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Differential impacts of brain stem oxidative stress and nitrosative stress on sympathetic vasomotor tone

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ABSTRACT

Based on work-done in the rostral ventrolateral medulla (RVLM), this review presents four lessons learnt from studying the differential impacts of oxidative stress and nitrosative stress on sympathetic vasomotor tone and their clinical and therapeutic implications. The first lesson is that an increase in sympathetic vasomotor tone because of augmented oxidative stress in the RVLM is responsible for the generation of neurogenic hypertension. On the other hand, a shift from oxidative stress to nitrosative stress in the RVLM underpins the succession of increase to decrease in sympathetic vasomotor tone during the progression towards brain stem death. The second lesson is that, by having different cellular sources, regulatory mechanisms on synthesis and degradation, kinetics of chemical reactions, and downstream signaling pathways, reactive oxygen species and reactive nitrogen species should not be regarded as a singular moiety. The third lesson is that well-defined differential roles of oxidative stress and nitrosative stress with distinct regulatory mechanisms in the RVLM during neurogenic hypertension and brain stem death clearly denote that they are not interchangeable phenomena with unified cellular actions. Special attention must be paid to their beneficial or detrimental roles under a specific disease or a particular time-window of that disease. The fourth lesson is that, to be successful, future antioxidant therapies against neurogenic hypertension must take into consideration the much more complicated picture than that presented in this review on the generation, maintenance, regulation or modulation of the sympathetic vasomotor tone. The identification that the progression towards brain stem death entails a shift from oxidative stress to nitrosative stress in the RVLM may open a new vista for therapeutic intervention to slow down this transition.

Contents

Abbreviations: Akt, serine/threonine protein kinase; Ang II, angiotensin II; AT_1R, angiotensin receptor subtype 1; BDNF, brain-derived neurotrophic factor; BH4, tetrahydrobiopterin; CoQ_{10}, coenzyme Q_{10}; CREB, cAMP response element binding protein; Cu/ZnSOD, cupper/zinc SOD; EPSCs, excitatory postsynaptic currents; ERK, extracellular signal-regulated kinase; ETC, electron transfer chain; GABA, γ-amino butyric acid; H_2O_2, hydrogen peroxide; HIF-1, hypoxia-inducible factor; HO-1, heme oxygenase 1; HSPs, heat shock proteins; IkB, inhibitors of NF-kB; IL, interleukin; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK kinase 1/2; MNK1/2, MAPK signal-interacting kinase 1/2; MnSOD, manganese SOD; mtNOS, mitochondrial nitric oxide synthase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS I, neuronal NOS; NOS II, inducible NOS; NOS III, endothelial NOS; Nrf2, nuclear factor erythroid 2-related factor 2; $O_2^{\bullet-}$, superoxide; $^{\bullet}OH$, hydroxyl radical; $ONOO^-$, peroxynitrite; PDK, 3′-phosphoinositide-dependent kinase; PI3K, phosphoinositide 3-kinase; PIN, protein inhibitor of nNOS; PIP_2, phosphorylating phosphatidylinositol-4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PKG, protein kinase G; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Rac1, Ras-related C3 botulinum toxin substrate 1; RNS, reactive nitrogen species; ROS, reactive oxygen species; RVLM, rostral ventrolateral medulla; sEPSCs, spontaneous EPSCs; sGC, soluble guanylyl cyclase; SHR, spontanenously hypertensive rats; SHRSP, stroke-prone SHR; SOD, superoxide dismutase; SUMO, small ubiquitin-related modifier; TFAM, mitochondrial transcription factor A; TrkB, tropomyosin receptor kinase B; UCH-L1, ubiquitin carboxyl-terminal hydrolases isozyme L1; UCPs, uncoupling proteins; UPS, ubiquitin-proteasome system.

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1. Introduction

Whereas the existence of free radicals was known in chemistry since 1900 (Gomberg, 1900) and their roles identified in biological systems in the 1950s (Commoner, Townsend, & Pake, 1954), the concept of oxidative stress was only proposed towards the end of the twentieth century as an imbalance between oxidants and antioxidants in favor of the oxidants, with the ratio of oxidants to antioxidants >1 that potentially leads to cellular damage (Sies, 1997). A parallel process is nitrosative stress, which is defined as the ratio of nitrosants to antioxidants >1 (Klandorf & Van Dyke, 2012). Based on those concepts, oxidative stress or nitrosative stress represents an imbalance between the production and elimination of reactive oxygen species (ROS) or reactive nitrogen species (RNS).

One common drawback in contemporary biomedicine is the inclination to create a stereotypic generalization on an identified physiological phenomenon or cellular mechanism and inadvertently assumes its universal applicability. A case in point is ROS and RNS. Oxidative stress and nitrosative stress are frequently depicted as the common culprit for cellular damage. In addition, the expression "ROS/RNS" has often appeared in the literature as if they represent a singular moiety, usually as the surrogate for ROS. As will be elaborated in this review using differential impacts of brain stem oxidative stress and nitrosative stress on sympathetic vasomotor tone as an illustrative example, these generalized assumptions require modification.

2. ROS and RNS are not the same moiety

It is beyond the scope of this review to expand detailed narratives on ROS and RNS, which have been the subject of many excellent reviews (Chiesa, Baidanoff, & Golombek, 2018; Daiber et al., 2017; Di Meo, Reed, Venditti, & Victor, 2016; Finkel & Holbrook, 2000; Ghimire, Altmann, Straub, & Isenberg, 2017; Kavdia, 2011; Klandorf & Van Dyke, 2012; Moldogazieva, Mokhosoev, Feldman, & Lutsenko, 2018; Novo & Parola, 2008; Radi, 2018; Shadel & Horvath, 2015; Vanhoutte, Zhao, Xu, & Leung, 2016). However, a brief account of the key components of ROS and RNS is provided below to set the stage for our elaboration on the thesis that ROS and RNS are not only not the same moiety, but possess disparate functional fates with clinical and therapeutic implications.

2.1. Reactive oxygen species

ROS are highly reactive oxygen moieties that include the hydroxyl radical ($^{\bullet}OH$) whose reactivity is so high that it reacts very close to its site of formation (Halliwell, 1987), and other species such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), which are less reactive (Di Meo et al., 2016). ROS are generated in cells (Zalba et al., 2007) mainly from the conversion of a small fraction of the inspired oxygen by electrons leaked from the mitochondrial electron transfer chain (ETC) during the generation of energy via oxidative phosphorylation (Murphy, 2009; Quinlan, Perevoshchikova, Hey-Mogensen, Orr, & Brand, 2013). Other sources of ROS include incomplete reduction of molecular oxygen through enzymatic reactions with intracellular oxidases such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase, or through cyclooxygenases, lipoxygenases or cytochrome P450 reductases. However, NADPH oxidase and mitochondrial ETC are the two major sources of ROS in the central nervous system (Chan & Chan, 2012a, 2012b; Hirooka, 2008, 2011; Kishi & Hirooka, 2012; Zimmerman & Davisson, 2004). The synthesis of ROS is balanced by their enzymatic degradation by superoxide dismutase (SOD), catalase, glutathione peroxidase, thioredoxin, glutaredoxin, peroxiredoxin, heme oxygenase or paraoxonase (He et al., 2017; Lei et al., 2016); as well as nonenzymatically by glutathione, vitamins, lipoate, urate or ubiquinone (Zadák et al., 2009). Cellular ROS homeostasis, therefore, depends on a dynamic balance between production and degradation of the free radicals.

2.2. Reactive nitrosative species

RNS include at least nitric oxide (NO), which is relatively unreactive, and its derivative peroxynitrite ($ONOO^-$), a powerful oxidant that is able to damage many biological molecules (Radi, 2013). NO is synthesized from its precursor, L-arginine, by a group of enzymes referred to as nitric oxide synthase (NOS). The four members of the NOS family are neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), endothelial NOS (eNOS or NOS III), and mitochondrial NOS (mtNOS), all of which are present in brain.

The constitutively expressed NOS I is abundantly present in neurons (Ferrari & Fior-Chadi, 2005; Lin, Taktakishvili, & Talman, 2007; Martins-Pinge, Garcia, Zoccal, Crestani, & Pinge-Filho, 2007; Qadri, Carretero, & Scicli, 1999; Talman & Dragon, 2004), and NOS III is localized within endothelial cells in brain capillaries (Touyz & Briones, 2011). NOS II, as the name denotes, is originally thought to be activated in macrophages, astrocytes, and microglia by immunological or inflammatory stimuli (Iadecola, Zhang, Xu, Casey, & Ross, 1995). Further evidence supports the expression of NOS III in astrocytes (Lin et al., 2007; Park et al., 2011) and NOS II is expressed constitutively in neurons and microglia (Chan, Wang, Wang, & Chan, 2001; Chan, Wang, & Chan, 2003; Chang, Chan, & Chan, 2003; Forstermann et al., 1995). The mtNOS is a new member of the NOS family and was identified as an isoform of NOS I present in the inner mitochondrial membrane (Elfering, Sarkela, & Giulivi, 2002). NO can be produced by mtNOS locally in the mitochondria (Giulivi, Poderoso, & Boveris, 1998), and excessive intramitochondrial NO production inhibits the activity of Complexes I and IV in the mitochondrial ETC to yield mitochondrial ROS (Ghafourifar & Cadenas, 2005).

$O_2^{\bullet-}$ reacts 3 to 6 times faster with NO to form the strong oxidant peroxynitrite than with SOD to form H_2O_2 (Beckman & Koppenol, 1996; Koppenol, Moreno, Pryor, Ischiropoulos, & Beckman, 1992). Further to the depletion of NO bioavailability, peroxynitrite itself can directly react with lipids (lipid peroxidation), thiols (oxidation of thiols), amino acid residues (nitration of tyrosine residues in protein), DNA bases (DNA strand breaks), and low-molecular weight antioxidants (Bartesaghi & Radi, 2018).

