Research Perspective

The NADPH Oxidase Nox4 and Aging in the Heart

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Abstract: Oxidative stress in mitochondria is believed to promote aging. Although passive leakage of electron from the mitochondrial electron transport chain has been considered as a major source of oxidative stress in the heart and the cardiomyocytes therein, enzymes actively producing reactive oxygen species may also exist in mitochondria. We have shown recently that Nox4, a member of the NADPH oxidase family, is localized on intracellular membranes, primarily at mitochondria, in cardiomyocytes. Mitochondrial expression of Nox4 is upregulated by cardiac stress and aging in the heart, where Nox4 could become a major source of oxidative stress. This raises an intriguing possibility that Nox4 may play an important role in mediating aging of the heart. Here we discuss the potential involvement of Nox4 in mitochondrial oxidative stress and aging in the heart.

INTRODUCTION

Oxidative stress is defined as an excessive accumulation of reactive oxygen species (ROS) beyond the capacity of antioxidants [1]. ROS usually emerge as superoxide (O2•−), which is dismutated immediately to hydrogen peroxide (H2O2) by superoxide dismutase (SOD). H2O2 is further converted into water by catalase or several types of peroxidases, including glutathione peroxidases and peroxiredoxin (Prx) (thioredoxin peroxidases) [2]. However, this sequential conversion from O2•− to water is not 100% efficient. Residual O2•− acts directly as an oxidant or reacts immediately with NO to produce peroxynitrite (ONOO−), a very harmful ROS, in NO-producing cells, such as endothelial cells [3] and macrophages [4]. H2O2 is stable and diffusible, but it can also be converted to hydroxyl radical (OH•), a potent oxidant, by the Fenton reaction (Fe2+ + H2O2 → Fe3+ + OH• + OH•−) [5]. In the heart, nearly 90% of ROS in cardiomyocytes is produced by leakage of electrons from Complexes I [6] and III [7] of the mitochondrial electron transport chain (ETC) during oxidative phosphorylation, an important process that generates ATP [8]. Since electrons are by-products of energy production by the ETC, O2•− production through this mechanism does not appear to be a regulated process. Although increased oxidative stress during heart failure is also largely mediated by the leakage of electrons from the mitochondrial ETC, whether or not the leakage of electrons is really the predominant source of oxidative stress in the heart under stress conditions remains to be elucidated. Besides the leakage from the ETC, ROS can be also generated through ROS-producing enzymes [9]. In particular, the NADPH oxidase family proteins are unique enzymes which purposefully produce O2•− [1, 10, 11]. An emerging hypothesis is that Nox4, a member of the NADPH oxidase family, is localized in mitochondria and actively produces ROS under pathological conditions and during aging [12, 13]. Here we discuss the role of Nox4 in mediating oxidative...
stress in mitochondria and during aging in the heart.

**Regulation of aging and lifespan by oxidative stress in mitochondria**

Aerobic cells need antioxidants localized in mitochondria in order to overcome inevitable ROS production following energy generation. A functional decline in the antioxidants or increased production of ROS in mitochondria causes accumulation of ROS, thereby leading to mitochondrial dysfunction. Accumulation of oxidative stress in mitochondria is highly relevant to aging and the development of various aging-related common diseases, including cardiovascular diseases. This hypothesis is referred to as the “free radical theory of aging” [8]. However, the involvement of specific forms of ROS and each antioxidant and/or ROS-producing enzyme in the process of aging remains obscure. Recently, generation of mouse models in which the level of specific ROS and/or enzymes modifying the level of ROS is altered has provided us with valuable information regarding the role of ROS in mediating aging in mammalian hearts.

