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Forum Review

Thioredoxin System in Premature and Newborn Biology

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ABSTRACT

Thioredoxin is an important redox protein that is ubiquitously distributed. Thioredoxin exists in dynamic equilibrium between the oxidized and reduced forms, making it an ideal redox-regulatory protein. Thioredoxin, together with thioredoxin reductase and peroxiredoxins, forms a complete redox system that is similar to the glutathione system, but with distinct and divergent functions. This review provides a brief general summary of the thioredoxin system with particular emphasis on its role in premature birth and newborn physiology and disease states. Although extensive studies have examined the role of the thioredoxin system in antioxidant defense, cell proliferation, and signal transduction, further studies are needed to understand its role in embryogenesis and development. Such studies will facilitate our understanding of how thioredoxin may modulate newborn diseases via redox regulation. *Antioxid. Redox Signal.* 6, 177–184.

INTRODUCTION

As air-breathing organisms, we inhale oxygen levels of 20–21%. However, neonatal development occurs in an environment that is much more hypoxic; the oxygen concentration *in utero* is <3% (31). At birth, newborn infants are therefore suddenly exposed to significantly higher levels of inspired oxygen. As all aerobic organisms require oxygen for energy production and maintenance of cellular functions, the efficiency of energy metabolism increases after birth. However, neonates must also withstand the associated generation of toxic oxygen radicals, which can oxidize critical macromolecules. A protective mechanism occurs during the third trimester of fetal life, in the form of increased antioxidant enzyme levels (24, 39, 73, 79). This adequately prepares the newborn to face higher levels of oxygen at birth. However, premature infants frequently suffer from oxidative injury, due to their insufficient ability to protect against oxidative insult. The redox balance during fetal life would be expected to differ from that in neonatal life, due to the dramatic differences in oxygen levels in these two environments. However, the role of redox in embryogenesis and fetal development has not yet been delineated.

The thioredoxin (Trx) system is a major cellular redox system that is similar to the glutathione system, but with different and divergent functions. The shift of balance between the oxidized and reduced forms of these proteins provides the cell with a redox milieu that regulates various responses, including gene expression. However, the mechanisms by which these redox systems are modulated during development and in newborn diseases are not yet understood. There are several excellent reviews summarizing the role of the Trx system in antioxidant defense, redox regulation, and cell growth (60–63, 77). In this review, I will discuss the role of the Trx system in development and neonatal life and its possible role in premature newborn disease. A brief overview of the Trx system has been included to introduce this very important redox protein.

THIOREDOXIN

Trx is a low-molecular-weight protein (12 kDa) that is localized in the cytoplasmic, membrane, and mitochondrial cell fractions, as well as in the extracellular space (36, 37). This protein was originally identified in *E. coli* as a hydrogen donor for ribonucleotide reductase, an essential enzyme that provides deoxyribonucleotide for DNA replication (36, 37). The active site of Trx (Trp-Cys-Gly-Pro-Cys) is highly conserved across species (36, 37). Trx expression has been shown to be

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increased in response to a variety of oxidative stress conditions (2), including retinal ischemia–reperfusion (55), phorbol 12-myristate 13-acetate, interferon (81), retinol (9), exposure to UV light (81), hydrogen peroxide (H_2O_2 (68), and hyperoxia (17). A schematic presentation of the Trx system and some of the important functions is shown in Fig. 1.

Trx has been shown to regenerate oxidatively damaged proteins in lens epithelial cells (74) and in endothelial cells (22). Trx was found to be resistant to H_2O_2-mediated oxidation as compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in porcine endothelial cells (22). Trx overexpression also prevented nitric oxide (NO)-induced reduction of NO synthase activity in lung endothelial cells (58). It has been shown that Trx expression is increased in response to hypoxia (38). In addition, increased expression of Trx was also shown in many types of cancer cells (38, 63). Trx is secreted from normal and neoplastic cells, as well as cells transfected with Trx expression vectors in a leaderless pathway (67). Besides acting as an antioxidant itself (15), Trx also regulates the expression of other important antioxidant genes, such as manganese superoxide dismutase (MnSOD) (16). Trx can regulate the activation of redox-responsive transcription factors, such as nuclear factor-κB (14, 33, 34, 50). It has been shown that Trx can inhibit apoptosis signal regulating kinase (ASK) (70). The redox-active nature of Trx is important in cell proliferation of lymphoid cells, as well as in many types of malignant cells (63). Trx was shown to enhance the DNA binding of the hypoxia inducible transcription factor (HIF-1) in hypoxic cell extracts, and overexpression of Trx potentiated hypoxia-inducedinduction of a HIF-1-dependent reporter construct (38). A recent study showed that the redox regulatory