2.3. NOS uncoupling

In addition to the well-known example of peroxynitrite, NOS uncoupling is another form of ROS-RNS interaction that is of cellular consequences. Although well-established in NOS III (Montezano & Touyz, 2012), depending on substrate (L-arginine) and cofactor (tetrahydrobiopterin, BH4) availability (Montezano & Touyz, 2012; Sun, Druhan, & Zweier, 2010), all NOS isoforms can in theory be in an uncoupled state that generates $O_2^{\bullet-}$ instead of NO, resulting in oxidative stress and decreased NO bioavailability (Turrens, 2003). The most prominent cause for the catalytic activity of NOS to become functionally uncoupled to produce $O_2^{\bullet-}$ is reduction or loss of BH4, the bioavailability of which is regulated by the enzyme GTP cyclohydrolase I (Crabtree & Channon, 2011; Moens & Kass, 2006). Alternatively, upregulation of

protein inhibitor of nNOS (PIN), an 89 amino-acid protein, may underlie the uncoupling of NOS I and generation of ROS. Stability of homodimer is critical for functional NOS I activity (Moens & Kass, 2006), and PIN destabilizes the formation of enzyme homodimer (Hemmens et al., 1998; Jaffrey & Snyder, 1996) by binding to the contact region in the oxygenase domain (Ghosh & Stuehr, 1995). On increase in PIN level, the homodimer structure of NOS I is altered such that electrons normally flowing from the reductase domain to the oxygenase domain of the enzyme are diverted to molecular oxygen rather than to the substrate L-arginine (Rochette et al., 2013). Under these conditions, the catalytic activity becomes functionally uncoupled, and $O_2^{\bullet-}$ rather than NO is produced.

2.4. Misconceptions on ROS/RNS and oxidative stress and nitrosative stress

By having different cellular sources and regulations, it is obvious that using the expression "ROS/RNS" as if they represent a singular moiety is conceptually inaccurate. Likewise, the supposition that oxidative stress and nitrosative stress are interchangeable phenomena also requires modification. Using sympathetic vasomotor tone as the example, this review will proselytize the thesis that oxidative stress and nitrosative stress in brain stem in fact exert differential impacts on cardiovascular regulation that are with clinical and therapeutic implications.

3. Differential roles of brain stem ROS and RNS on sympathetic vasomotor tone

3.1. Both increase and decrease in sympathetic vasomotor tone bear clinical consequences

Results from human and animal studies uneqivocally concluded that overexcitation of the sympathetic nervous system plays a pivotal role in the pathogeneis of myraid cardiovascular diseases (Biancardi & Stern, 2016; Esler, 2010; Giannoglou et al., 2008; Grassi, Seravalle, Dell'Oro, & Mancia, 2011; Wang, Wang, Cornish, Rozanski, & Zucker, 2014). For example, sympathetic overactivation is involved in the development, staging, progression and end-organ damage of neurogenic hypertension (Malpas, 2010). In contrast, a reduction in sympathetic vasomotor tone causally underpins pathological conditions such as brain stem death (Chan, Chang, & Chan, 2005).

3.2. Rostral ventrolateral medulla is a key brain stem site in the generation of sympathetic vasomotor tone

An extensive neural network, including the brain stem, hypothalamus and cortical and limbic regions, is known to be involved in the generation, maintenance, regulation or modulation of sympathetic nerve activity (Dampney, 1994; Spyer, 1994). Since it is beyond the scope of this review to discuss this extensive neural network, we will restrict our narratives on the rostral ventrolateral medulla (RVLM), a key brain stem site that is known since the mid-1970s to be engaged in the generation of sympathetic vasomotor tone. Bilateral lesion or inhibition of the RVLM reduces arterial pressure to a level that resembles that produced by spinal cord transection (Granata, Ruggiero, Park, Joh, & Reis, 1985; Guertzenstein & Silver, 1974). On the other hand, activation of the RVLM increases arterial pressure (Ciriello, Caverson, & Polosa, 1986; Granata et al., 1985; Ross et al., 1984; Varner, Grosskreutz, Cox, & Brody, 1989). Anatomical (Minson, Chalmers, Caon, & Renaud, 1987; Ross et al., 1984) and electrophysiological (Barman & Gebber, 1985; Guyenet, Haselton, & Sun, 1989; Morrison, Milner, & Reis, 1988) results indicate that the implicated tonic sympathetic vasomotor outflow from the RVLM is mediated via direct bulbo-spinal projections to the intermediolateral column in the thoracic spinal cord. The neurotransmitters released at the spinal cord include at least epinephrine (Granata et al., 1985; Minson et al., 1987; Morrison et al., 1988; Ross et al., 1984) and glutamate (Allen, Adams, & Guyenet, 1993; Bazil & Gordon, 1991; Hong & Henry, 1992; Morrison, Callaway, Milner, & Reis, 1991).

3.3. ROS in the RVLM and basal sympathetic vasomotor tone

The first evidence which suggests that ROS signaling in the RVLM contributes to sympathetic vasomotor tone came from a study (Zanzinger & Czachurski, 2000) in which microinjection of SOD into the RVLM of normotensive anesthetized pigs decreases basal sympathetic nerve activity and blood pressure. Similarly, application of an $O_2^{\bullet-}$ scavenger, tempol, reduces basal sympathetic nerve discharges and promotes vasodepressor response (Campese et al., 2004). In addition, ROS enhances glutamatergic excitatory inputs and attenuates GABAergic inhibitory inputs to the RVLM, thereby increasing sympathoexcitatory inputs to RVLM neurons (Nishihara, Hirooka, Matsukawa, Kishi, & Sunagawa, 2012; Shinohara, Hirooka, Kishi, & Sunagawa, 2012).

3.4. RNS in the RVLM and basal sympathetic vasomotor tone

All isoforms of NOS, except mtNOS, are present in the RVLM (Chan, Wang, Wu, & Chan, 2001; Chan, Wang, Chao, & Chan, 2003; Chang et al., 2003; Hirooka et al., 2003; Martins-Pinge et al., 2007; Topel, Stanarius, & Wolf, 1998), with differential actions on sympathetic vasomotor outflow. Central to the role of NO at the RVLM in cardiovascular regulation is the intriguing finding that both NOS I and II are tonically active in this medullary site (Chan, Wang, Wang, & Chan, 2001). Whereas NO derived from NOS I in the RVLM causes sympathoexcitation via activation of glutamatergic neurotransmission, NOS II-derived NO promotes sympathoinhibition by facilitation of γ-amino butyric acid (GABA)ergic neurotransmission (Chan, Wang, & Chan, 2003; Huang, Chan, & Hsu, 2003, 2004). Under physiological conditions, the prevalence of NOS I over NOS II activity is responsible for the maintenance of sympathetic vasomotor outflow and stable arterial pressure by the endogenous NO in the RVLM (Chan, Wang, Wang, & Chan, 2001; Chan, Wang, & Chan, 2003). These results, which were obtained in anesthetized rats, were later confirmed in rats studied under conscious conditions (Martins-Pinge et al., 2007). In contrast, Mayorov (2005) reported that NOS I and II in the RVLM play little role in tonic regulation of sympathetic vasomotor outflow in conscious rabbits, possibly because of species differences. Chan, Wang, Wang, and Chan (2001); Chan, Wang, and Chan (2003) also reported that NOS III in the RVLM does not appear to contribute to basal sympathetic vasomotor tone.

4. Oxidative stress in the RVLM underpins neurogenic hypertension

4.1. Neurogenic hypertension

Neurogenic hypertension is defined as chronic elevation of the 24-h average blood pressure that is not caused by defects in peripheral organs (e.g. kidney) or blood vessels. As demonstrated in several animal models, a hallmark of neurogenic hypertension is brain oxidative stress induced by angiotensin II (Ang II). Indeed, the ROS level is increased in the RVLM of spontanenously hypertensive rats (SHR) or stroke-prone SHR (SHRSP) (Chan, Tai, Li, & Chan, 2006; Kishi et al., 2004; Kishi, Hirooka, Konno, Ogawa, & Sunagawa, 2010; Tai, Wang, Wu, & Chan, 2005; Wu et al., 2017), or in animals that received brain infusion of Ang II (Chan, Wu, Chang, Tai, & Chan, 2009; Su, Tsai, Chang, Chan, & Chan, 2016; Tsai et al., 2013). We present below (Fig. 1) the impact of oxidative stress on this brain stem site that leads to augmented sympathetic vasomotor tone and hypertension, based primarily on investigations using Ang II as the inducer of ROS, particularly $O_2^{\bullet-}$, via activation of the angiotensin receptor subtype 1 (AT_1R).

4.2. Oxidative stress in the RVLM underpins augmented sympathetic vasomotor tone in neurogenic hypertension

An augmented sympathetic vasomotor outflow from the RVLM is a major contributor to neurogenic hypertension (Dampney et al., 2003; Osborn, Fink, Sved, Toney, & Raizada, 2007). Ample evidence implicates that this sympathoexcitatory action results from oxidative stress in the RVLM via an imbalance between the production (impairment of mitochondrial bioenergetics or biogenesis and activation of NADPH oxidase) and degradation (reduction in antioxidant capacity) of ROS.