There are 3 forms of superoxide dismutase (SOD 1-3), enzymes dismutating O$_2^-$, in mammals. SOD2 is localized specifically in the mitochondrial matrix. Since SOD2 has manganese in its reactive center, it is referred to as MnSOD. Systemic ablation of MnSOD in mice is accompanied by dilated cardiomyopathy and neurodegeneration leading to early postnatal death [14]. Cardiomyocyte-specific deletion of MnSOD was sufficient to induce mitochondrial dysfunction and the development of dilated cardiomyopathy. In this model, mitochondrial O$_2^-$ content was increased by 40% while H$_2$O$_2$ was decreased by 70% [15]. Thus, this model would be useful for determining the role of O$_2^-$ in mediating mitochondrial dysfunction and aging in the heart. Although overexpression of MnSOD extends lifespan in *Saccharomyces cerevisiae* [16] and *Drosophila melanogaster* [17], neither overexpression nor heterozygous knockout of MnSOD affects lifespan in mice [18, 19], suggesting that mitochondrial O$_2^-$ may not directly affect lifespan in mammals. However, MnSOD overexpression slows the replicative growth rate in human cancer cells [20]. Overexpression of MnSOD also protects the heart from mitochondrial dysfunction and heart failure in a mouse model of diabetic cardiomyopathy [21]. Thus, it is possible that O$_2^-$ may accelerate the aging processes by stimulating mitochondrial dysfunction in some organs and cell types.

In contrast to the ambiguous effect of O$_2^-$ upon lifespan/aging in mammals, mitochondrial H$_2$O$_2$ appears more clearly correlated with lifespan. In transgenic mice overexpressing catalase in mitochondria, maximal lifespan was extended by 20%, and cardiac pathology and development of cataracts were significantly delayed [22]. Possible underlying mechanisms include direct beneficial effects of reduced oxidative stress in mitochondria, *i.e.* preservation of mitochondrial functions, and indirect effects upon cell signaling modulated by the redox status. Catalase is primarily localized in peroxisomes, and is present in mitochondria only at low concentrations. Thus, mitochondrially-localized peroxiredoxin (Prx3 and 5) may be more critical in H$_2$O$_2$ detoxification [2]. Although the relevance of Prx5, but not Prx3, to lifespan has been demonstrated in *Drosophila melanogaster* [23], a gain-of-function allele of peroxiredoxin (thioredoxin peroxidase, Tsa1p) reduces oxidative stress but stimulates premature aging in yeast [24]. Thus, further investigation is required to elucidate how H$_2$O$_2$ is regulated in mitochondria, as well as the role of this mechanism in aging in the heart.

**Nox4 is a major superoxide-producing enzyme localized in mitochondria in the cardiovascular system**

The NADPH oxidases are membrane-spanning proteins with NAD(P)H and FAD binding domains in their C-terminal tails that produce O$_2^-$ by transferring an electron from NADPH (or NADH) to molecular oxygen [11]. The NADPH oxidase was thought to be a phagocyte-specific enzyme and to play a critical role in mediating bacterial killing by producing a burst of O$_2^-$ [10], until its family protein Nox1 was discovered in smooth muscle cells and colonic epithelium [25]. There are now 7 known proteins in the NADPH oxidase (Nox) family, *i.e.* Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2, which, with the exception of Nox2, were all identified in this decade [1, 10, 11]. The initial cloning paper reported that Nox4 was expressed highly in the kidney [26, 27]. However, in contrast to other Nox proteins that are expressed only in specific tissues or cells, Nox4 is ubiquitously expressed at high levels, including in cardiovascular systems, such as endothelial cells [28], smooth muscle cells [29], and cardiomyocytes [12, 30]. As we discuss below, Nox4 has unique characteristics compared to the other members of the Nox family. In particular, its localization on intracellular membranes, including mitochondria, makes it an important candidate for regulating mitochondrial oxidative stress during aging in the heart.

**Mitochondrial localization of Nox4 in cardiomyocytes**

Although Nox2, a prototypical NADPH oxidase, is localized primarily on the plasma membrane, Nox4...
appears to be localized on intracellular membranes. Although the intracellular localization of Nox4 remains controversial, Nox4 appears to be localized in mitochondria in mesangial cells [12], the nucleus [31] or endoplasmic reticulum [32] in vascular endothelial cells, and the plasma membrane, especially at focal adhesions, in vascular smooth muscle cells [33]. The subcellular localizations of Nox4 may be truly cell type-dependent. However, different results may also be due to the distinct specificities of Nox4 antibodies used for analyses.