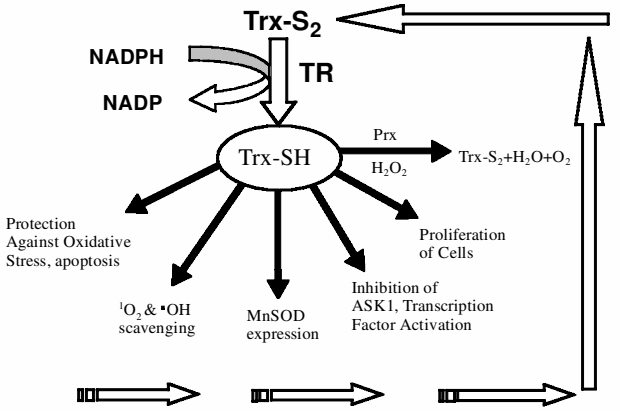

FIG. 1. A schematic presentation of the Trx redox system and its major functions. The reduced form of Trx protects against oxidative stress-induced cell injury and apoptosis. The protection against oxidative stress can be achieved directly through scavenging of reactive oxygen species, increased expression of MnSOD, or removal of peroxides through the action of Prx. In addition, reduced Trx can also inhibit ASK1, resulting in inhibition of apoptosis. Trx can also influence the repair or regeneration of injured tissue by increasing cell proliferation. All of these functions of Trx may be redox-dependent and are expected to generate the oxidized form of Trx that can be reduced at the expense of NADPH.

and antiapoptotic functions of Trx depend on its *S*-nitrosylation at Cys69 (32). Despite these new findings, the role of Trx in growth, signal transduction, and gene expression is only beginning to emerge.

TRX REDUCTASE

Trx reductase (TR) (EC 1.6.4.5) is member of the pyridine nucleotide-disulfide oxidoreductase family that includes glutathione reductase, lipoamide dehydrogenase, mercuric reductase, and NADH peroxidase (46, 71, 82, 83). Members of this family are homodimeric proteins, in which each subunit has a redox-active disulfide bond and a tightly bound FAD group that mediates the transfer of reducing equivalents from NADPH to its own disulfide bond, and then to the disulfide bond of the substrate (83). Physiological substrates for TR include Trx and protein disulfide isomerase (48). TR has diverse functions in the cell. Among these, it provides reducing equivalents through Trx for ribonucleotide reductase, which is the first unique step in DNA synthesis.

PEROXIREDOXINS

Peroxiredoxin (Prx) is a 25-kDa enzyme, initially identified in yeast, that reduces H_2O_2 by transferring electrons from Trx (8). In yeast, Prx exists as a homodimer and contains two essential cysteine residues, Cys47 and Cys170, in each subunit (8). The Cys47-SH group is the primary site of oxidation by H_2O_2, and upon oxidation, Cys47 rapidly reacts with the Cys170-SH group on the other subunit to form an intermolecular disulfide bond. This disulfide bond is subsequently reduced by Trx.

Mammalian Prx can be divided into six distinct groups, designated as Prx1 through Prx6, on the basis of their amino acid sequence and immunological properties (23, 40, 56, 72). Whereas Prx1–4 require Trx to act as an electron donor, Prx5 and Prx6 can use other cellular reductants, such as glutathione, as their electron donors (23, 72). Prx1–4 have two conserved cysteines involved in peroxidase activity and share a high level of amino acid homology. In contrast, the peroxidase activity of Prx5 and Prx6 requires only a single cysteine residue (40, 41, 47). Recombinant proteins from each group are able to reduce peroxides with the use of electrons from Trx. Until recently, catalase and glutathione peroxidase have been viewed as the major enzymes responsible for removing cytotoxic H_2O_2. However, Prx has recently been shown to play a role in the removal of H_2O_2 (40).