4.2.1. Impairment of mitochondrial bioenergetics

Impairment of a redox-sensitive feedforward stimulation of mitochondrial ETC activity contributes to oxidative stress in the RVLM of SHR, leading to augmented sympathetic vasomotor tone and hypertension (Chan, Wu, Chang, et al., 2009; Zimmerman & Zucker, 2009). The mitochondrial ETC activity in the RVLM of SHR, particularly the enzyme activity of Complexes I and III, as well as electron coupling capacity between Complexes I and III or II and III, is significantly reduced (Chan, Wu, Chang, et al., 2009). Overexpression of SOD or catalase in the RVLM by gene transfer reverses the mitochondrial bioenergetics dysfunctions and blunts mitochondrial ROS generation (Chan, Wu, Chang, et al., 2009). On the other hand, preservation of the mitochondrial electron transport capacity by microinjection of coenzyme Q_{10} (CoQ_{10}), a mobile mitochondrial electron carrier, into the RVLM reverses the depressed ETC activity, mitochondrial oxidative stress and hypertensive phenotypes in SHR and attenuates the pressor response of normotensive Wistar-Kyoto rats to Ang II infusion (Chan, Wu, Chang, et al., 2009). Long-term treatment with AT_1R blocker losartan prevents hypertension and improves mitochondrial ETC function and CoQ_{10} content in brain mitochondria of SHR (Sumbalová, Kucharská, & Kristek, 2010). In addition, antioxidant supplement with oral treatment of alpha-lipoic acid enhances intracellular antioxidant capacity and increases levels of mitochondrial bioenergetic enzymes in the RVLM during the development of high salt-induced hypertension (Huang, Jin, & Yu, 2017).

4.2.2. Impairment of mitochondrial biogenesis

Much less is known on the impact of mitochondrial biogenesis in ROS-associated neurogenic hypertension. A recent study (Wu, Wu, Chao, Hung, & Chan, 2016) provided the first evidence of defective mitochondrial biogenesis in the pathophysiology of brain oxidative stress-associated hypertension. These authors showed that under *E. coli* lipopolysaccharide-evoked neuroinflammation, the key signaling cascade involved in mitochondrial biogenesis, including expression of nuclear factor erythroid 2- related factor 2 (Nrf2) and mitochondrial

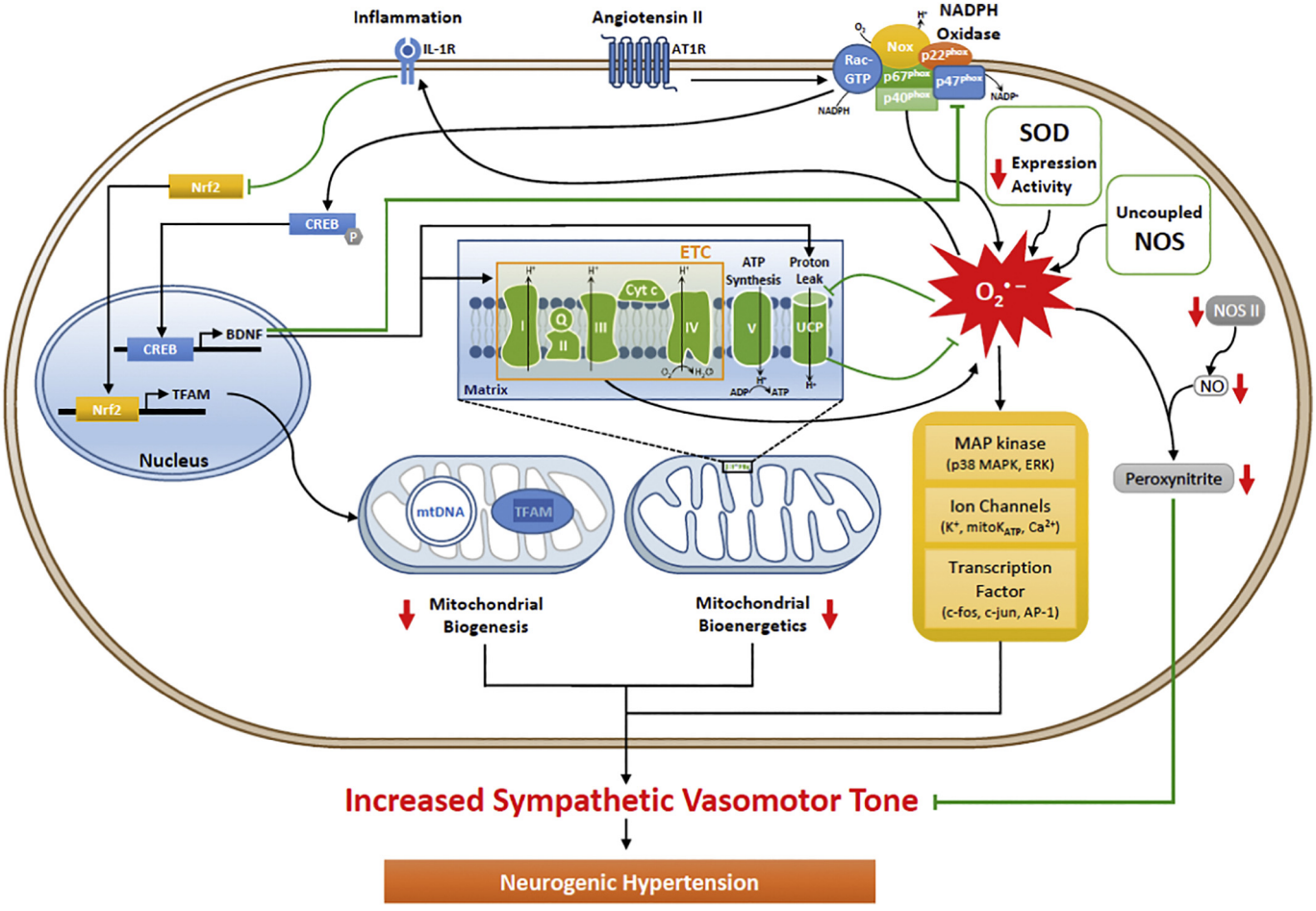

Fig. 1. Summary of key cellular events associated with oxidative stress and nitrosative stress in the RVLM during neurogenic hypertension. An increase in sympathetic vasomotor tone because of oxidative stress in the RVLM leads to neurogenic hypertension. One pivotal inducer of $O_2^{\bullet -}$ is Ang II acting on the AT1R in the RVLM. The source of the enhanced $O_2^{\bullet -}$ production includes impairment of mitochondrial bioenergetics by a reduction in the enzyme activity and electron coupling capacity of the ETC; and impairment of mitochondrial biogenesis by depressed Nrf2 and TFAM expression and reduced mitochondrial DNA copy number. Production of $O_2^{\bullet -}$ is also contributed by upregulation of the subunits and enhancement of enzyme activity of NADPH oxidase. At the same time, there is a reduction in antioxidant capacity because of reduced expression and enzyme activity of Cu/ZnSOD, MnSOD and catalase. Mechanistically, by activating various MAPK, ion channels and transcription factors and inducing inflammation, oxidative stress induces overall excitation of RVLM neurons that leads to augmented sympathetic vasomotor tone. Of interests is that a reduction in nitrosative stress because of downregulation of NOS II in the RVLM, as is uncoupling of NOS I and II that produces $O_2^{\bullet -}$, further tilts the balance in favor of oxidative stress-associated increase in sympathetic vasomotor tone in neurogenic hypertension. Transcriptional upregulation of three redox-sensitive endogenous antioxidants, UCP2, BDNF and Nrf2, provides feedback regulations of $O_2^{\bullet -}$ production in the RVLM.

transcription factor A (TFAM), as well as nuclear translocation of phosphorylated Nrf2 in RVLM neurons and binding of the activated Nrf2 to the promoter region of the *Tfam* gene, is significantly depressed, alongside reduced mitochondrial DNA copy number, augmented tissue ROS level in the RVLM, and elevated blood pressure. On the other hand, neurogenic hypertension in response to neuroinflammation is prevented on induction of mitochondrial biogenesis and alleviation of oxidative stress in the RVLM by treatment with intracisternal infusion of an interleukin (IL)-1β blocker, IL-1Ra; a mobile mitochondrial electron carrier, CoQ_{10}; or a Nrf2 inducer, tert-butylhydroquinone.

4.2.3. Activation of NADPH oxidase

In the RVLM of SHR (Chan et al., 2009; Koga et al., 2008) or SHRSP (Konno et al., 2008), both gene and protein expressions of the NADPH oxidase subunits, including $gp91^{phox}$ and $p22^{phox}$, are upregulated, along with augmented enzyme activity of NADPH oxidase in the RVLM of SHR (Hirooka, Sagara, Kishi, & Sunagawa, 2010; Kishi, Hirooka, Konno, & Sunagawa, 2010; Peterson, Shrama, & Davisson, 2006). Gene knock down of $gp91^{phox}$, $p22^{phox}$ and $p47^{phox}$ subunits in the RVLM (Chan, Wu, Wu, et al., 2009; Chan, Wu, Kung, & Chan, 2010; Hirooka, 2008; Nozoe et al., 2008) blunts the enhanced sympathetic vasomotor outflow and promotes antihypertension in SHR or SHRSP; as is treatment with apocynin to reduce the augmented NADPH oxidase activity in the RVLM (Kishi, Hirooka, Konno, & Sunagawa, 2010; Zhong et al., 2009). These results indicate that sympathetic overexcitation in neurogenic hypertension is attributable to the enhanced production of ROS generated by NADPH oxidase in the RVLM. In addition, a cross-talk between NADPH oxidase-derived $O_2^{\bullet-}$ and mitochondrial ETC enzymes (Chan, Wu, Chang, et al., 2009) that contribute to chronic oxidative stress in the RVLM of SHR has been put forth as a mechanism that underpins the sustained augmentation in sympathetic vasomotor tone and maintenance of neurogenic hypertension.

