Commentary

Thioredoxins, Mitochondria, and Hypertension

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Endothelial dysfunction, often demonstrated by the loss of the endothelial cell's ability to cause vasodilation in response to appropriate stimuli, is one of the earliest events in the development of atherosclerosis. This has led to intense investigation of the factors affecting both the production and the degradation of NO, the endothelium-derived relaxing factor and a primary mediator of endothelial function. Reactive oxygen species (ROS), particularly superoxide anion, are well known to inhibit NO, and therefore the mechanisms by which endothelium regulates production of ROS are also of high interest. In this issue of *The American Journal of Pathology*, Zhang et al¹ demonstrate regulation of such events by a mitochondria-specific thioredoxin, which reduces oxidative stress and increases NO bioavailability, thus preserving vascular endothelial cell function and preventing atherosclerosis development. (*Am J Pathol* 2007, 170:805–808; DOI: 10.2353/ajpath.2007.061243)

Regulation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species in Endothelial Function

It has long been described that superoxide rapidly reacts with NO to inactivate it—in fact, this was a major clue to the original identification of endothelial-derived relaxation factor as NO.² Superoxide reacts directly with NO with high affinity to form the reactive nitrogen species peroxynitrate. In addition to inactivating NO, ROS have a host of potentially deleterious effects, and antioxidants are widely touted as having some potential for heart disease prevention. However, initial results from clinical trials of antioxidants have been disappointing, suggesting that we have insufficient understanding of the specific roles of ROS and antioxidants to design effective therapies.

Although oxidative stress is broadly considered to be pathogenic, ROS are not always unwanted by-products. Cells actively regulate ROS with both antioxidant and pro-oxidant enzyme systems, and in fact, production of

superoxide by endothelial cells is an essential part of the signaling that leads to vasoconstriction. The superoxide-producing NADPH oxidases were once thought to function solely as cell-killing engines in phagocytes, but they are also present in endothelial cells.³ Mice lacking the NADPH oxidase subunit p47 no longer generate endothelial superoxide in response to angiotensin II and have a blunted hypertensive response to angiotensin *in vivo*.⁴

Although NADP(H) oxidases have been established as an important pathway for ROS generation in response to angiotensin, the p47-null mouse does not show any differences in resting blood pressure or in progression of atherosclerosis on the background of the highly atherogenic ApoE-deficient mouse.⁵ Perhaps dysregulation of other pathways is important for the development of dysfunction.

Thioredoxins: Beyond Scavenging?

Thioredoxin proteins are classically defined by their ability to reduce disulfides (an S—S bond) to dithiols (two —SH groups), and in the process the thioredoxins are oxidized from a dithiol to a disulfide. The thioredoxin disulfide is then cycled back to its active form by the enzyme thioredoxin reductase, using NADPH as the electron donor. Thioredoxin's properties derive from the special features of two neighboring cysteine residues in its active site that are particularly prone to giving up electrons. These active-site cysteines are arranged in the sequence motif Cys-Pro-Gly-Cys (or more generally, the CxxC motif) that is conserved in thioredoxins throughout evolution.

The role of thioredoxin proteins as an antioxidant that reduces protein thiols and various forms of ROS is widely appreciated. However, thioredoxin is not the only type of antioxidant that reduces disulfides and ROS—glutathione also performs this function. In many ways, the glutathione and thioredoxin systems are similar. Both use NADPH as the ultimate electron source, and in *Drosophila*

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melanogaster, the thioredoxin system supports glutathione reduction because the glutathione reductase gene is absent.⁶ However, glutathione levels are typically at least 100-fold higher than thioredoxin.^{7,8} If ROS scavenging were nonspecific, glutathione could be a far more effective antioxidant system than thioredoxin, based on its abundance. Yet there are several lines of evidence that these two systems operate independently. In yeast, deletion of the glutathione reductase causes a major shift in the glutathione oxidation state: the concentration of reduced glutathione is normally 60 times higher than the concentration of oxidized glutathione, and this shifts to only a threefold excess in the mutant yeast. Surprisingly, despite the oxidation of glutathione in the absence of glutathione reductase, there is no compensatory change in the oxidation state of thioredoxin.⁹ Despite their similarities, then, the glutathione and thioredoxin systems can operate independently, potentially serving separate roles within the cell. Defining the roles specific to each system is likely to provide key advances in our understanding of antioxidant functions.

