behind the increase in *Drosophila* resistance to ROS caused by loss of DOA function. We also found that when human CLK-1 is knocked down by treatment with siRNA in HeLa and MCF-7 human tumor cells, there is an increase in SOD1 protein, activity and transcript. While the sequence of the conserved catalytic domain of CLK-2 suggest that it is the closest human ortholog to *Doa* (75% identity), there is still considerable conservation between the catalytic domains of *Doa* and CLK-1 (62% identity). From these data we can not discount the possibility that CLK-2 or one of the other two LAMMER kinase genes contribute to SOD regulation. In fact our results using the pan LAMMER kinase inhibitor TG003 support the possibility that more then one of the human LAMMER kinases is involved in SOD regulation. Our findings in *Drosophila* and human cell lines contrast with reports in *S. pombe* where the LAMMER kinase homolog *lkh1*$^+$ appears to positively regulate SOD, and mutations in *lkh1+* decrease resistance to ROS and decrease *sod1*$^+$ mRNA [16]. Although the direction of regulation we observed in *Drosophila* and HeLa and MCF-7 cells was opposite to that reported in yeast, taken together these data suggest that the ability of LAMMER kinases to regulate SOD is evolutionarily conserved between humans and *Drosophila*.

When newly eclosed adult *Drosophila* were exposed to oxidative stress by feeding them the $O_2{\cdot}^-$ generator paraquat [28], animals carrying mutations in the LAMMER kinase homolog *Doa* survived significantly longer than wild-type controls. One possible explanation for this resistance to paraquat is the coincident increase in SOD protein and activity seen in *Doa* mutants. This is in concert with previous reports that *Drosophila* SOD loss of function mutants are more sensitive to paraquat [34]. While overexpression of bovine SOD in *Drosophila* is sufficient to increase resistance to paraquat [35,36] overexpression of transgenic *Drosophila* SOD1 was insufficient to increase resistance to paraquat in two different studies [34,37]. The difference in paraquat resistance between *Doa* mutants and the *Drosophila* SOD1 transgenic

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lines may be due to differences in SOD activity. For example, while we saw a greater than two fold increase in SOD1 activity in *Doa*$^{\gamma 3B}$and *Doa*^{HD} mutants, the transgenic lines in the previous studies increased SOD activity by at most 70% [34]. Alternatively, paraquat resistance in *Doa* mutants might due to the simultaneous increase in both SOD1 and SOD2 activity or in the regulation of other stress resistance genes by *Doa*.

We found that SOD2 is negatively regulated by the *Doa* gene in *Drosophila*, and that SOD2 protein levels are increased in HeLa cells treated with the pan LAMMER kinase inhbitorTG003, but not in TG003 treated MCF-7 cells, nor in HeLa or MCF-7 cells treated with siRNA against the CLK-1. In *S. pombe*, the regulation of the two SOD genes is different; SOD1 transcript is increased by Atf1 in response to H_2O_2, while SOD2 is Atf1 independent [38,39], and there is no report of SOD2 being regulated by *lkh1*+. While there is only one LAMMER kinase gene in *Drosophila* [17], encoded by *Doa*, there are four reported LAMMER kinase genes in humans. At 10 μM TG003, as used in our study, there would most likely be inhibition of ATP binding to CLK-1, CLK-4 (and possibly CLK-2) [29]. Therefore, we may not have detected regulatory activities that CLK-3 or CLK-2 may exert on SOD. This suggests that in MCF-7 and HeLa cells, SOD2 is regulated by a LAMMER kinase other then CLK-1, or a combination of LAMMER kinases.

The mechanism for the increase in SOD1 by CLK-1 remains unclear. CLK-1 phosphorylates SR proteins to regulate pre-mRNA splicing, specifically influencing the activity of SR protein splicing factors [23]. It was possible that CLK-1 regulates SOD1 through alternate splicing, at least five alternatively spliced forms of human SOD1 have been reported [40,41], but we found no evidence of that in our studies. Examination of *Drosophila* genomic sequence and EST data suggest that the *Drosophila* SOD1 and SOD2 genes are not alternatively spliced. There may be indirect effects of CLK-1 on SOD1 expression since the promoter of human SOD1 is regulated by Egr-1 and two splicing variants of the Egr-related protein WT1 exist [5].