In cardiomyocytes, Nox4 significantly affects the level of oxidative stress in mitochondria [12, 34]. Nox4 has a mitochondrial localization signal-like motif in the N-terminal region, which potentially directs expression of Nox4 to mitochondria [12]. Furthermore, many redox-sensitive mitochondrial proteins, including aconitase and components of Complex I and the MPTP, are significantly more oxidized in the mouse heart overexpressing Nox4, and less in the cardiac-specific Nox4 KO heart [12,34]. Thus, Nox4 not only produces \( O_2^- \), thereby directly contributing to oxidative stress in mitochondria, but also induces oxidative damage of mitochondrial proteins and causes leakage of \( O_2^- \) from mitochondria, which triggers a response known as ROS-induced ROS release [35]. Using cardiac-specific Nox4 KO mice, we have shown recently that Nox4 is an important source of oxidative stress in mitochondria during cardiac hypertrophy and failure [34]. The results suggest that electron leakage may not be the sole source of oxidative stress in the heart and that Nox4 in mitochondria could be an active source under stress. Since expression of Nox4 is upregulated by cardiac stress, including pressure overload, heart failure and aging, upregulation of Nox4 may allow it to be a significant source of mitochondrial oxidative stress under stress conditions.

The NADPH oxidases receive electrons from either NADPH or NADH and transfer them to molecular oxygen. The proto-type NADPH oxidase, Nox2, which is localized at the plasma membrane, utilizes NADPH as an electron donor [10]. During phagocytosis, the pentose-cycle supplying NADPH in the cytosol is drastically activated in phagocytes. In contrast, Nox4 appears to utilize NADH as an electron donor to produce \( O_2^- \), at least in vitro assays [27,36,37]. Consistently, NADH produces a greater amount of ROS than NADPH in mitochondria isolated from hearts overexpressing Nox4 [12]. Since NADH is produced abundantly in the TCA cycle, Nox4 localized in mitochondria may directly utilize NADH derived from the TCA cycle to produce \( O_2^- \). Thus, an exciting hypothesis is that Nox4 is an integral component of the mitochondrial TCA cycle and directly regulates the activity of NADH-generating enzymes as a negative feedback mechanism. When the TCA cycle operates, the resultant generation of NADH may increase ROS production through Nox4, which could in turn suppress the activity of mitochondrial proteins through oxidative modification.

**Aging, lifespan, and Nox4**

The localization of Nox4 in mitochondria and its upregulation during aging support the hypothesis that Nox4 plays an important role in mediating ROS production during aging and controlling the aging process in the heart. Overexpression of Nox4 induces cellular senescence in fibroblasts [26, 27] and apoptosis in cardiomyocytes [12]. In vascular smooth muscle cells, Nox4 upregulation plays a causal role in mediating the accumulation of polyploid cells, a biomarker of aging [38]. Cardiac-specific overexpression of Nox4 in mice exacerbates aging-associated cardiac phenotypes, such as left ventricular dysfunction, apoptosis, and fibrosis, accompanied by mitochondrial oxidative stress and dysfunction [12].

If endogenous Nox4 is a major source of oxidative stress and mediates aging in the heart, it could become an ideal target for pharmacological interventions to prevent age-associated complications in the heart. It will, therefore, be important to evaluate the role of endogenous Nox4 and Nox4-derived ROS in mediating aging of the heart, using Nox4 KO mice. We expect that downregulation of Nox4 should reduce the amount of \( O_2^- \) in mitochondria, thereby inhibiting the aging process in the heart. On the other hand, since Nox4 consumes NADH as an electron donor to produce \( O_2^- \), downregulation of Nox4 could lead to accumulation of NADH, a condition opposite from that induced by caloric restriction, an established intervention attenuating aging. Thus, the overall effect of Nox4 downregulation upon aging could be more complex. Although Nox4 does not have an obvious SOD-like motif in its structure, recent evidence suggests that Nox4 may directly produce \( H_2O_2 \) rather than \( O_2^- \) [39, 40]. If this observation is true, Nox4 may affect aging through production of \( H_2O_2 \). The involvement of specific ROS and the molecular mechanism mediating stimulation of aging by Nox4 in the heart remain to be elucidated. It should be noted that the mouse heart exhibits a remarkably negligible aging phenotype in response to Nox4 overexpression in young mice [12]. Increased expression of Nox4 in the heart upregulates antioxidants, such as catalase [12], indicating that the heart has adaptive mechanisms antagonizing the action of Nox4. Elucidating these mechanisms would also provide us with valuable information regarding how to...
Effects of redox modification & Nox4 may be cell-type dependent

