Expression and regulation of antioxidant enzymes in the developing limb support a function of ROS in interdigital cell death

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Abstract

Vertebrate limb development is a well-studied model of apoptosis; however, little is known about the intracellular molecules involved in activating the cell death machinery. We have shown that high levels of reactive oxygen species (ROS) are present in the interdigital ‘necrotic’ tissue of mouse autopod, and that antioxidants can reduce cell death. Here, we determined the expression pattern of several antioxidant enzymes in order to establish their role in defining the areas with high ROS levels. We found that the genes encoding the superoxide dismutases and catalase are expressed in autopod, but they are downregulated in the interdigital regions at the time ROS levels increased and cell death was first detected. The possible role of superoxide and/or peroxide in activating cell death is supported by the protective effect of a superoxide dismutase/catalase mimetic. Interestingly, we found that peroxidase activity and glutathione peroxidase-4 gene (Gpx4) expression were restricted to the non-apoptotic tissue (e.g., digits) of the developing autopod. Induction of cell death with retinoic acid caused an increase in ROS and decrease in peroxidase activity. Even more inhibition of glutathione peroxidase activity leads to cell death in the digits, suggesting that a decrease in antioxidant activity, likely due to Gpx4, caused an increase in ROS levels, thus triggering apoptosis.

Introduction

During development, abundant cell death is associated with several morphogenetic processes (Clarke, 1990). Despite this observation, the actual function of this cell death has only been experimentally established in few cases. Furthermore, although some growth factors that appear to participate in controlling the death fate in morphogenetic processes have been identified, the complete set of molecules defining the ‘death environment’ in no case has been defined (see for instance Zuzarte-Luis and Hurle, 2002). Even less well characterized is the transducing signals that emerge in response to extracellular factors that trigger death within cells. In the limb, we proposed that cell death influences digit individualization by restricting interdigit growth and promoting regression of interdigital tissue in contrast to the traditional purely sculpting model (Salas-Vidal et al., 2001). Among the molecules regulating the death fate of interdigital cells are members of the fibroblast growth factor (Fgf) and bone morphogenetic protein (Bmp) families, as well as retinoic acid (RA), though the mechanism by which they turn on the death process is not known (Zuzarte-Luis and Hurle, 2002).

Reactive oxygen species (ROS) are generated in all aerobic organisms during normal respiratory metabolism. ROS are known to affect cells in different ways by directly oxidizing and damaging diverse cellular components like DNA, proteins, and lipids (Bokov et al., 2004). However, recent findings show that ROS also act as major intracellular signals involved in the regulation of key cellular process such as cell
proliferation, cell differentiation, senescence, and apoptosis (Esposito et al., 2004; Jabs, 1999). Intracellular concentration of ROS can be modulated by controlling the sites and amounts of their synthesis or by inactivating them with antioxidant molecules (e.g., glutathione, thioredoxin) directly or with the help of antioxidant enzymes. Due to the difficulty in determining ROS concentration in vivo, frequently, the redox state of a cell or tissue is estimated indirectly by measuring the amount of oxidized molecules or the activity of different antioxidant enzymes, such as superoxide dismutase (Sod), catalase, and peroxidases (see for instance Brandes and Janiszewski, 2005 and references herein).

Pro-oxidant states are known to occur previous to the onset of cell death in several cell types like neurons (Greenlund et al., 1995), monocytes (Baran et al., 2004), tumor cells (Djavaheri-Mergny et al., 2004), and embryonic stem cells (Castro-Obregón and Covarrubias, 1996) among many other cell types. It is also known that overexpressing antioxidant enzymes can suppress apoptosis; for example, neurons overexpressing Sod1 display delayed apoptosis induced by Ngf removal (Greenlund et al., 1995), and Tnfo-induced apoptosis is completely suppressed by overexpressing Sod2 (Manna et al., 1998). These observations indicate that intracellular modulation of ROS concentration is of major importance for cell survival.