Increased formation of ROS plays an important role in various human pathologies [3] Mutations in SOD1 are associated with 20% of familial amyotrophic lateral sclerosis cases [42,43]. In human tumor cells, overexpressing SOD2 inhibits cell proliferation, increases differentiation, and can reverse a malignant phenotype to a nonmalignant phenotype[44–46]. Given the role of SOD in reducing the damaging effects of ROS, increasing SOD activity through inhibition of CLK-1 may provide an effective way for treating some of these diseases.

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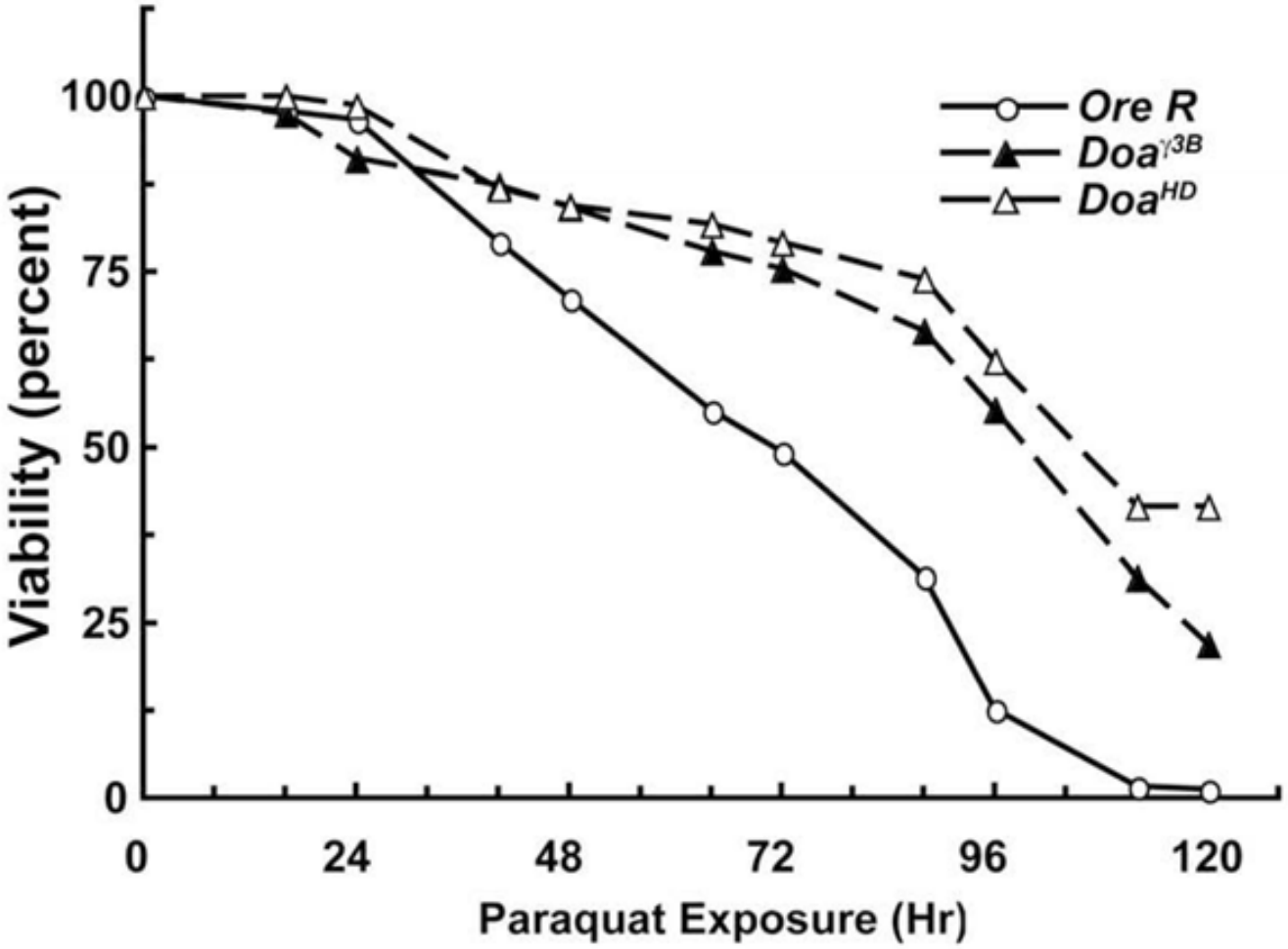