THE MITOCHONDRIAL TRX SYSTEM

The mitochondrial Trx system, designated as Trx-2, includes Trx-2, mitochondrial Trx reductase-2 (Trx-R2), and Prx3. Trx-2, Trx-R2, and Prx3 each are encoded by the nuclear genome and contain a mitochondrial localization leader sequence (52). The mitochondrial Trx-2 system is ubiquitously expressed, and

the reader may wish to refer to the more extensive review of Miranda-Vizuette *et al.* for details regarding this system (52). Recent studies have demonstrated that overexpression of Trx-2 *in vitro* can protect cells against oxidative stress-induced apoptosis (13, 78). Moreover, cells deficient in Trx-2 exhibit increased cellular reactive oxygen species (ROS) and apoptosis (78). A recent report has shown that mouse embryos homozygous for deletion of the Trx-2 gene die due to massive apoptosis in the neural tube (54). This study is the first report demonstrating that Trx-2 plays a critical role in embryonic development. However, the specific function of Trx-2 and Prx3 in embryogenesis and fetal development has not yet been delineated.

ROLE OF TRX IN IMPLANTATION AND EMBRYOGENESIS

Trx and TR are present in the cytosol and mitochondria of both early and term placenta (20, 21). In addition, the mitochondrial soluble fraction in early placenta was more highly enriched in TR and Trx protein than in term placenta (21). The tissue distribution of Trx in placenta has been described by Perkins *et al.* (59). TR is localized in cytotrophoblasts, deciduas, and stromatal cells in the stem villi (21). These studies suggest that the Trx-TR system in placenta may protect against oxidative stress in mitochondria. Although the Trx-TR system has been localized in the placenta, the redox status of Trx in early versus term placenta has not yet been established. A differential distribution of the various redox forms of Trx may be related to the oxidative load in fetal life at various stages of development. In addition, analysis of the redox state of Trx would provide clues as to how Trx-mediated redox signaling may affect embryogenesis.

Attempts to generate knockout mice by targeted disruption of the Trx gene resulted in early embryonic lethality, demonstrating the importance of Trx in embryonic development (49). This study also showed that Trx plays a role in preimplantation; the inner cell mass of homozygous mutant embryos failed to proliferate, and the embryos died shortly after implantation (49). Culturing mouse embryos in the presence of Trx increased the frequency of embryos that developed into blastocysts (26, 27, 44). Interestingly, these effects were observed only from the pronuclear stage to the two-cell stage. Trx or Trx-like molecules have been identified as so-called "early pregnancy factors" and are present for at least the first two-thirds of pregnancy (10, 76). Thus, it is clear that Trx plays a role in mouse embryogenesis, and this can be demonstrated indirectly by measuring the frequency of blastocyst development. Furthermore, Trx has also been shown to enhance the development of bovine embryos to the blastocyst stage, although the effect was observed exclusively in embryos during the 24–44-h culture period after insemination (3). These studies indicate an important role of Trx in implantation and embryogenesis, which clearly requires cell proliferation. However, although the role of Trx has been clearly shown in embryonic development to the blastocyst stage, the mechanisms by which this occurs are as yet unknown. Thus, understanding how the redox state of Trx may modulate embryogenesis awaits further investigation.

DEVELOPMENTAL REGULATION OF THE TRX SYSTEM

The expression of Trx has been investigated in embryonic and fetal mice by immunohistochemical localization (42, 43). Trx expression begins at embryonic day 11 (E11) or E13 and appears to increase later during development in most of the tissues examined (42). However, in another study, E8.5 embryos were also found to be positive for Trx (43). Trx exhibited a heterogeneous localization in normal human placenta, suggesting that its expression may reflect the variable functional states of the cell (21).

We have investigated the developmental regulation of the Trx system in a primate model, the fetal baboon. Our findings showed that there was no change in the expression of Trx or TR mRNA in the fetal baboon lung during the final third of gestation (18). Thus, there was no evidence for developmental regulation of Trx or TR gene expression in the low oxygen tension of the late gestation fetal environment. A significant increase in Trx, but not TR, mRNA in the lung occurred by day 2 of life in the air-breathing term newborn baboons, as compared with that measured in late gestation (175 day) fetal control lungs (18). To determine whether Prx1 is developmentally regulated in baboons, we compared the Prx1 mRNA levels in the lungs of fetal baboons at 125, 140, 160, or 175 days of gestation by northern blotting (19). There was no significant change in the expression of Prx1 mRNA in fetal baboon lung during the various gestational ages, unlike other antioxidant enzymes, which are altered during the final third of gestation (24, 39, 73, 79). We also measured Prx1 levels in baboon lung at term birth. There was a significant increase in Prx1 mRNA at day 2 following term birth. However, at day 3, Prx1 mRNA levels were back to the levels observed at 175 days, suggesting that sudden exposure to ambient oxygen only transiently increased Prx1 mRNA expression. In contrast, Prx2 mRNA expression was not significantly altered in response to term birth (19). These studies indicate that although the Trx system is an efficient system for removal of toxic oxygen radicals, it does not follow a pattern of developmental regulation similar to that exhibited by other antioxidant enzymes. Thus, these studies and others in mouse and bovine embryos suggest that although Trx plays a critical role in embryonic implantation and development, its role as an antioxidant in aerobic adaptation to newborn life differs from that of other antioxidant enzymes.