Similar findings were obtained in Ang II-dependent model of neurogenic hypertension. Ang II upregulates NADPH oxidase subunits $gp91^{phox}$, $p67^{phox}$, $p47^{phox}$, and $p40^{phox}$ in the RVLM (Chan et al., 2005; de Oliveira-Sales et al., 2009; Gao et al., 2004, 2005; Patel, Mayhan, Bidasee, & Zheng, 2011; Zucker, Schultz, Patel, Wang, & Gao, 2009), and enhances its enzymatic activity (Gao et al., 2004) through activation of Ras-related C3 botulinum toxin substrate 1 (Rac1; Nozoe et al., 2007) or phosphorylation of $p47^{phox}$ (Chan, Hsu, et al., 2005; Su et al., 2016), leading to generation of ROS in the RVLM (Chan, Hsu, et al., 2005; Chan, Wang, Tseng, & Chan, 2007; Chan, Wu, Kung, & Chan, 2010; Gao et al., 2004, 2005; Patel et al., 2011; Zimmerman et al., 2004; Su et al., 2016). Furthermore, gene knockdown with antisense (Chan, Hsu, et al., 2005; Chan, Wu, Wu, et al., 2009; Chan, Wu, Kung, & Chan, 2010) or dominant negative mutant of the NADPH oxidase subunits (Zimmerman et al., 2004), genetic deletion of components of the NADPH oxidase (Gavazzi et al., 2006; Landmesser et al., 2002; Mayorov, 2007), and inhibition of enzyme activity of NADPH oxidase (Chan, Hsu, et al., 2005) in the RVLM, markedly attenuate the Ang II-induced increase in sympathetic vasomotor tone and blood pressure (Gao et al., 2005; Hirooka, 2011; Hirooka et al., 2010; Hirooka, Kishi, Sakai, Takeshita, & Sunagawa, 2011; Oliveira-Sales et al., 2009).

4.2.4. Reduction in antioxidant capacity

The expression and enzyme activity of antioxidants, in particular cupper/zinc SOD (Cu/ZnSOD) and manganese SOD (MnSOD), are decreased in the RVLM of SHR or SHRSP (Chan et al., 2006; Hirooka, 2008; Kishi et al., 2004; Konno et al., 2008; Nozoe et al., 2007; Peterson et al., 2006). Overexpression of MnSOD in the RVLM by gene transfer reduces sympathoexcitation and hypertension in SHR or SHRSP (Chan et al., 2006; Kishi et al., 2004; Nishihara et al., 2012), and blunts the augmented cardiovascular responses elicited by microinjection of Ang II to the RVLM (Chan et al., 2006; Nozoe et al., 2008). Ang II-induced hypertension is also attenuated in transgenic mice overexpressing MnSOD (Dikalova et al., 2010). In addition, the expression of catalase in the RVLM of SHR is decreased and transfection of adenoviral vectors expressing catalase decreases the blood pressure of SHR (Chan et al., 2006); administration of catalase into the RVLM reduces the elevated ROS level in SHRSP (Kishi et al., 2004). Finally, $O_2^{\bullet-}$- and H_2O_2-dependent feedforward impairment of mitochondrial ETC complexes because of predisposed reduction in synthesis or activity of SOD or catalase (Chan, Wu, Chang, et al., 2009) has been proposed to underlie chronic oxidative stress in the RVLM of SHR that sustains the augmented sympathetic vasomotor tone in neurogenic hypertension.

4.3. Oxidative stress-associated signaling pathways in the RVLM that mediate the augmented sympathetic vasomotor tone in neurogenic hypertension

There are indications that cellular signals associated with augmented sympathetic vasomotor tone generated by oxidative stress in the RVLM at least include mitogen-activated protein kinase (MAPK), ion channels, transcription factors and inflammation.

4.3.1. Mitogen-activated protein kinase

As a major class of redox-regulated signaling molecules, the activities of the MAPK family in the RVLM, in particular p38 MAPK and extracellular signal-regulated kinase (ERK), are potentiated by Ang II via an AT_1R-depedent signaling cascade that includes sequential activation of protein kinase C, NADPH oxidase and ROS production (Chan, Hsu, et al., 2005; Chan et al., 2007). The AT_1R-ROS-p38 MAPK/ERK pathway in the RVLM is also activated in SHRSP (Kishi, Hirooka, Konno, Ogawa, & Sunagawa, 2010). Of interest is that whereas redox activation of p38 MAPK signaling mediates the short-term pressor response to Ang II by enhancing glutamatergic neurotransmission in the RVLM (Chan, Hsu, et al., 2005), $O_2^{\bullet-}$-dependent ERK phosphorylation leads to long-term pressor response to Ang II via transcriptional upregulation of AT_1R mRNA expression (Chan et al., 2007). Furthermore, AT_1R-activated caspase-3 is downstream to p38 MAPK/ERK pathway in the RVLM and is involved in sympathoexcitation in SHRSP (Kishi, Hirooka, Konno, Ogawa, & Sunagawa, 2010). In addition to MAPK signaling, an overactivation of the phosphoinositide 3-kinase (PI3K)/serine/threonine protein kinase (Akt) signaling because of ROS-dependent inactivation of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Wu et al., 2013) in the RVLM is involved in the manifestation of sympathoexcitation and hypertension (Veerasingham, Yamazato, Berecek, Wyss, & Raizada, 2005) in SHR.

4.3.2. Ion channels

Several classes of ion channels in the RVLM have been linked to Ang II-induced oxidative stress and the associated augmented sympathetic vasomotor tone. Ang II increases RVLM neuronal discharges by increasing voltage-gated Ca^{2+} current (Wang et al., 2004; Zimmerman, Sharma, & Davisson, 2005) or inhibiting K^+ current, in particular the delayed rectifier K^+ current (Sun, Sumners, & Raizada, 2002; Sun et al., 2009). Blockade of voltage-gated K^+ channels in the RVLM elicits sympathoexcitation and hypertension (Gao et al., 2010); a mitochondrial ATP-sensitive K^+ channel blocker reverses Ang II-induced ROS production (Rodriguez-Pallares, Parga, Joglar, Guerra, & Labandeira-Garcia, 2011). Interestingly, several calcium channel blockers have been shown to promote sympathoinhibition via a reduction in brain stem oxidative stress. For example, oral intake of amlodipine (Hirooka et al., 2006) or azalnidipine (Konno et al., 2008) reduces oxidative stress in the RVLM by inhibiting NADPH oxidase activity and activating MnSOD activity, resulting in sympathoinhibition in SHRSP. Whether ROS modulates channel activity by direct oxidation of amino acid residues in channel proteins or indirect modulation of protein kinases that control gating properties of the channels remains to be confirmed.

4.3.3. Transcription factor

Redox-sensitive upregulation of transcription factors, including activator protein-1 (AP-1), c-*jun* or c-*fos*, is observed in the RVLM of animals with hypertension (Chan et al., 2007; Gao et al., 2005), alongside an increase in DNA binding activity of AP-1 (Zucker et al., 2009). Of note is that, in the RVLM, transcriptional upregulation of AT_1R mRNA expression because of activation of c-*fos* by Ang II-NADPH oxidase-dependent ERK phosphorylation leads to long-term pressor response to Ang II (Chan et al., 2007).

4.3.4. Inflammation

Activation of brain stem inflammatory system, including activation of perivascular macrophages (Yu et al., 2010), production of microglial cytokines (Shi et al., 2010; Zubcevic, Waki, Raizada, & Paton, 2011), and upregulation of proinflammatory nuclear factor-κB (NF-κB) (Cardinale, Sriramula, Mariappan, Agarwal, & Francis, 2012; Zubcevic et al., 2011), as well as altered T-cell function (Zubcevic et al., 2011), has been postulated to be additional contributors to ROS-associated neurogenic hypertension, although the underlying mechanisms await further delineation. For example, chronic Ang II infusion increases the number of activated microglial cells, and upregulates the expression of proinflammatory cytokines, including IL-1β, IL-6, and tumor necrosis factor-α in the RVLM (Shi et al., 2010). These responses are accompanied by an increase in tissue level of ROS, and are alleviated by blockade of proinflammatory cytokines (Wu, Chan, & Chan, 2012). Of note is that infusion of cyclooxygenase-2 inhibitor to the cisterna magna reduces the oxidative stress-associated increase in sympathetic vasomotor activity and neurogenic hypertension.

4.4. Reduced nitrosative stress in the RVLM further favors augmented sympathetic vasomotor tone in neurogenic hypertension

Under physiological conditions, the prevalence of NOS I over NOS II activity is responsible for the maintenance of sympathetic vasomotor outflow and stable arterial pressure by the endogenous NO in the RVLM. It is therefore reasonable to speculate that a shift of this equilibrium towards reduction in the synthesis and activity of NOS II (Chan, Wang, Wu, & Chan, 2001; Chan, Wang, Chao, & Chan, 2003) or heightened expression of NOS I (Edwards et al., 2004) in the RVLM will result in augmented sympathetic vasomotor tone exhibited during neurogenic hypertension. However, verification of this speculation has met with difficulties because in the RVLM of SHR with established hypertension, NOS I has been reported to remain unchanged (Chan, Wang, Wu, & Chan, 2001) or upregulated (Edwards et al., 2004; Plochocka-Zulinska & Krukoff, 1997), and transcription of NOS II was found to be downregulated (Chan, Wang, Wu, & Chan, 2001; Chan, Wang, Chao, & Chan, 2003) or upregulated (de Oliveira-Sales et al., 2010; Kimura et al., 2009).

In two studies (Chan, Wang, Wu, & Chan, 2001; Chan, Wang, Chao, & Chan, 2003) designed to settle this issue, genomic and phenotypic documentations are presented to substantiate the notion that it is the innate abnormality in molecular synthesis and functional expression of NOS II at the RVLM the underlies the augmented sympathetic vasomotor tone seen during hypertension. Specifically, significant reduction in basal and lipopolysaccharide- induced NOS II mRNA and protein expression is shown to be already present in young, prehypertensive SHR, and that this trend persists after normalizing the arterial pressure of adult SHR to the level of the control Wistar-Kyoto rats. Also demonstrated is the tonically active sympathoinhibitory action of endogenous NOS II in the RVLM is reduced in the SHR. In contrast, measurements in all parallel experiments revealed unaltered molecular synthesis and functional expression of NOS I at the RVLM. It follows that a reduction in nitrosative stress primarily because of downregulation of NOS II in the RVLM further tilts the balance in favor of oxidative stress-induced augmented sympathetic vasomotor tone in neurogenic hypertension.