Figure 1.

Mutations in *Doa* cause resistance to paraquat. *Ore R*, $Doa^{\gamma 3B}$/+ and Doa^{HD}/+ animals 1–3 days post eclosion were starved for 6 hr, and then fed continuously with 10 mM paraquat dissolved in 5% sucrose. The number of dead animals was counted twice each day for 5 days and the data was expressed as percent survival. The estimated times of 50% mortality were 74.3 h for *Ore R*, 109.2 h for $Doa^{\gamma 3B}$/+ and 116.2 h for Doa^{HD}/+. Less than 1% of the sibling control animals for each genotype that were fed 5% sucrose without paraquat died during the 5-day counting period. $n \geq 100$ for each genotype.

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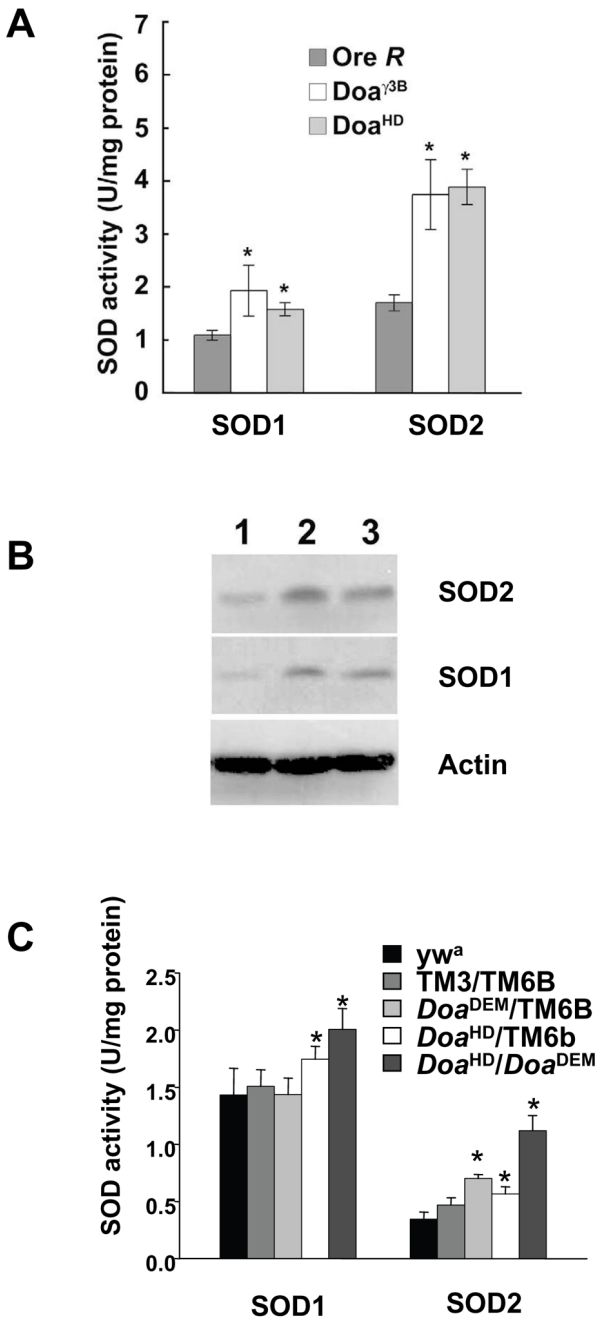

Figure 2.