The relevance of the redox state in cell survival in vivo is hard to establish. However, the observation that mutations in mouse genes encoding molecules involved in typical antioxidant systems are embryonic lethal could indicate that strict control of ROS level is required for the survival of the highly metabolically active developing cells (Imai et al., 2003; Matsui et al., 1996). ROS have been proposed to participate in several metabolic processes. More importantly, inhibition of glutathione peroxidase activity down-regulation. More importantly, inhibition of glutathione peroxidase activity dramatically increased cell death in the normally non-dying distal digital tissue. These data suggest that peroxidase activity, likely due to Gpx4, determines ROS and cell death patterns in the developing autopod.

### Materials and methods

#### Animals

Mouse strain CD-1 was used in this study. Pregnant females were sacrificed by cervical dislocation from 12.5 to 14.5 days post-coitus (dpc; day 0.5 of coitus was the day on which vaginal plug was found). The embryos were removed, and the forelimbs were dissected in L15 medium (Microlab, México) and staged according to the limb bud development staging system of Wanek et al. (1989).

#### Limb culture

The protocol used to culture embryonic limbs is based on the embryonic gonad organotypic culture described by Taketo and Koide (1981) with slight modifications (Salas-Vidal et al., 1998). The staged mouse embryonic forelimbs were cultured on 0.45-μm pore Durapore polycarbonate membrane filters (Millipore) floating on Mc-CoY’s 5a modified medium (Microlab, México) without serum and supplemented 200 UI/ml penicillin G sodium, 200 mg/ml streptomycin sulfate, and 2 mM glutamine (GIBCO, USA). In some cases, RA (1 μM), mercaptoascorbic acid (MS; 1–100 mM), or hydroperoxide (H2O2; 5–300 mM) was added to the culture medium.

#### In vivo peroxidase activity, cell death, and ROS detection

Peroxidase activity was determined by incubation of forelimbs for 30 min at 37°C in PBS pH 6.5 with 50 mM H2O2 (hydroperoxide) and either 40 μM diclorofluorescein (DCF; Molecular Probes) (Buraw and Valet, 1987) or diaminobenzidine followed by two washes in PBS. Regions of cell death in freshly dissected embryonic limbs were visualized with acridine orange (AO; Sigma) staining by the protocol described by Abrams et al. (1993) and validated to detect cell death in mouse embryos by Salas-Vidal et al. (1998).

Briefly, the tissues were rinsed in phosphate-buffered saline (PBS: 0.2% KH2PO4, 0.115 Na2HPO4, 0.8% NaCl, 0.02% KCl, pH 7.4) stained with AO at 5 μg/ml in PBS, for 30 min at 37°C and observed under standard fluorescence microscopy. For more detailed studies, the tissues were analyzed by confocal microscopy as described below. To determine apoptosis and ROS levels, forelimbs were incubated 20 min in 5 μg/ml acridine orange (AO) and 1 μM dihydroethidium (DHE) or dihydrodihydrodiamine (DHR), respectively, in PBS pH 6.5 at room temperature, followed by two washes in PBS. Detection of fragmented DNA was performed on 4% paraformaldehyde-fixed paraformin-embedded 10-μm serial sections of complete forelimbs with the TdT-mediated dUTP nick end labeling (TUNEL) method, as described by the manufacturer (Roche). Limbs were sectioned perpendicular to the proximo-distal axis such that the dorso-ventral cell death pattern could be observed. The complete series were analyzed (representative images are shown).

#### Catalase activity

Equal amounts (150 μg) of protein were loaded onto a 10% native polyacrylamide gel. After electrophoresis, gel was soaked in 5% methanol for 3 min and then incubated with 0.03% hydrogen peroxide. Catalase activity was revealed by incubating gel in a fresh mixture of 40 mg/ml Iron (III) chloride and 40 mg/ml potassium ferricyanide.