In addition to differential regulation of the intracellular redox state, another difference between glutathione and thioredoxin is that they can play specific signaling roles. Plant metabolism provides a clear example of how thioredoxin participates in signaling: chloroplast thioredoxins coordinate an entire network of enzymes involved in photosynthesis. Energy from light causes reduction of ferredoxin, which in turn reduces thioredoxin. Reduced thioredoxin then modifies the activity of the enzymes of the photosynthetic machinery by reducing regulatory disulfides. The importance of thioredoxins as signaling molecules in plants is perhaps reflected in the number of thioredoxin proteins: *Arabidopsis* has 19 of them.¹⁰ If thioredoxins were simply a nonspecific scavenger of ROS, there would be little need for multiple thioredoxins.

In mammals, the thioredoxin story initially seemed less complicated because only one form of thioredoxin (Trx1) was known for some time. However, research has since revealed at least seven human proteins containing a thioredoxin-fold structure with the CPGC motif, although it is not clear that all of these proteins are active thioredoxins. In addition, multiple roles for thioredoxin have now been identified, from reduction of 2-Cys-peroxiredoxins¹¹ to regulation of binding partners such as ASK1, PTEN, and Txnip.¹²

Mitochondrial Thioredoxin: A Distinct System for Mitochondrial Redox Regulation?

Mammals have an entirely independent set of thioredoxin-coupled enzymes in mitochondria. Mitochondria contain unique versions of thioredoxin (Trx2), thioredoxin reductase (TR2), and peroxiredoxin (a peroxidase that is recycled by thioredoxin-2). Mitochondria are a major source of superoxide as a result of electron leakage from the electron transfer chain. This has led many to hypothesize that the extra antioxidant systems are needed to protect the mitochondria from damage by ROS. Indeed, measurements of the redox state within the mitochondria

demonstrate that they are maintained at a more highly reducing state than in the cytosol. Still, the reason for the specialized Trx2 and associated antioxidants remains unclear.

Trx2-knockout mice die as early embryos because of a massive apoptotic response just as the cells begin cellular respiration.¹³ Thus it would seem that Trx2 is required to prevent apoptosis from mitochondrial superoxide production; however, it is not yet clear whether Trx2 function is decreased as a coordinated part of the apoptotic program or whether apoptosis is simply a result of excess ROS.

Control of Endothelial Dysfunction by Trx2

Transgenic mouse models suggest that regulation of the mitochondrial redox state in particular may play an important role *in vivo*. An intriguing study by Schriener et al¹⁴ showed that mice with catalase targeted to the mitochondria exhibited extended lifespan, whereas there was no effect when catalase was targeted to the peroxisomes or to the nucleus. Another recent study has found that mice overexpressing peroxiredoxin-3, the mitochondria-specific peroxidase linked to thioredoxin-2, have improved survival after MI.¹⁵ Further *in vivo* investigation of the mitochondrial redox system is needed.

In this issue of the *AJP*, Zhang et al¹ expressly address this issue by creating a Trx2-transgenic mouse that overexpresses Trx2 specifically in the endothelium by the use of a vascular endothelial cadherin promoter.¹ Intriguingly, the authors noticed that the mice had higher NO levels and lower resting blood pressure. To investigate whether this association was causal, they performed aortic ring experiments demonstrating that the aortas of the transgenic mice have more dilation because of increased NO levels.