SOD activity is elevated in adult flies carrying mutations in *Doa*. (A) Total SOD activity was measured in homogenates of age-matched $Doa^{\gamma 3B}$/+ and Doa^{HD}/+ males. *Ore R* males were assayed as wild type controls. SOD2 activity was measured in parallel aliquots containing 5 mM NaCN. SOD1 activity was then estimated by subtracting the SOD2 activity from the total activity. (B) Representative Western blot of homogenates of adult males 1–3 days post eclosion. Lane 1, wild type (*Ore R*); lane 2, $Doa^{\gamma 3B}$/+; lane 3, Doa^{HD}/+. Staining for actin is included on all Western blots as a control for loading. (C) SOD activity in *trans*-heterozygous Doa^{Dem}/Doa^{HD} flies along with heterozygous $Doa^{Dem}/TM6B$ and $Doa^{HD}/TM6B$ siblings. Because the $Doa^{Dem}/TM6B$ and $Doa^{HD}TM6B$ animals were in *yw* and w^a backgrounds,

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respectively, stocks of *yw* and *TM6B/TM3* animals were used as controls. Error bars indicate standard error. * SOD activities significantly different from control values ($p < 0.05$).

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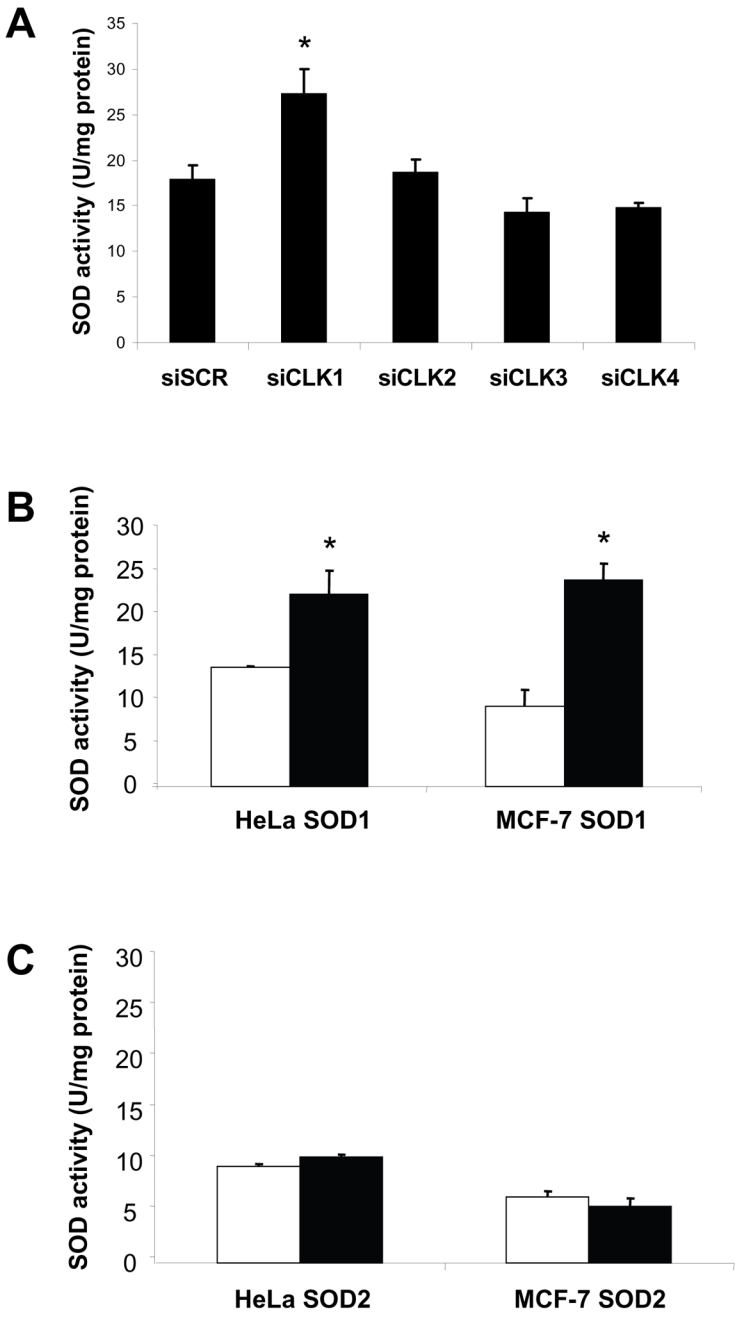