#### Confocal laser scanning microscopy

Developing limbs were observed in a Bio-Rad MRC-600 confocal laser scanning system equipped with a krypton/argon laser and coupled to an Axioskope microscope (Zeiss, Germany) with PlanNeofluar 5× (aperture 0.15), 10× (aperture 0.30), and 20× (aperture 0.5) objectives. BHS filter and a 488-nm excitation wavelength were used for detection of oxidized DCF, the RRS filter, and a 488-nm excitation wavelength for AO, and the YHS filter and 568-nm excitation wave length for oxidized DHE. The pinhole aperture was maintained at 5. Serial optical sections were produced at 15 and 30 μm as the z-step.
Cartilage staining with Alcian blue

Skeletal preparations were made following the procedure described by Wallin et al. (1994). Briefly, limbs were fixed in 99% ethanol for 24 h and in acetone for another 24 h with constant shaking. Afterwards, they were incubated in the staining solution (1 vol. of 0.3% Alcian blue in 70% ethanol, 1 vol. of 0.1% alizarin red S in 96% ethanol, 1 vol. absolute acetic acid, and 17 vol. of 70% ethanol) at 37°C for 4–6 h and then overnight at room temperature with constant shaking. Samples were rinsed in water and cleared in 1% potassium hydroxide and 20% glycerol in water at room temperature. For long-term storage, specimens were transferred into 50%, 80%, and finally 100% glycerol.

Image analysis

Image analysis by superimposition of two-dimensional (2D) limb confocal images was performed as previously described by Salas-Vidal et al. (1998). Briefly, images were displayed on a Macintosh G3 computer (Apple, USA) using Adobe Photoshop software, version 3.02. Images of interest were superimposed, and the overlying image opacity was reduced to 50%. The overlying image was rotated and moved vertically and horizontally until completely aligned with the underlying image. In some superimpositions, the cartilage condensations were used for the alignment; images were first aligned using the wrist and middle digit and then improved image alignment using the cartilage condensation. In some images where the limbs were treated with the glutathione peroxidase inhibitor, MS, it was necessary to highlight the limb contour by delineating it with dots, since for comparative purposes, the confocal sensitivity was maintained during the image acquisition and when the glutathione peroxidase activity was completely abolished the signal was extremely low.

Detection of gene expression by RT-PCR

Reverse transcriptase (RT) reaction was performed with 1 μg of total RNA, and the resultant cDNA was subjected to PCR. To amplify a region corresponding to the mRNA of Gpx1, Gpx2, Gpx3, Gpx4, Sod1, Sod2, Sod3, and Cas1, the following primers were used: Gpx1 forward 5′-ACACCGCT-TACTTCTCTCTTCT and Gpx1 reverse 5′-TCCCTCTACCGTTCTTCTC (expected product size, 185 bp); Gpx2 forward 5′-ACATGCAAGTGCTTC-TACG and Gpx2 reverse 5′-GAGCAGCTCTCATCTAGATGG (expected product size, 580 bp); Gpx3 forward 5′-CATTTGCTGTGCTTATTGG and Gpx3 reverse 5′-CTGCCCCCTTCTTCTGCTT (expected product size, 169 bp); Gpx4 forward 5′-GCAATGAGGCAAAACTGA and Gpx4 reverse 5′-GGACGCACAGTAGAGAT (expected product size, 382 bp); Sod1 forward 5′-AGTATGGGGAACACACTACAGG and Sod1 reverse 5′-CAGCTCAAGGCGCTCACAGTAC (expected product size, 439 bp); Sod2 forward 5′-TCAACCGCCAACCGGAGA and Sod2 reverse 5′-CCCCAGCACGGGAATAAG (expected product size, 358 bp); Sod3 forward 5′-GAAGAATGGCGCAAGCAGCA and Sod3 reverse 5′-CTCCCCGCGCCGCTTTCG (expected product size, 600 bp); Cas1 forward 5′-TACCTGTGAACTGTCCCTACCG and Cas1 reverse 5′-GAATTGCGTTCTTAGGCTTCT (expected product size 411 bp). Polymerase chain reaction conditions used were 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min for up to 30 cycles. The RT-PCR conditions were set in control tissues (i.e., liver, intestine, kidney, and testis) where the different antioxidant enzymes are expressed (data not shown).