To determine whether the increased NO levels were caused by increased synthesis or decreased degradation by superoxide, the authors isolated endothelial cells from the mice. No increase in eNOS expression or activity was observed, but as expected, ROS levels were significantly decreased in endothelial cells from transgenic animals as measured by two redox-sensitive fluorescent dyes. One of the dyes is designed to be targeted to the mitochondria, and this suggests that superoxide levels in the mitochondria had indeed been reduced with Trx2 overexpression.

Zhang and colleagues¹ extended their findings to show that the Trx2 transgenic mice have slower progression of atherosclerosis when crossed with ApoE-null mice. This result is particularly exciting given prior studies showing that gene deletion of p47 NADPH oxidase did not affect resting blood pressure or atherosclerosis progression in ApoE-null mice. The *in vitro* and *in vivo* studies of Zhang et al¹ therefore represent a significant advance for the hypothesis that mitochondrial redox state plays an important and controlling role in endothelial dysfunction as well as in atherosclerosis.

As with most transgenic mouse models, the model of Zhang and colleagues¹ is unable to address whether a

phenotype resulting from a sustained fivefold overexpression of thioredoxin provides true insight into normal physiology. However, a recent *in vitro* study by Liang and Pietrusz¹⁶ provides further support for the hypothesis of Zhang et al.¹ They performed a complementary set of experiments using siRNA-mediated Trx2 knockdown in human umbilical vein endothelial cells and found decreased eNOS expression and decreased nitrite/nitrate accumulation associated with increased ROS.¹⁶ Because Trx2^{+/-} mice are viable,¹³ investigation of these mice is also likely to be important for understanding the physiological importance of Trx2 in vessel reactivity.

A Question of Specificity

The exciting results described by Zhang et al.¹ raise significant questions about the effects of Trx2 and its normal physiological role. Although an effect on NO and *in vivo* pathophysiology is clearly demonstrated here, how exactly does Trx2 overexpression affect NO levels? A major difficulty with the hypothesis that mitochondrial superoxide regulates NO levels is that because of its high reactivity, superoxide's short lifespan probably limits its diffusion to within the mitochondria. In contrast, endothelial NADPH oxidases can generate superoxide both within the cell and on the cell membrane.¹⁷ How does mitochondrial superoxide significantly affect global NO output? For this to be attributable to direct interaction of superoxide and NO, the levels of NO within the mitochondria would have to be quite high. On the other hand, superoxide is rapidly dismutated to hydrogen peroxide. Hydrogen peroxide, which is diffusible and membrane-permeable, could transmit a signal out of the mitochondria, but endogenous hydrogen peroxide does not seem to oppose NO—it actually may have an independent relaxant effect on vessels.¹⁸ Finally, it is also possible that the change in superoxide levels observed here is not causally related to the mechanism for Trx2's effect on NO production.¹⁹

To answer this question, the mechanism that needs to be defined is whether Trx2 overexpression has specific effects, either in location, in its effects on other antioxidant systems, or in its interactions with other proteins. Zhang et al.¹ measured an increase in overall cell redox stress as well as mitochondrial redox stress, suggesting that Trx2 overexpression in these mice was sufficient to alter nonspecifically the redox state of the entire cell. Thus, a key question for assessing the importance of Trx2 will be whether a change in redox state that is isolated to the mitochondria controls endothelial cell function.

In general, the difficulty in directly characterizing the local and/or specific effects of ROS and antioxidants has been a major limitation in understanding redox signaling. On the other hand, new techniques are emerging. For example, to address the question of whether Trx2 has specific signaling roles, Jones and colleagues²⁰ have developed a modified Western analysis technique for measuring the oxidation state of Trx2 and Trx1, demonstrating that extracellular signals can indeed affect redox state differently in separate compartments of the cell. In

response to tumor necrosis factor, Trx2 was oxidized, but Trx1 was not, in HeLa cells. In contrast, epidermal growth factor oxidizes only Trx1 in human keratinocytes.²¹ Although much remains to be defined, this series of experiments suggests that Trx2 in the mitochondria will play a physiological role in signaling pathways that are independent of cytosolic Trx1.

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