4.5. NOS uncoupling contributes to oxidative stress in the RVLM

There is now extensive evidence demonstrating that uncoupling of NOS, in particular NOS III, decreases NO bioavailability, leading to endothelial dysfunction and increase in vascular ROS production in a variety of peripheral cardiovascular diseases (Montezano & Touyz, 2012). However, relatively few studies report on the role of NOS uncoupling in the generation of brain stem ROS and its implication to neurogenic hypertension. Kimura et al. (2005) showed that overexpression of NOS II in the RVLM elicits sympathoexcitation via an increase in oxidative stress in normotensive Wistar-Kyoto rats. Intriguingly, they found that the induced nitrite/nitrate in the RVLM dialysate amounts to only two folds instead of the theoretical five-fold increase induced by NOS II overexpression (Pannu & Singh, 2006). Because the induced sympathoexcitation and pressor response are abolished by NOS II inhibitor and tempol, the authors interpret their results to suggest that L-arginine and/or BH4 might be insufficient when NOS II is overexpressed (Hirooka, 2011; Kishi, Hirooka, & Sunagawa, 2012), and the resultant ROS production may promote NOS II uncoupling to further sustain oxidative stress in the RVLM.

In rats fed with high fat diet (Wu et al., 2014), the discrepancy in reduced tissue NO levels and increased NOS I protein expression in the RVLM prompted the authors to speculate that NOS I uncoupling is responsible for the generation of ROS in this brain stem site that leads to hypertension. Instead of a loss of BH4, the most prominent cause for NOS uncoupling, upregulation of PIN in the RVLM was found to underlie NOS I uncoupling and the generation of ROS. There is a significant decrease in NOS I dimer/monomer ratio that is negatively correlated with PIN upregulation, decrease in NOS activity and tissue NO level. Furthermore, stabilizing NOS I dimer formation by silencing the PIN gene resulted in the restoration of tissue NO level and a significant reversal of the augmented ROS level in the RVLM of rats fed with high fat diet.

4.6. Endogenous antioxidants in the RVLM

As part of the cellular redox-homeostasis, endogenous antioxidants are present in the RVLM to alleviate the impending oxidative stress. Although outside the scope of this review, three examples are given below to illustrate this aspect of feedback regulation of oxidative stress.

4.6.1. Uncoupling protein 2

The uncoupling proteins (UCPs) have emerged as important natural antioxidants in the maintenance of ROS homeostasis (Arsenijevic et al., 2000). UCPs belong to a superfamily of mitochondrial anion transporters that uncouple ATP synthesis from oxidative phosphorylation by causing proton leakage across the mitochondrial inner membrane, leading to energy dissipation and heat production (Korshunov, Skulachev, & Starkov, 1997). The resultant decrease in proton electrochemical gradient across the inner mitochondrial membrane elicited by the UCPs mitigates mitochondrial ROS production (Arsenijevic et al., 2000; Porter, 2001). Among the five homologues, UCP1 to UCP5, cloned in mammals (Kim-Han & Dugan, 2005), dysfunction of UCP2 is suggested to be of considerable importance in cardiovascular pathophysiology associated with oxidative stress (Blanc et al., 2003; Duval et al., 2002; Lee et al., 2005). Specifically, transcriptional upregulation of mitochondrial UCP2 activated by an elevation in mitochondrial and NADPH oxidase-derived extra-mitochondrial $O_2^{\bullet-}$ plays an active role in feedback regulation of ROS production in the RVLM and hypertension associated with chronic oxidative stress (Chan, Wu, Wu, et al., 2009). Both basal and Ang II-induced expressions of mitochondrial UCP2 are reduced in the RVLM of SHR. On the other hand, an increase in UCP2 expression in the RVLM of SHR following oral intake of rosiglitazone, a synthetic ligand of UCP2 transcription factor peroxisome proliferator-activated receptor-γ, reduces mitochondrial oxidative stress and promotes a central antihypertensive effect (Chan, Wu, Kung, & Chan, 2010).

4.6.2. Brain-derived neurotrophic factor

Superimposed on its classic trophic functions in proliferation, differentiation, and survival of neurons in the peripheral and central nervous system during development (Oppenheim, 1991) or in synaptic activity and plasticity of mature neurons (Schinder & Poo, 2000), brain-derived neurotrophic factor (BDNF) is now known to exhibit nontrophic neuroprotective actions (Macias et al., 2007) by defending neurons against injury and disease (Zacchigna, Lambrechts, & Carmeliet, 2008). In this context, another endogenous antioxidant against oxidative stress in neurogenic hypertension is redox-sensitive BDNF in the RVLM (Chan, Wu, Chang, Hsu, & Chan, 2010). Ang II induces NADPH oxidase-derived $O_2^{\bullet-}$-dependent upregulation of BDNF in the RVLM via phosphorylation of cAMP response element binding protein (CREB) at Ser133. The endogenously activated BDNF in turn exerts negative feedback alleviation of the induced elevation of tissue $O_2^{\bullet-}$ levels in the RVLM, resulting in protection against Ang-II-induced oxidative stress in the RVLM and long-term pressor response, by at least three mechanisms. First, BDNF suppresses Ang II-induced phosphorylation of $p47^{phox}$ subunit of NADPH oxidase. Second, BDNF preserves the mitochondrial ETC coupling capacity in the RVLM. Third, BDNF upregulates mitochondrial UCP2 expression in the RVLM by targeting tropomyosin receptor kinase B (TrkB) in the mitochondria.

4.6.3. Nuclear factor erythroid 2-related factor 2

Nrf2 is a master transcriptional regulator of redox homeostasis that impacts antioxidant gene expression (Nguyen, Sherratt, Nioi, Yang, & Pickett, 2005). It activates cellular antioxidant responses by inducing the transcription of a large array of cytoprotective genes that encode antioxidant proteins. This process is mediated by binding of Nrf2 to a DNA motif known as antioxidant response element in the promoter region of multiple genes, leading to the upregulation of a battery of antioxidant enzymes, including SOD, NAD(P)H:quinone oxidoreductase 1,heme oxygenase 1, and catalases (Kensler, Wakabayashi, & Biswal, 2007; Ma, 2013). In the RVLM of rats with peripheral inflammation, the expression of both total and activated Nrf2 is reduced because of elevated tissue $O_2^{\bullet-}$ levels, alongside reduction of its DNA binding ability to the promotor regions of its target genes in the nucleus (Wu et al., 2016). Activation of Nrf2 with its inducer tert-butylhydroquinone, on the other hand, increases mitochondrial antioxidant activity, alleviates tissue oxidative stress and reduces the inflammation-associated pressor response. In contrast, selective gene deletion of the *Nrf2* in the RVLM decreases the expression of MnSOD and catalase, increases sympathetic outflow and elevates blood pressure (Gao, Zimmerman, Biswal, & Zucker, 2017).

5. Nitrosative stress in the RVLM underpins brain stem death

5.1. Brain stem death

One often forgotten fact in medicine is that it is the functionality of the brain stem, rather than the forebrain, that defines life-and-death. The importance of the brain stem to brain death is reflected in a memoranum issued in 1976 by the Conference of Medical Royal Colleges and their Faculties in the UK (1976), which emphasizes "permanent functional death of the brain stem constitutes brain death". A second memorandum issued in 1979 (Conference of Medical Royal Colleges and their Faculties in the United Kingdom, 1979) specifically identifies brain stem death with death itself. The observation that asystole invariably takes place within hours or days after the diagnosis of brain stem death (Pallis, 1983) implies that permanent impairment of the brain stem cardiovascular regulatory machinery is intimately associated with this fatal phenomenon.

Previous clinical studies (Kuo et al., 1997; Yen, Yien, Wang, Lee, & Chan, 2000; Yien et al., 1997) identified a life-and-death signal detected from blood pressure that disappears to reflect failure of central cardiovascular regulation before the onset of brain stem death in comatose patients. Intriguingly, this life-and-death signal exhibits a biphasic waxing (pro-life) followed by waning (pro-death) pattern in animal models of brain stem death (Chan, Chang, & Chan, 2005) that mirrors the increase and decrease in sympathetic vasomotor tone. The demonstration that this signal takes origin from the RVLM (Kuo, Yang, & Chan, 1997) further suggests that pro-life and pro-death cellular and molecular programs in the RVLM are activated during the progression towards brain stem death. We elaborate below (Fig. 2) that nitrosative stress in the RVLM plays an intricate role in the temporal interplay between the pro-life and pro-death programs that leads to the final fate of diminished sympathetic vasomotor tone and death. Our narratives will be based primarily on investigations using NO derived from NOS I and NOS II and peroxynitrite as the representative RNS, employing the endotoxemia (Chan, Wang, & Chan, 2001; Chan, Chang, & Chan, 2005), mevinphos (an organophosphate insecticide) intoxication (Chan, Chang, & Chan, 2005; Yen et al., 2001), and hepatic encephalopathy (Su et al., 2016; Tsai, Su, Chan, & Chan, 2017) models of brain stem death.