The effects of Nox4-derived ROS appear to be cell type-dependent. Nox4 stimulates apoptosis in endothelial cells [41] and cardiomyocytes [12], while it induces cell proliferation in smooth muscle cells [42] and cardiac fibroblasts [12]. Although the molecular mechanisms through which Nox4 exerts different effects in different cell types are currently unknown, it is possible that ROS generated by Nox4 affect distinct targets in each cell type. Interestingly, the response of the heart to pressure overload differs substantially between "cardiomyocyte-specific" [34] and "global" [30] Nox4 KO mice. Although cardiomyocytes are the major cell type in the heart, the heart also consists of other cell types, including cardiac fibroblasts, endothelial cells, and smooth muscle cells. The absence of Nox4 differentially affects each cell type and, thus, the overall response of the heart differs when Nox4 is deleted only in cardiomyocytes from when it is deleted in every cell type. By inference, the role of Nox4 in regulating aging could differ from tissue to tissue, and the role of Nox4 in regulating lifespan in the whole animal is probably complex. In this regard, the role of endogenous Nox4 in mediating aging in each organ should be addressed with both tissue-specific and systemic Nox4 KO mice.

CONCLUSION

Unlike other ROS-producing enzymes, the Nox family proteins produce O$_2^-$ and/or H$_2$O$_2$ purposefully in a regulated manner. Nox4 is localized in the peri-nuclear region, especially in mitochondria, in cardiomyocytes. Due to its close proximity to mitochondrial proteins, ROS generated by Nox4 oxidize mitochondrial proteins, which in turn trigger mitochondrial dysfunction and electron leakage. We speculate that when ROS production via Nox4 is beyond the capacity of antioxidants, ROS are accumulated in mitochondria, thereby triggering the aging process. Aging not only upregulates Nox4 but also downregulates antioxidant mechanisms in mitochondria. We speculate that, as in the failing heart, Nox4 could be an important source of mitochondrial oxidative stress in the aging heart. If Nox4 is shown to be involved in the aging process in the heart, it could be a promising target of pharmacological intervention because aging-induced cardiomyopathy remarkably enhances the patient’s risk of developing heart failure in response to many cardiac conditions, including high blood pressure, ischemia, and diabetes.

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REFERENCES

1. Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol. 2004; 4: 181-9.
2. Cox AG, Winterbourn CC, Hampton MB. Mitochondrial peroxiredoxin involvement in antioxidant defence and redox signalling. Biochem J. 425: 313-25.
3. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, et al. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci U S A. 1998; 95: 9220-5.
4. Xia Y and Zweier JL. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc Natl Acad Sci U S A. 1997; 94: 6954-8.
5. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002; 82: 47-95.
6. Ide T, Tsusui H, Kinugawa S, Utsumi K, Kang D, Hattori N, et al. Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. Circ Res. 1999; 85: 357-63.
7. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesniewsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem. 2003; 278: 36027-31.
8. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. Cell. 2005; 120: 483-95.
9. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res. 2000; 87: 840-4.
10. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev. 2007; 87: 245-313.
11. Sumimoto H. Structure, regulation and evolution of Nox family NADPH oxidases that produce reactive oxygen species. Febs J. 2008; 275: 3249-77.
12. Ago T, Kuroda J, Pain J, Fu C, Li H, Sadoshima J. Upregulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes. Circ res. 2010; 106: 1253-64.
13. Block K, Gorin Y, Abboud HE. Subcellular localization of Nox4 and regulation in diabetes. Proc Natl Acad Sci U S A. 2009; 106: 14385-90.
14. Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, et al. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nat Genet. 1995; 11: 376-81.
15. Nojiri H, Shimizu T, Funakoshi M, Yamaguchi O, Zhou H, Kawakami S, et al. Oxidative stress causes heart failure
with impaired mitochondrial respiration. J Biol Chem. 2006; 281: 33789-801.