Whole-mount in situ hybridization

Mouse embryonic forelimbs were dissected in cold PBS and fixed overnight in 4% paraformaldehyde dissolved in PBS. Whole-mount in situ hybridization was performed according to Wilkinson and Nieto (1993). Using digoxigenin-labeled antisense riboprobes, specific hybridization was detected with an antidigoxigenin antibody coupled to alkaline phosphatase. Proteinase K treatment was titrated to prevent damage to superficial ectoderm structures such as the apical ectodermal ridge and was adjusted, for comparative analysis, to simultaneously obtain the best detection at the different limb developmental stages. For all genes tested, an in situ hybridization with a specific sense or not related riboprobes was performed as a control of procedure specificity (data not shown). The probes used correspond to the regions between positions: 213 to 576 of Gpx4 mRNA (gene bank accession number: BC083137); 128 to 609 of Gpx3 mRNA (gene bank accession number: BC057027); 618 to 1140 of Cas1 mRNA (gene bank accession number: BC013447); 152–490 of Sod1 mRNA (gene bank accession number: BC048874); 235–574 of Sod2 mRNA (gene bank accession number: BC010548); 405–1004 of Sod3 mRNA (gene bank accession number: NM_011435).

Results

Peroxidase activity pattern in limbs is complementary to that of ROS levels and cell death

During mouse limb development, at stages 9 through 11 (S9–S11; as classified by Wanek et al. (1989)), apoptosis is located in the interdigital regions as detected by TUNEL or by

![Fig. 1. Cell death, ROS, and peroxidase activity patterns during autopod development. Forelimbs at stages 9, 10, and 11 were stained with AO to detect cell death (A; red in E), with DHE to detect ROS levels (B; green in D), or with DCF in the presence of hydroperoxide to detect peroxidase activity (C; blue in D and E). Peroxidase activity was observed in the digital tissue with the most intense level at the tips. No peroxidase activity was detected at the interdigitalgins such that its pattern is complementary with that of ROS and cell death as shown by the superimpositions of the corresponding patterns (D and E, respectively). All limbs are oriented with the anterior side to the left. Scale bar = 500 μm.](image-url)
staining with acridine orange (Salas-Vidal et al., 2001; Fig. 1A). Here, using the redox-sensitive dye dihydroethidium, we confirmed our previous observations showing oxidative stress in the regions undergoing cell death (Salas-Vidal et al., 1998; Fig. 1B). Furthermore, in agreement with the participation of superoxide and peroxide in promoting cell death in the interdigital regions, a Sod/catalase mimetic, EUK-134, reduced cell death as measured by acridine orange staining and TUNEL, and developing limbs displayed a reduction in interdigital tissue regression (Fig. 2).

Peroxidase activity (the reduction of peroxide using a reducing substrate) is the major antioxidant activity found in cells. This activity could be due to different classes of peroxidase, each characterized by the distinct substrate it uses. In order to determine whether this activity participates in defining the ROS pattern, we assessed general peroxidase activity in situ. Peroxidase activity was assayed by using either diaminobenzidine (data not shown) or 2′,7′-dichlorodihydrofluorescein-diacetate (DCF) as substrates, as it has been previously described by Burow and Valet (1987). As these are small substrates, it is expected that several peroxidases could use them to reduce hydroperoxide. As shown in Fig. 1C, in S9 through S11 limbs, peroxidase activity is restricted to the future digital tissue and is excluded from the interdigital tissue. This distinct pattern is complementary to the ROS level and cell death patterns (Figs. 1D and E, respectively). Interestingly by S9, peroxidase activity is already strong along the whole digital rays, whereas the cell death is still restricted to the most distal interdigital region.

To analyze in more detail the pattern of peroxidase activity, we superimposed images of Alcian blue stained limbs (Fig. 3A), which use cartilage condensations as reference for the position of peroxidase activity images. These superimpositions show that interdigital peroxidase activity decreased at up to the distal level of phalange 1 at S9, up to the proximal level of phalange 1 by S10, and up to the level of articulation between phalange 1 and metacarpals by S11. Thus, endogenous peroxidase activity down regulation occurs in a disto-proximal pattern, similar to the one that follows cell death (Salas-Vidal et al., 2001). Additionally by analyzing the dorsal and the ventral sides of the same limbs, we observed that peroxidase activity exhibit a dorso-ventral asymmetry, covering most of the dorsal interdigital tissue in the dorsal side compared to the ventral one; a v-shaped region devoid of activity is observed, same area in which cell death takes place (Fig. 3B). This dorso-ventral pattern of peroxidase activity is opposite to the dorso-ventral cell death pattern [see also Salas-Vidal et al. (2001)].