Figure 3.

Knockdown of CLK-1 increases SOD activity in HeLa and MCF-7 cells. (A) Total SOD activity in HeLa cells treated with siRNA against CLK-1, CLK-2, CLK-3 and CLK-4. (B) SOD1 activity in HeLa and MCF-7 cells treated with CLK-1 siRNA. (C) SOD2 activity in HeLa and MCF-7 cells treated with CLK-1 siRNA.. White bars indicate non-targeting siSCR controls; black bars indicate siCLK-1. In each assay RT-PCR confirmed that the target transcript level was reduced at least 80%. Error bars indicate standard error. *SOD activities significantly different from control values ($p < 0.05$).

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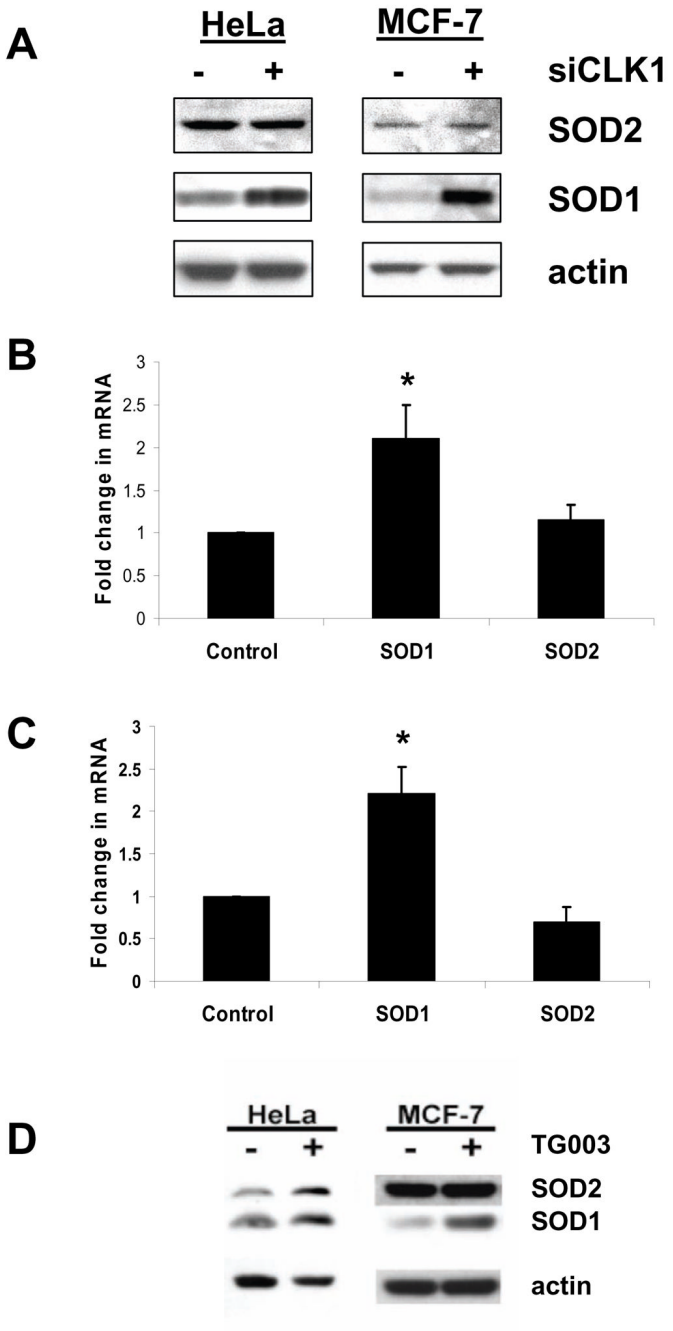

Figure 4.