5.2. NO generated by NOS I or II in the RVLM plays a pro-life or pro-death role during brain stem death

As discussed under Section 3.3, under physiological conditions, the prevalence of NOS I over NOS II activity is responsible for the maintenance of sympathetic vasomotor outflow by the endogenous NO in the RVLM. On the other hand, a tilt of the balance towards NOS II leads to a decrease in sympathetic vasomotor tone and eventual death. It is therefore intriguing to note that the progression towards death in our models of experimental brain stem death is associated with an initial but shorter-lasting maintained or increase in NOS I expression in the RVLM (Chan, Wang, & Chan, 2001; Chan, Chan, & Chang, 2004; Chan, Chan, Li, Cheng, & Chang, 2005; Li, Chan, Chan, & Chang, 2005), to be followed by a delayed but longer-lasting augmentation of molecular synthesis and functional expression of NOS II. By causally related respectively to the augmentation and reduction of the life-and-death signal (Chan et al., 2001, 2002, 2003b, 2004, b, 2005, d; Chang et al., 2001; Li et al., 2005), the tonically active NOS I- and NOS II-derived NO has been implicated to play a pro-life and pro-death role during brain stem death.

5.3. Mechanisms for the dual cardiovascular actions of NO generated by NOS I or II in the RVLM

5.3.1. NOS I and II exhibit differential enzyme kinetics

The sympathoexcitatory actions of endogenous NO generated by NOS I in the RVLM exhibit a pattern of fast onset and short duration; the prolonged sympathoinhibitory responses to NO induced by NOS II develop slowly (Chan et al., 2001, 2003). Differences in enzyme kinetics may dictate the relative prevalence of NOS I and NOS II activity, which in turn determines the duration and concentration of NO present in the RVLM that impact sympathetic vasomotor tone. Activated NOS I releases short puffs of NO, and is responsive to an increase in intracellular Ca^{2+} and subsequent binding of Ca^{2+} to calmodulin (Bredt and Snyder, 1990; Mayer et al., 1990). In contrast, NOS II produces long-lasting generation of NO (Green et al., 1991; Nathan, 1992), but is not regulated by intracellular Ca^{2+} and is under constant activation (Yui et al., 1991). Furthermore, the amount of NO generated by NOS II may be up to 1000-fold of that derived from NOS I (Nathan, 1997).

In brain stem slices coupled with whole-cell patch-clamp recording, NO donors differentially affect the evoked excitatory postsynaptic currents (EPSCs) or spontaneous EPSCs (sEPSCs) in RVLM neurons in a concentration-dependent manner (Huang et al., 2003, 2004). Whereas a low concentration of NO donors augments the amplitude of EPSCs and the frequency of sEPSCs, a higher concentration significantly inhibits the same synaptic events.

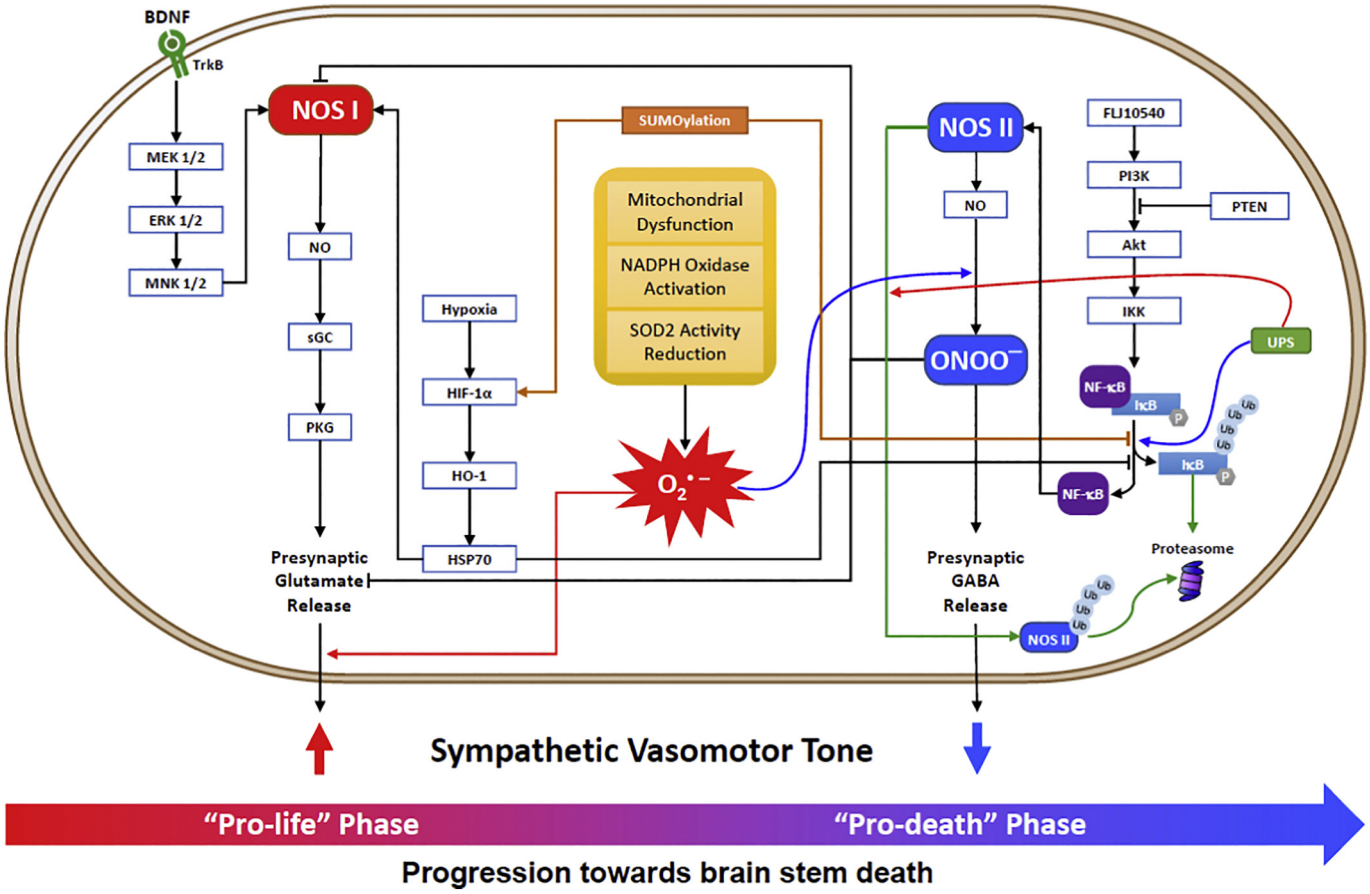

Fig. 2. Summary of key cellular events associated with oxidative stress and nitrosative stress in the RVLM during brain stem death. The progression towards brain stem death entails an increase ("pro-life" phase) followed by a decrease ("pro-death" phase) of sympathetic vasomotor tone. An initial but shorter-lasting increase in NOS I expression, which activates sGC/cGMP/PKG signaling that leads to enhancement of presynaptic glutamate release in the RVLM is the key player for the pro-life phase. On the other hand, a delayed but much longer-lasting upregulation of NOS II and subsequent formation of peroxynitrite in the RVLM, which facilitates presynaptic GABA release, underpins the pro-death phase. NOS I expression in the RVLM is positively modulated by HSP70 activated via the HIF-1α/HO-1 cascade in response to hypoxia, and BDNF/TrkB via the MEK 1/2, ERK 1/2 and MNK 1/2 cascade; and negatively by peroxynitrite. NOS II expression is positively modulated by the FLJ10540/PI3K/Akt/NF-κB signaling cascade; and negatively modulated by PTEN. Of note is that SUMOylation plays a pro-life role by acting on HIF-1α to upregulate NOS I and on IκB to dowregulate NOS II expression. At the same time, two cellular factors that exhibit double-edge sword actions during brain stem death are oxidative stress and UPS. Oxidative stress because of augmented $O_2^{\bullet -}$ production elicited by impairment of mitochondrial bioenergetics, activation of NADPH oxidase and reduction in SOD2 activity is playing initially a pro-life role by increasing sympathetic vasomotor tone. However, oxidative stress becomes pro-death on reaction of $O_2^{\bullet -}$ with the progressively available NOS II-derived NO to form peroxynitrite. The UPS promotes NOS II synthesis via ubiquitination of IκB, and reduces NOS II contents by continuous degradation coupled with de-ubiquitination.

5.3.2. NOS I and II activate different signaling pathways

As an important neurotransmitter involved in tonic excitatory drive to RVLM neurons (Ito and Sved, 1997; Sved et al., 2001), glutamate presents itself as a logical cellular target for the dual actions of NO on RVLM neurons. Both subtypes of ionotropic glutamate receptors (Rao et al., 1997) are distributed in the RVLM, and activation of these receptors elicits sympathoexcitation (Ito and Sved, 1997; Morrison et al., 1991; Willette et al., 1983). In addition, GABA receptors (Bowery et al., 1987) are also distributed in the RVLM, and activation of those receptors elicits inhibition of sympathetic vasomotor tone (Coleman and Dampney, 1998; Willette et al., 1984).

The soluble guanylyl cyclase (sGC)/cGMP/protein kinase G (PKG) cascade is a well-known intracellular signaling pathway for the physiological actions of NO (Arnold et al., 1977; Knowles et al., 1989). Mediation of the cytotoxic cardiovascular actions of NO by peroxynitrite was also reported (Beckman et al., 1990; Blough and Zafiriou, 1985; Garcia-Estan et al., 2002). It is thus of interest to note that these two signaling cascades are differentially associated with the concentration-dependent bi-directional modulation of glutamatergic neurotransmission by NO on RVLM neurons (Huang et al., 2003, 2004). At lower concentrations, NO elicits a reversible potentiation of presynaptic glutamate release through a NO-sensitive sGC/cGMP/PKG-coupled signaling pathway. On the other hand, the overproduced NO acts presynaptically to inhibit glutamatergic neurotransmission via the formation of peroxynitrite. An increase in NO production also elevates the concentration of GABA in the RVLM (Kishi et al., 2001). Moreover, persistent overexpression of NO in the RVLM by transfection of adenovirus vectors (Kishi et al., 2001, 2002) promotes hypotension and bradycardia via an increase in GABA release. As such, NO may also act as a retrograde messenger and interact with GABAergic neurotransmission via a positive feedback mechanism (Stern and Ludwig, 2001).