REDOX STATE OF TRX IN DEVELOPING EMBRYO AND NEONATAL LIFE

Although the Trx system has been shown to be localized in the placenta and in various stages of development, the redox state of Trx is unknown. As the *in utero* environment is highly hypoxic (>3% oxygen), it will be of considerable interest to examine the redox state of Trx. The production of ROS is essential for normal embryogenesis in mice, and an increase in ROS in specific cell types mediates apoptosis, which is essential for cell elimination during morphogenesis. Therefore,

it could be hypothesized that the redox state of Trx should be modulated during embryogenesis. This modulation may induce distinct signaling events leading to fetal development and "turning on" or "turning off" a specific set of genes. These studies are essential to understanding how Trx redox modulation may contribute to fetal development.

As the physiological effects of Trx are mediated predominantly by its reduced form, we determined the redox status of Trx in respiratory distress, as well as in lung explant cultures exposed to various levels of oxygen tension. Human Trx contains five cysteine residues that can exist in different states of oxidation, including oxidized monomers or homodimers (25). Oxygen exposure increased the relative proportion of oxidized-to-reduced Trx levels in premature infant baboons exposed to PRN (pro re nata, or as occasion requires) or 100% oxygen. Increased oxidized Trx levels were also observed in lung explants exposed to 95% oxygen (18). Fetal baboon lung showed a comparable distribution of oxidized and reduced Trx. However, after 6 days of PRN oxygen *in vivo*, most of the total Trx was found to be in the oxidized state. Newborn baboon lung exposed to 95% oxygen *in vivo* expressed increased levels of Trx protein, and most of the Trx present was in the oxidized state (18). Similar results were obtained in 140-day lung explant cultures. Explants exposed to 1% oxygen showed a similar distribution of Trx as did *in vivo* fetal baboon lungs, whereas 95% oxygen shifted the Trx redox balance toward the oxidized state. The relative proportion of reduced Trx is considerably greater in fetal lung tissue and in fetal lung explant tissue incubated in 1% oxygen, as compared with what is observed at higher oxygen tensions *in vivo* or *in vitro* (18). The oxidized dimeric form of Trx was detectable at the highest oxygen tensions both *in vivo* and *in vitro*. The Trx active site is blocked by dimer formation, and the dimer is not a substrate for TR. These studies indicate that the Trx redox state could play an important role in regulating the pathogenesis of oxidant-related diseases in the premature infant or in newborns. However, it remains to be determined whether the Trx redox state is modulated in various pathological conditions of newborns.

TRX SYSTEM AND DISEASES OF PREMATURE AND NEWBORNS

To characterize further the role of Trx in implantation and embryogenesis, we have recently examined the expression and activities of Trx, TR, and Prx in premature newborn primates exposed to high levels of inspired oxygen with mechanical ventilation in a bronchopulmonary dysplasia (BPD) model (18, 19). Not only were Trx and TR mRNA levels elevated in response to oxygen *in vivo*, but Trx protein and both Trx and TR enzymatic activities were also increased in the lungs of newborn baboons exposed to oxygen. At 6 days after birth in 140-day gestation animals, the increase in both mRNA and enzyme activity occurred to a comparable extent in animals that were exposed to 100% oxygen and were therefore destined to develop BPD (11, 12), and in those exposed only to PRN oxygen who do not acquire chronic lung disease. In newborns exposed to 100% oxygen, Trx and TR mRNA levels remained elevated even after 10 days, whereas Trx mRNA expression declined in animals exposed to PRN oxygen as the inspired oxygen tension was weaned (18). Although only a small number of newborns were available for our study at this time point, newborn baboons exposed to 100% or PRN oxygen for only 24 h also exhibited increased levels of Trx and TR mRNA (18).