RA has been proposed to mediate interdigital cell death (Dupe et al., 1999; Rodriguez-Leon et al., 1999). In addition, RA increases interdigital cell death and causes ectopic cell death at the tip of digits (Crocoll et al., 2002; Rodriguez-Leon et al., 1999; data not shown). Using whole-mount staining with DHR, we observed that limbs treated with RA for 8 h showed increased ROS levels within the interdigital region and also at the tip of digits (Fig. 4). This increase in ROS correlated with a decrease in peroxidase activity, including the tip of digital areas. Therefore, as in intact limbs, low peroxidase activity is associated with high ROS levels and cell death in RA-treated limbs.

Gpx4 is responsible for most of the peroxidase activity in developing limbs

Several enzymes could be responsible for the observed peroxidase activity: any one belonging to the peroxidase groups and/or catalase (which, under certain conditions, can have peroxidase activity; LeHir et al., 1979). Catalase activity cannot be easily detected in situ, but a specific gel activity assay demonstrated significant levels of catalase activity during the S9 to S11 period (Fig. 5A). Some general but specific biochemical characteristics of peroxidases and catalases could allow us to determine which enzyme was responsible of the peroxidase activity detected in situ in whole developing limbs. For instance, different pH and hydroperoxide concentration are necessary to get the optimal activity of peroxidases or catalases. Peroxidases are active at acidic pH and low hydroperoxide concentration (1.5–50 mM); these enzymes are inactivated at high hydroperoxide concentration. On the other hand, catalase is active at basic pH, and the enzyme is not inhibited by high hydroperoxide concentration (up to 4 M) (Herzog and Fahimi,
We found that the activity we detected corresponds to that of a peroxidase since DCF fluorescence in the presence of hydroperoxide was evident at pH 6.5, whereas at pH 8 and 10, it was undetectable (Fig. 5B). Furthermore, at low hydroperoxide concentration (5 and 50 mM), the activity was readily detected, whereas at 500 mM, it was undetectable (Fig. 5B).

There are three major families of peroxidases: the hemo-containing group (e.g., iodoperoxidase, lactoperoxidase, and myeloperoxidase), the peroxiredoxins, and the group that uses glutathione as reducing agent (Gpxs). There are four known seleno-cysteine Gpxs, all sensitive to mercaptosuccinate (MS) (Arthur, 2000; Chaudiere et al., 1984). In order to determine which peroxidase was responsible for the activity, we cultured autopods in the presence of different concentrations of MS (1 to 200 mM) for 30 min and then determined peroxidase activity. Our results show a dose-dependent inhibition of the activity, which was completely abolished at 100 mM (Fig. 6A). When incubation was extended to 6 h, peroxidase activity was completely inhibited, even at concentrations as low as 1 mM of MS (Fig. 6B). With this experiment, we exclude the possibility that the activity detected was due to peroxidases from the hemo-family or the peroxiredoxins. Therefore, four criteria confirm that we were observing Gpx activity: the coupling of hydrogen peroxide metabolism with DCF oxidation, the optimal pH, the inhibition at high concentration of hydroperoxide, and the inhibition by MS.

To determine which of the four Gpxs was responsible for the activity observed, we performed a specific RT-PCR using RNA from 11 dpc mouse limbs; as a control for the different Gpxs, RNA from liver, intestine, kidney, and testis were used (data not shown). We observed expression of all of the Gpxs, but, interestingly, we found abundant Gpx4 expression as compared with the other three genes coding for Gpxs (Fig. 7A).

Whole-mount in situ hybridization was used in order to detect the expression pattern of Gpx4 in the whole mouse limb. We detected a strong expression of Gpx4 in the digits, mainly at the tips (Fig. 7B). This pattern of expression corresponded to the peroxidase activity pattern. Expression of Gpx3 was much lower and did not match the activity profile (data not shown). We did not perform in situ hybridization for Gpx1 and Gpx2 because they are hardly detectable by RT-PCR. Therefore, the results suggest that the activity we detected during limb development is due mainly to Gpx4.