5.3.3. The pro-life actions of NO generated by NOS I in the RVLM involve sGC/cGMP/PKG signaling

Application of a small amount of NO to the RVLM provided non-enzymatically by low doses of S-nitro-*N*-acetylpenicillamine elicits a transient increase in arterial pressure (Chan et al., 2001). The sympathoexcitatory actions of endogenous NO generated by NOS I in the RVLM exhibit a pattern of fast onset and short duration (Chan et al., 2001, 2003). Together with the narratives presented in Section 5.3.3, it is reasonable to conclude that NO generated by NOS I elicits presynaptic facilitation of glutamate release through the sGC/cGMP/PKG cascade in RVLM in the elicitation of the pro-life phase of cardiovascular responses in experimental brain stem death (Chan et al., 2004, 2005; Huang et al., 2003; Li et al., 2005). This conclusion is in line with the reports that the NO/sGC/cGMP pathway facilitates

glutamatergic neurotransmission (Fedele and Raiteri, 1999; Martins-Pinge et al., 1999; Morimoto et al., 2000) by acting presynaptically on N-type Ca^{2+} channels (Huang et al. 2003).

5.3.4. The pro-death actions of NO generated by NOS II in the RVLM involve peroxynitrite

Progressive and long-lasting hypotension is induced by microinjection of high doses of a NO donor to the RVLM (Chan et al., 2001). The prolonged sympathoinhibitory responses to NO induced by NOS II develop slowly (Chan et al., 2001, 2003). Together with the narratives presented in Section 5.3.3, it is also reasonable to conclude that overproduction of NO generated by massive activation of NOS II elicits presynaptic inhibition glutamate release through the formation of peroxynitrite in RVLM in the elicitation of the pro-death phase of cardiovascular responses in experimental brain stem death (Chan et al., 2002, 2004, 2005, d; Huang et al., 2004; Li et al., 2005).

Mechanistically, high concentrations of NO acts presynaptically to elicit synaptic depression on RVLM neurons through inhibition of presynaptic N-type Ca^{2+}-channel activity, leading to reduced glutamate release (Huang et al. 2004). This depressant action is mediated by the formation of peroxynitrite, which subsequently acts to release adenosine to activate presynaptic A_1 adenosine receptors. Peroxynitrite has also been reported to oxidize protein and non-protein sulfhydryls (Radi et al., 1991a) and induce membrane lipid peroxidation (Radi et al., 1991b; Rubbo et al., 1994). It also shuts down cellular energy production by inhibiting mitochondrial ETC (Cassina and Radi, 1996; Packer and Murphy, 1995) or inactivating mitochondrial respiratory enzymes (Pryor and Squadrito, 1995; Radi et al., 1994). Activation of poly (ADP)ribosyltransferase contributes to peroxynitrite toxicity by promoting excessive ADP ribosylation of nuclear protein (Szabo et al., 1996,b; Zingarelli et al., 1996), leading to ATP depletion and cellular injury (Szabo et al., 1996, Zingarelli et al., 1996). Overproduction of NO via activation of NOS II (Chan et al., 2003) also downregulates both synthesis and activity of AT_1R in the RVLM, alongside a reduction in sympathetic vasomotor tone and hypotension. NO may also act as a retrograde messenger and interact with GABAergic neurotransmission via a positive feedback mechanism (Stern and Ludwig, 2001). Of note is the GABA-mediated sympathoinhibitory actions of endogenous NO derived from NOS II are exerted primarily via $GABA_A$ receptors in the RVLM (Chan et al., 2003; Smith and Barron, 1990; Ying et al., 1998).

5.4. Cellular factors that modulate NOS I expression in the RVLM

Given the pro-life role of NOS I in the RVLM via augmentation of sympathetic vasomotor tone, it is understandable that several cellular factors are now known to sustain its expression at the level of transcription, translation and post-translational modification.

5.4.1. Heat shock factor 70

The heat shock proteins (HSPs) represent a group of intracellular proteins that are thought to participate in protective adaptation that spares cells from otherwise lethal consequences of exposure to various forms of cellular stresses (Craig, 1985; Lindquist and Craig, 1988; Morimoto and Santoro, 1998; Subjeck and Shyy, 1986; Welch, 1992). Because of their critical roles in intracellular processing, synthesis, transportation and degradation of proteins, HSPs have been termed molecular chaperones (Ellis and van der Vries, 1991). The chaperone activities of HSPs include prevention of protein denaturation and promotion of refolding of damaged proteins after stress (Benjamin and McMillan, 1998; Fink, 1999; Morimoto and Santoro, 1998), and sustaining proteins in the productive folding pathway or maintaining newly synthesized proteins in an unfolded conformation suitable for translocation across membranes (Beckman et al., 1990; Nelson et al., 1992). Whereas HSP70 is generally contended to be an inducible form of HSP (Lindquist and Craig, 1988; Welch, 1992), both proteomic and Western blot analyses detected the presence of HSP70 expression in the RVLM under basal conditions (Li et al., 2005). More importantly, augmented expression of HSP70 in the RVLM that resulted from de novo synthesis confers neuroprotection in the endotoxemia (Chan et al., 2004; Li et al., 2005) or mevinphos intoxication (Chang et al., 2004a) models of brain stem death by preventing the depression of sympathetic vasomotor tone via enhancement of the NOS I/PKG signaling pathway in the RVLM (Li et al., 2005).

5.4.2. Hypoxia-inducible factor 1/heme oxygenase 1

One clue in the search for the upstream signals that lead to HSP70 induction arises from the demonstration that hypoxia takes place in RVLM in our mevinphos model of experimental brain stem death (Yen et al., 2005). As a master regulator of cellular responses to hypoxia via activation of a multitude of oxygen-sensitive gene products (Pouysségur and Mechta-Grigoriou, 2006; Semenza, 1999), hypoxia stabilizes the transcription factor hypoxia-inducible factor 1α (HIF-1α), and nucleus-bound translocation of the stabilized HIF-1α allows for formation of the HIF-1αβ heterodimer that becomes transcriptionally active (Ema et al., 1999). Of note is that hypoxia also induces HSP70 expression (Iwaki et al., 1993; Lee et al., 2001), although *hsp70* gene lacks HIF-1 binding site in its promoter region. Also noted is that the activated HIF-1αβ complex binds to target genes at hypoxia regulatory element, which contains the core recognition sequence 5′-RCGTG-3′, leading to upregulation of hypoxia responsive gene products such as heme oxygenase 1 (HO-1) (Choi and Alam, 1996; Hellwig-Burgel et al., 2005; Lee et al., 1997; Yang and Zou, 2001). Since HSP70 and HO-1 are two of the most common stress-induced proteins (Das and Maulik, 2006), and both share common induction kinetics, including onset and duration, when exposed to hyperthermia, hypoxia, or inflammation (Das and Maulik, 2006; Turner et al., 1999), HO-1 is a logical intermediate between HIF-1 activation and HSP70 upregulation. Indeed, biochemical and pharmacological results (Chang et al., 2009; Dai et al., 2010) support the notion that the repertoire of cellular events in the RVLM during the pro-life phase of brain stem death triggered by hypoxia preferentially entail the sequential activation of HIF-1, HO-1, and HSP70, leading to upregulation of NOS I/PKG signaling that results in augmented sympathetic vasomotor tone. Of interest is that this signaling cascade is not engaged in the modulation of the pro-death NOS II/peroxynitrite cascade in the RVLM.

5.4.3. Sumoylation

Small ubiquitin-related modifier (SUMO) is a group of proteins (Mahajan et al., 1997; Matunis et al., 1996) that participates in post-translational modifications. Despite the name, SUMO shares only about 18–20% homology with ubiquitin (Matunis et al., 1996; Müller et al., 2001). Sumoylation involves the covalent attachment of a member of the SUMO proteins to lysine residues in the target proteins. The process of sumoylation is very similar to ubiquitination and other ubiquitin-like proteins (Johnson, 2004), and involves four enzymatic steps: maturation, activation, conjugation and ligation (Hilgarth et al., 2004). All SUMO proteins share the same activating (E1) and conjugating (E2; Ubc2) enzymes. Hypoxia increases SUMO-1 mRNA and protein levels in brain, and the induced SUMO-1 co-expresses with HIF-1α in neurons (Shao et al., 2004).

Results from Western blot analysis, EMSA, ELISA, confocal microscopy and immunoprecipitation (Chan et al., 2011a) demonstrated that drastic tissue hypoxia, elevated levels of proteins conjugated by SUMO-1, Ubc9 or HIF-1α, augmented sumoylation of HIF-1α, nucleus-bound translocation and enhanced transcriptional activity of HIF-1α in RVLM neurons take place preferentially during the pro-life phase of experimental brain stem death. Furthermore, loss-of-function manipulations of SUMO-1, Ubc9 or HIF-1α in the RVLM blunted the upregulated NOS I/PKG signaling cascade, which sustains sympathetic vasomotor tone during the pro-life phase. The return of SUMO-1, Ubc9 or HIF-1α expression in the RVLM to control levels during the pro-death phase further suggests that sumoylation of HIF-1α plays minimal

role in the modulation of NOS II/peroxynitrite cascade in RVLM during this phase of brain stem death.

5.4.4. Brain-derived neurotrophic factor

BDNF/TrkB signaling may also play a pro-life role in brain stem death (Chan et al., 2011b) via upregulation of NOS I in the RVLM through sequential activation of MAPK kinase 1/2 (MEK1/2), ERK1/2 and MAPK signal-interacting kinase 1/2 (MNK1/2) (Chan et al., 2010). There are two possible, though not necessarily mutually exclusive, underlying mechanisms. One is for ERK1/2 to upregulate NOS I transcriptionally. Candidates for ERK nuclear targeting in the mediation of gene transcription, including CREB, C/EBP and c-Myc (Bros et al., 2007; Jeong et al., 2000), are present in the promoter region of NOS I gene. Another possibility is for ERK to exert posttranslational modification by phosphorylation of NOS I protein. MNK1/2 may enhance phosphorylation of NOS I on activation by MEK/ERK.