Unlike Trx mRNA, which was consistently up-regulated, Trx enzyme activity exhibited a spectrum of activity in response to hyperoxia *in vitro*, as determined from fetal lung explants cultured under different oxygen concentrations. Specifically, lung tissue from the most premature baboon fetus studied (125 days, which is approximately equivalent to a 24–26-week human fetus) did not exhibit increased Trx protein in response to 95% oxygen over this interval. Lung tissue from animals at 140 days of gestation tended to exhibit increased levels of Trx protein, although this did not achieve statistical significance. In contrast, explants from animals approaching term gestation (175 days) showed substantially elevated Trx activity in response to hyperoxia. In addition to enabling us to evaluate the effects of acute exposures, the explant system allows us to isolate the effect of oxygen tension from other factors, such as bacterial or fungal colonization or infection, inflammation, exogenous cytokines, and barotrauma, which may be present in the intubated, artificially ventilated newborn. Data obtained from newborns *in vivo* after 6 days of exposure to 100% or PRN oxygen indicated that expression of Trx activity in response to elevated oxygen tension was not impaired in either group of 140-day animals (18). However, the various postnatal factors listed above could have contributed to elevation of Trx expression *in vivo* at any of these gestations. Considerable caution is necessary when attempting to extrapolate *in vitro* findings to the *in vivo* situation. Nevertheless, based on the data from the explant system, it appears that the regulation of Trx protein in response to hyperoxia could be delayed in the lungs of the most premature newborns.

We also evaluated the expression and activities of Prx1 and Prx2 in the baboon model of BPD (19). Our findings suggest that the gene for Prx1, an important component of the Trx system, is expressed at low levels in the prenatal period. In contrast, its expression is increased postnatally, primarily by ambient oxygen tension. This pattern of expression differs considerably from the pattern of regulation of a number of classical antioxidant enzymes (24, 39, 73, 77), although it is clear that oxygen can modulate the expression of some of these in the newborn (39). At 6 days after birth in 140-day gestation baboons, the increase in Prx1 mRNA levels occurred to a comparable extent in animals exposed to 100% oxygen, who were destined to develop BPD (11, 12), and in those exposed only to PRN oxygen who do not acquire chronic lung disease. In newborns exposed to 100% oxygen, elevation of lung Prx1 mRNA persisted even after 10 days. Furthermore, Prx1 mRNA expression declined in animals exposed to PRN oxygen as the inspired oxygen tension was weaned. Although a smaller number of newborns were studied at this time point, newborn baboons exposed to 100% or PRN oxygen for only 24 h also exhibited strong increases in Prx1 mRNA (19).

Unlike Prx1 mRNA, there was no increase in Prx enzyme activity in response to hyperoxia after 48 h in lung tissue derived from animals at 140 days of gestation. In contrast, there was a 40% *decrease* in Prx activity after 48 h of exposure to hyperoxia in lung explant cultures. One potential mechanism

for this decline in enzyme activity is that Cys47 of Prx1 could react with H_2O_2, causing its oxidation during prolonged exposure to hyperoxia. The regeneration of this sulfhydryl group may not occur in hyperoxia, due to the oxidation of Trx itself, which normally regenerates the Cys47 of Prx1 (8). Data obtained from 140-day premature newborns *in vivo* after 6 days of exposure to hyperoxia indicated that Prx1 activity had still not increased in response to elevated oxygen tension. However, 140-day premature infant baboons exposed to PRN oxygen exhibited a 2.6-fold increase in Prx activity. Hence, this increase in Prx activity may offer protection against oxygen-mediated injury in PRN animals. On the other hand, the failure of animals exposed to 100% oxygen significantly increase lung Trx activity that may contribute to the subsequent development of lung injury.

Elevated expression of Trx and TR in response to oxygen could be beneficial through (a) direct and indirect antioxidant effects of Trx or TR (4, 5, 22, 35, 53, 60), (b) supply of reducing equivalents to Trx-dependent peroxidases, and/or activation of MnSOD gene expression (16). Perhaps more importantly, the Trx system assures the maintenance of reduced thiol groups that are essential for activity of numerous enzymes. Among these, the endothelial cell NO synthase (57, 58), which is upregulated at least in part by oxygen at birth (6), plays a key role in pulmonary vasodilatation during the perinatal transition (1). In addition, the Trx system can reactivate sulfhydryl-containing enzymes that are inactivated by oxidative stress (7, 36, 37), such as the glycolytic enzyme GAPDH (22, 53). A possible effect of alteration of Trx redox state on cellular metabolism that could result in the pathogenesis of BPD is shown in Fig. 2.