Interestingly, although the peroxidase activity detected in whole limbs was not due to catalase (see also data below), the gene expression level (Fig. 8A) and pattern (Fig. 8B) of this enzyme were similar to those determined for Gpx4, being highest around digits and low in interdigital regions at the time.
cell death is occurring (Fig. 8). In addition, the three genes encoding the three Sods known, *Sod1*, *Sod2*, and *Sod3*, also show significant expression level and similar expression pattern (Fig. 8). Thus, down-regulation of several antioxidant enzymes contributes to generate oxidative stress in the interdigital area.

**Inhibition of Gpx activity induces an increase of apoptosis in the digits**

If the Gpx activity modulates ROS levels, which in turn regulates apoptosis, we expected to affect the apoptosis pattern by inhibiting this peroxidase activity. With this aim, we cultured limbs for 24 h with MS and stained them afterwards with acridine orange to visualize the apoptosis pattern. We found that apoptosis increased in a dose-dependent manner, specifically in the tips of digits, where peroxidase activity is inhibited (Fig. 9). These results suggest that peroxidase activity, likely due to Gpx4, could be involved in the down-regulation of ROS levels in the digits, therefore preventing the activation of the apoptotic process by ROS, normally occurring in the interdigits during autopod development.

**Discussion**

We previously showed that many embryonic regions with increased ROS levels coincide with apoptotic areas, including the interdigital regions of the developing limb (Salas-Vidal et al., 1998). In the limb, interdigital cell death can be reduced by chemical antioxidants such as phenol and dimethylsulfoxide. In the present work, we confirmed the presence of high ROS levels in interdigital areas and showed that an antioxidant with very low toxicity (EUK134, a sod/catalase mimetic; Jung et al., 2001; Rong et al., 1999) can prevent cell death. More important, our data suggest that Gpx4 is an essential antioxidant enzyme responsible for setting the ROS levels and, consequently, the cell death pattern in the autopod, supporting the critical contribution of oxidative stress in triggering interdigital cell death.

By definition, ROS are molecules that react with nearby cellular components (e.g., proteins, lipids, and DNA), resulting in damage that can affect their function. ROS include a number of chemically reactive molecules derived from oxygen. Among the major ROS are superoxide, nitric oxide, hydroxyl radical, hydroperoxide, and peroxynitrite. Some of these species can be produced by redox reactions between them. For instance, superoxide can generate hydroperoxide when reacts with water (reaction catalyzed by Sod), and peroxynitrite when it reacts with nitric oxide. Peroxide can produce the very reactive hydroxyl radical in the presence of reduced metal ions such as ferrous ion. Catalase and peroxidases catalyze the oxidation of hydroperoxide to produce water. Most cellular ROS are produced as by-products of oxygen metabolism (e.g., incomplete reduction of oxygen to water) and are highly toxic to cells.
When the level of ROS is relatively high, cells defend themselves by activating antioxidant and repair mechanisms. In the limb, the expression of several antioxidant enzymes in the distal region previous to the onset of interdigital cell death may reflect this defense response to the oxidative stress condition present in proliferating cells (Niswander et al., 1994). The presence of superoxide, hydroperoxide, or both is supported by the protective effect of the antioxidant sod/catalase mimic used in the present work, resulting in similar phenotype (i.e., sydactyly) as we previously reported with other antioxidants (see Materials and methods). All Sods were expressed along limb development but, apparently, the Cas1 was expressed at higher levels. (B) In situ hybridization analysis. Whole-mount in situ hybridization for the genes indicated was performed on S9-S12 autopods. Initially, expression was detected in all distal regions, but downregulation was evident at the time interdigital cell death appears; the latter event limited the expression around the digits.