Knockdown of CLK-1 increases SOD transcript and protein. (A) Western blots of lysates from HeLa cells and MCF-7 cells treated with CLK-1 siRNA. (B) SOD1 and SOD2 transcript levels in MCF-7 cells treated with CLK-1 siRNA were measured using Taqman real-time PCR. (C) SOD1 and SOD2 transcript levels in HeLa cells treated with CLK-1 siRNA were measured using Taqman real-time PCR. (D) Western blots of lysates from HeLa cells and MCF-7 cells grown for 48 hr in 10 μM TG003. In each assay RT-PCR confirmed that the target transcript level was reduced at least 80%. Error bars indicate standard error. *SOD- transcripts significantly different from control values ($p < 0.05$). Staining for β-actin was done to control for sample loading.

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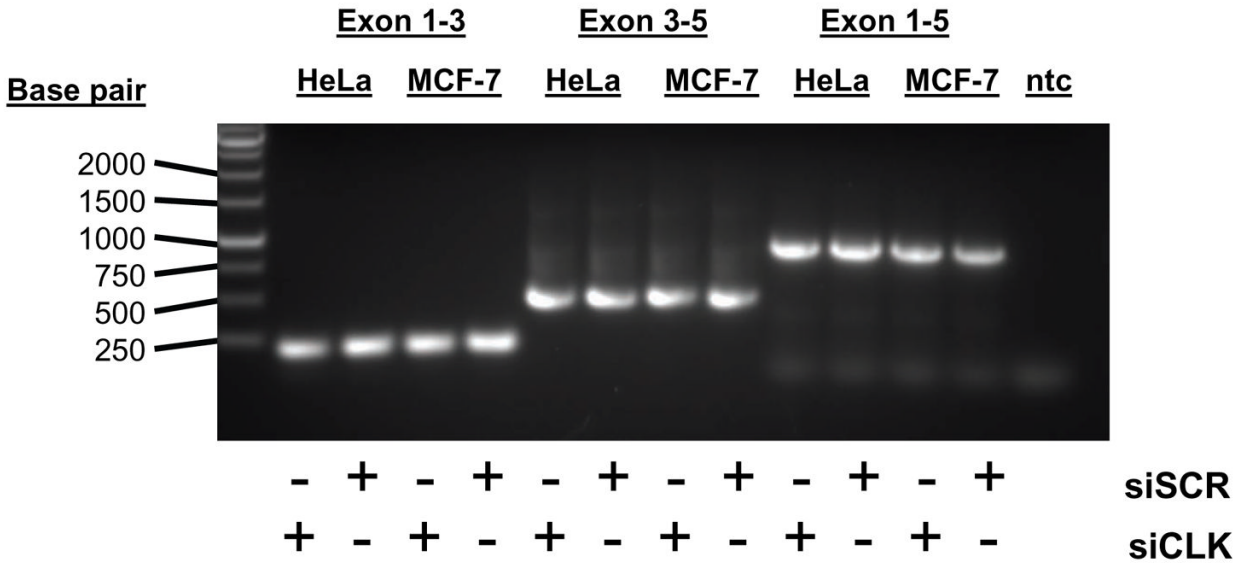

Figure 5.

RT-PCR analysis of SOD transcripts in HeLa and MCF-7 cells treated with siRNA against CLK-1. (A) Results of amplification of SOD1 cDNA using primers spanning exon 1 through 3, exon 3 through 5 and and exon 1 through 5 (ntc: no template control).