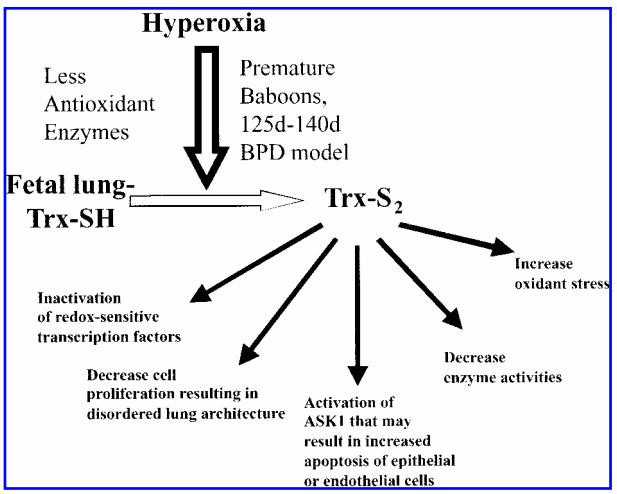

FIG. 2. A possible role of Trx in pathogenesis of BPD. Premature infant baboons ventilated with higher oxygen concentration (50–100%) develop BPD. Ventilation with hyperoxia results in oxidation of the total pool of Trx. Additionally, due to less antioxidant enzymes in fetal premature baboons, the oxidation of Trx could occur in a more accelerated manner. Oxidation of Trx could have deleterious effects such as inactivation of transcription factors, resulting in decreased expression of important genes required for antioxidant defense or repair of oxidatively injured tissue. Less availability of reduced Trx could also result in decreased cell proliferation that can directly affect the repair process. The activation of ASK1 could also induce apoptosis of various cells. The other effects include decreased activities of SH-containing enzymes affecting energy metabolism and increased oxidative stress due to inefficient peroxide removal by Prxs.

ROLE OF TRX IN PREECLAMPSIA

Preeclampsia is a disorder of human pregnancy and a leading cause of premature delivery and fetal growth retardation (64). Preeclampsia is characterized by hypertension, reduced uteroplacental blood flow, and proteinuria, and is considered to be a major health problem in human pregnancy (69). It has been suggested that preeclampsia is an endothelial cell disorder, as significant evidence indicates that most clinical aspects of the disease can be explained by endothelial cell dysfunction in different vascular beds (45, 51, 66, 80). Oxidative stress has been implicated in preeclampsia (65). Circulating markers for oxidative stress are generally increased in both normal pregnancies and preeclampsia (28–30). Some markers, such as lipid peroxides in serum and placenta, are selectively increased in preeclampsia, as compared with normal pregnancy (29). Recent studies have indicated lower levels of Trx and glutaredoxin in conditions of preeclampsia. Although this finding supports the hypothesis of Stark (75), a causal relationship has not yet been established.

CONCLUSION

Although the functional role of Trx in antioxidant defense, cell proliferation, and gene expression has been intensely investigated, its role in premature birth and newborn biology has not been adequately examined. Studies on the role of Trx on preimplantation, implantation, and fetal development suggest that Trx is an important protein in developmental biology. Furthermore, the emerging role of redox signaling and the oxidant environment in embryonic development suggest a likely role for redox signaling in embryogenesis. Recent studies of mitochondrial Trx also suggest that this protein plays an important role in embryonic apoptosis, which is immensely important in development. Thus, further studies are clearly required in these directions. The fetal Trx redox balance would be expected to be modulated at birth, and the fetal–neonatal transition could mediate a redox-signaling event leading to expression of genes that are essential for aerobic life. Such studies are expected to be highly complex; nevertheless, they would provide a fundamental understanding of redox processes in the etiology of premature newborn diseases.

ABBREVIATIONS

ASK1, apoptosis signal regulating kinase 1; BPD, bronchopulmonary dysplasia; E, embryonic day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF, hypoxia inducible factor; H_2O_2, hydrogen peroxide; MnSOD, manganese superoxide dismutase; NO, nitric oxide; PRN, pro re nata (as occa-

sion requires); Prx, peroxiredoxin; ROS, reactive oxygen species; TR, thioredoxin reductase; Trx, thioredoxin; Trx-R2, thioredoxin reductase-2.

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