Fig. 8. Superoxide dismutases and catalase gene expression in the developing autopod. (A) RT-PCR analysis. An RT-PCR protocol was performed for each antioxidant enzyme indicated using total mouse autopod RNA. Samples of the RT-PCR reaction were taken at the number of cycles indicated (25, 30, 35). The specific RT-PCR products were obtained for the mRNAs of the enzymes tested (see Materials and methods). All Sods were expressed along limb development but, apparently, the Cas1 was expressed at higher levels. (B) In situ hybridization analysis. Whole-mount in situ hybridization for the genes indicated was performed on S9-S12 autopods. Initially, expression was detected in all distal regions, but downregulation was evident at the time interdigital cell death appears; the latter event limited the expression around the digits.

Fig. 9. Effect of seleno-cystein Gpx activity inhibition on autopod cell survival. Autopods were cultured in the absence (Control) or presence of 1 mM or 10 mM MS for 24 h. After this period, autopods were stained with AO to detect cell death. Observe the MS dose-dependent increase in cell death. Scale bar = 500 μm.
of a common gene (Imai and Nakagawa, 2003); they are located in different cellular compartments: cytosol, mitochondria, and nucleolus. In contrast to the other three Gpx that are active as tetramers, Gpx4 is active as a monomer. Gpx4 is the only Gpx able to directly reduce the peroxidized lipids produced in cell membranes. This property may be due to the small size of the active enzyme. In support of the essential role of Gpx4 in membranes, this property may be due to the small size of the active enzyme. In support of the essential role of Gpx4 in membranes, this property may be due to the small size of the active enzyme.

We propose that Gpx4 provides at least one likely essential antioxidant activity that controls ROS level and cell death in the developing autopod. Other antioxidant enzymes contributing to this process are Sods, catalase (this work), and peroxiredoxin 1 (Shan et al., 2005), though none of these appear to be essential (Carlsson et al., 1995; Ho et al., 2004; Lebovitz et al., 1996; Reaume et al., 1996); D. H.-G. and L. C., unpublished observations). Lipid peroxidation is a common characteristic of cells under oxidative stress; high levels of peroxidized lipids can disrupt both the plasma membrane and membranes that are components of cellular organelles, such as the mitochondria, causing cell death (Keller and Mattson, 1998). In concordance with this proposal, several reports have stressed the relevance of Gpx4 in cell survival (Nomura et al., 2001). For instance, embryonic fibroblast cells derived from Gpx4−/− mice are highly sensitive to oxidative insults (Yant et al., 2003). On the other hand, overexpression of the mitochondrial Gpx4, but not the cytosolic isoform, is able to reduce the appearance of typical markers of apoptosis like cytochrome c release, caspase activation, and DNA fragmentation in response to glucose deprivation and treatment with etoposide, staurosporine, UV irradiation, cycloheximide, and actinomycin D, stimuli that induce apoptosis by the intrinsic pathway (Nomura et al., 1999). Interestingly, this phenomenon was associated with the activity of the mitochondrial Gpx4 that appears to be responsible for the reduction of peroxidized cardiolipin (Nomura et al., 2000).

Oxidative stress in the autopod could be generated as a consequence of the high metabolism associated with the rapid growth occurring in its distal region (Niswander et al., 1994). Alternatively, enzymes that produce ROS such as NADPH oxidase could be activated in restricted regions in response to specific growth factors (Balaban et al., 2005; Kreutzer et al., 2003). The present work did not investigate the source of ROS but established that antioxidant enzymes, mainly Gpx4, modulate the ROS levels in specific areas of the autopod. Several molecules have been shown to participate in the control of cell death in the developing limbs. Among the most important are retinoic acid and members of the Tgfβ (e.g., Bmps) and Fgf (e.g., Fgfl) superfamilies (Zuzarte-Luis and Hurle, 2002). These factors could regulate directly or indirectly the production of ROS and/or the levels of antioxidant enzymes. In our study, we show that RA can cause cell death in digit tips, and this effect is associated with an increase in ROS level and peroxidase activity down-regulation. Therefore, in the limb, RA could promote cell death by generating oxidative stress, perhaps by down-regulating Gpx4 expression. Previous work from our group has shown that death of embryonic stem cells caused by RA correlates with increase in ROS levels and can be prevented by addition of antioxidants to the culture medium (Castro-Obregón and Covarrubias, 1996).

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