16. Harris N, Costa V, MacLean M, Mollapour M, Moradas-Ferreira P, Piper PW. Msod overexpression extends the yeast chronological (G0) life span but acts independently of Sir2p histone deacetylase to shorten the replicative life span of dividing cells. Free Radic Biol Med. 2003; 34: 1599-606.

17. Sun J, Folk D, Bradley TJ, Tower J. Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult Drosophila melanogaster. Genetics. 2002; 161: 661-72.

18. Jang YC, Perez VI, Song W, Lustgarten MS, Salmon AB, Mele J, et al. Overexpression of Mn superoxide dismutase does not increase life span in mice. J Gerontol A Biol Sci Med Sci. 2009; 64: 1114-25.

19. Van Remmen H, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, et al. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiol Genomics. 2003; 16: 29-37.

20. Ough M, Lewis A, Zhang Y, Hinkhouse MM, Ritchie JM, Oberley LW, et al. Inhibition of cell growth by overexpression of manganese superoxide dismutase (MnSOD) in human pancreatic carcinoma. Free Radic Res. 2004; 38: 1223-33.

21. Shen X, Zheng S, Metreveli NS, Epstein PN. Protection of cardiac mitochondria by overexpression of MnSOD reduces diabetic cardiomyopathy. Diabetes. 2006; 55: 798-805.

22. Schirner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, et al. Extension of murine life span by overexpression of catalase targeted to mitochondria. Science. 2005; 308: 1909-11.

23. Radyuk SN, Michalak K, Klichko VI, Benes J, Rebrin I, Sohal RS, et al. Peroxiredoxin 5 confers protection against oxidative stress and apoptosis and also promotes longevity in Drosophila. Biochem J. 2009; 419: 437-45.

24. Timmermann B, Jarolim S, Russmayer H, Kerick M, Michel S, Kruger A, et al. A new dominant peroxiredoxin allele identified by whole-genome re-sequencing of random mutagenized yeast causes oxidant-resistance and premature aging. Aging (Albany NY). 2: 475-86.

25. Suh YA, Arnold RS, Lassgue B, Shi J, Xu X, Sorescu D, et al. Cell transformation by the superoxide-generating oxidase Mox1. Nature. 1999; 401: 79-82.

26. Geizts M, Kopp JB, Varnai P, Leto TL. Identification of renox, an NAD(P)H oxidase in kidney. Proc Natl Acad Sci U S A. 2000; 97: 8010-4.

27. Shiode A, Kuroda J, Tsuruya K, Hirai M, Hirakata H, Naito S, et al. A novel superoxide-producing NAD(P)H oxidase in kidney. J Biol Chem. 2001; 276: 1417-23.

28. Aco T, Kitazono T, Oboshi H, Iyama T, Han YH, Takada J, et al. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. Circulation. 2004; 109: 227-33.

29. Clempus RE, Sorescu D, Dikalova AE, Pounkova L, Jo P, Sorescu GP, et al. Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. Arterioscler Thromb Vasc Biol. 2007; 27: 42-8.

30. Zhang M, Brewer AC, Schroder K, Santos CX, Grieve DJ, Wang M, et al. NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis. Proc Natl Acad Sci U S A. 2010; 107: 18121-6.

31. Kuroda J, Nakagawa K, Yamasaki T, Nakamura K, Takeya R, Kuribayashi F, et al. The superoxide-producing NAD(P)H oxidase Nox4 in the nucleus of human vascular endothelial cells. Genes Cells. 2005; 10: 1139-51.

32. Van Buul JD, Fernandez-Borja M, Anthony EC, Hordijk PL. Expression and localization of NOX2 and NOX4 in primary human endothelial cells. Antioxid Redox Signal. 2005; 7: 308-17.

33. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2004; 24: 677-83.