5.5. Cellular factors that modulate NOS II expression in the RVLM

Likewise, given the pro-death role of NOS II in the RVLM via depression of sympathetic vasomotor tone, several cellular factors are now known to positively or negatively modulate its expression at the level of transcription, translation and post-translational modification.

5.5.1. Nuclear factor-κB

The NOS II gene is regulated transcriptionally in part by activation of nuclear factor-κB (NF-κB) (Nathan and Xie, 1994; Piaggio et al., 2001; Pingle et al., 2003; Xie et al., 1994). In mammals, the NF-κB family consists of five proteins, p65 (RelA), RelB, c-Rel, p50 and p52, which can form transcriptionally active homo- or heterodimers (Baeuerle and Baltimore, 1996; Karin and Ben-Neriah, 2000; Oeckinghaus and Ghosh, 2009; Whiteside et al., 1997). NF-κB is sequestered in the cytoplasm of non-stimulated cells in a latent form because of its association with inhibitory molecules collectively termed inhibitors of NF-kB (IkB) (Baeuerle and Henkel, 1994). Under stress conditions (Armstead et al., 1999; Maulik et al., 1999), phosphorylation of IκB by IκB kinase leads to degradation of IκB via a phosphorylation-dependent ubiquitination process (Baldwin, 1996) (see Section 5.6.2 below) and disruption of the NF-κB/IκB complex. Following IkB degradation, the dissociated NF-κB subsequently translocates from the cytoplasm to the nucleus, where this transcription factor binds to the κB promotor region of target genes, including NOS II (Nathan and Xie, 1994; Xie et al., 1994). This repertoire of cellular events was found to occur in the RVLM in the endotoxemia model of brain stem death (Chan et al., 2004). In addition to the demonstration of augmented protein level and DNA binding activity of NF-κB, blockade of NF-κB binding to its cognate site in the RVLM significantly attenautes the surge in NOS II expression and the depression of sympathetic vasomotor tone.

5.5.2. PI3K/Akt signaling

The PI3K/Akt signaling, which is well-established to be involved in tumorigenesis (Mayer and Arteaga, 2016; Nicholson and Anderson, 2002), is now known to participate in the modulation of NOS II expression in the RVLM in experimental brain stem death (Tsai et al., 2018). PI3K can be divided into classes I, II and III subfamilies (Jean and Kiger, 2014), although class I PI3K subfamily is the only kinase that generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3) by phosphorylating phosphatidylinositol-4,5-bisphosphate (PIP_2). The most common form of class I PI3K is a heterodimer composed of a catalytic subunit (p110) and a regulatory subunit (p85). Akt, also referred to as protein kinase B, is a serine/threonine kinase with a pleckstrin homology domain that preferentially binds PIP_3. As a downstream target of PI3K (Wymann et al., 2003), Akt is activated via binding PIP_3 and subsequent phosphorylation of two specific sites, one in the kinase domain (Thr308) and the other in the C-terminal regulatory region (Ser473), by 3′-phosphoinositide-dependent kinase 1 (PDK1) and PDK2 at the membrane.

In the mevinphos-intoxication model of brain stem death (Tsai et al., 2014), the enzymatic activity of PI3K and Akt in the RVLM is increased. The augmented Akt activity or phospho-Akt(Thr308) expression is antagonized by local application of PI3K inhibitors into the RVLM. Results from biochemical and pharmacological experiments confirmed that PI3K/Akt signaling is upstream to NF-κB activation, and activation of PI3K/Akt/NF-κB signaling cascade preferentially upregulates NOS II or peroxynitrite in the RVLM. It was concluded that selective activation of class I PI3K/Akt signaling in the RVLM, leading to upregulation of the NF-κB/NOS II/peroxynitrite cascade, underpins the impairment of the sympathetic vasomotor tone and the loss of sympathetic vasomotor tone and the life-and-death signals in experimental brain stem death.

5.5.3. FLJ10540, a PI3K-association protein

FLJ10540, also named Cep55 (Zhao et al., 2006) or C10orf3 (Sakai et al., 2006), was originally identified as a microtubule-association protein that plays critical roles in cell division (Zhao et al., 2006). More recently, FLJ10540 has been found to be significantly associated with human cancer (Chen et al., 2009; Hwang et al., 2013; Jeffery et al., 2016). As a PI3K-association protein (Hwang et al., 2013), FLJ10540 forms a complex with PI3K and enhances PI3K activity, resulting in increased Akt activation. Both FLJ10540 mRNA and protein levels are significantly elevated in RVLM neurons in experimental brain stem death. Immunoneutralization of FLJ10540 in the RVLM blunts the augmented formation of p110-p85 heterodimeric PI3K, phosphorylation of Akt, and NOS II or peroxynitrite expression in the RVLM, and antagonizes the hypotension and decrease in sympathetic vasomotor tone during the pro-death phase. It follows that upregulation of FLJ10540 is upstream to activation of PI3K/Akt/NOS II/peroxynitrite cascade in the RVLM, leading to a reduction in sympathetic vasomotor tone and the loss of the life-and-death signals during experimental brain stem death (Tsai et al., 2015).

5.5.4. PTEN

Classified as a tumor suppressor gene (Ali et al., 1999; Li et al., 1997) and a well-known negative regulator of PI3K (Maehama and Dixon, 1998), PTEN dephosphorylates the 3′-phosphate on PIP_3 to generate PIP_2, thereby directly antagonizes PI3K/Akt signaling. Augmentation of PTEN activity as reflected by a decrease in its oxidized form and an increase of its reduced form in the RVLM takes place in experimental brain stem death (Tsai et al., 2017). Loss-of-function manipulations of PTEN significantly aggravate the augmentation of phosphorylation of Akt, NOS II or peroxynitrite in the RVLM; and enhance the elicited hypotension and exacerbate the decreased sympathetic vasomotor tone during the pro-death phase. These results support the notion that PTEN plays a protective role by ameliorating the loss of sympathetic vasomotor tone in brain stem death as a negative regulator of PI3K/Akt signaling in the RVLM.

5.5.5. Sumoylation

Prevention of NF-κB activation inhibitis NOS II expression (Feinstein et al., 1996; Pingle et al., 2003), and sumoylation inactivates NF-kB by conjugation with IkB (Mabb and Miyamoto, 2007; Ulrich, 2005). The sumoylated pool of IkB is protected from ubiquitination, thereby blocking its degradation and inhibits subsequent activation and nuclear translocation of NF-kB (Desterro et al., 1998). In an endotoxemia model of brain stem death (Tsai et al., 2016), the SUMO-1 and Ubc9 mRNA or protein levels in the RVLM are augmented, alongside SUMO-1-conjugated proteins. Immunoneutralization of SUMO-1 or Ubc9 in the RVLM significantly potentiates the already diminished sumoylation of IkBα and intensifies NF-kB activation and NOS II/peroxynitrite expression in this brain stem substrate, together with exacerbated fatality, cardiovascular depression and reduction of sympathetic vasomotor tone. It follows that sumoylation plays a pro-life role by interacting with the NF-

kB/NOS II/peroxynitrite signaling pathway in the RVLM during brain stem death.

5.5.6. HSP70

Up-regulation of HSP70 may also play a pro-life role in brain stem death by holding NOS II expression and peroxynitrite formation in RVLM in check (Chang et al., 2004a; Li et al., 2005). There are at least two possible mechanisms. The first involves inhibiting IκB kinase activation (Kohn et al., 2002; Yoo et al., 2000); the second entails inhibiting degradation of cytoplasmic IκBα (Pritts et al., 2002).

5.6. Cellular factors in the RVLM that exhibit double-edge sword actions during brain stem death

In addition to cellular factors that undergo the tug-of-war events, another intriguing observation is that some factors are exhibiting double-edge sword actions in the RVLM during brain stem death. Oxidative stress and the ubiquitin-protesasome system represent two such cellular factors.

5.6.1. Oxidative stress

As discussed under Section 4, oxidative stress in the RVLM is generally associated with the hypertensive state. Nevertheless, rapid onset of elevations in $O_2^{\bullet-}$ level in the RVLM is consistently observed in animal models of brain stem death (Chan et al., 2005; Chang et al., 2009; Chuang et al., 2003; Li et al., 2012; Sheh et al., 2007; Su et al., 2016; Tsai et al., 2017, 2018; Yen et al., 2004, 2005). The source of this increase in $O_2^{\bullet-}$ includes reduction in SOD2 activity (Chan et al., 2005), dysfunction of mitochondrial ETC complexes (Chang et al., 2009; Chuang et al., 2003; Sheh et al., 2007; Yen et al., 2004, 2005) and activation of NADPH oxidase (Sheh et al., 2007; Su et al., 2016; Tsai et al., 2017).

An interesting corollary that arises from this paradox is that oxidative stress is exhibiting double-edge sword actions at the RVLM during brain stem death. By eliciting augmented sympathetic vasomotor tone, oxidative stress may in fact play a pro-life role during the early stage towards brain stem death. However, with the subsequent elevation in the molecular synthesis and functional expression of NOS II associated with experimental brain stem death (Chan et al., 2004, 2005,d; Chang et al., 2009; Chuang et al., 2003; Sheh et al., 2007; Yen et al., 2004, 2005; Tsai et al., 2014, 2015, 2016, 2017, 2018), oxidative stress becomes pro-death because of the formation of peroxynitrite by a reaction between NOS II-derived NO and $O_2^{\bullet-}$ in the RVLM. The ultimate result is progressive reduction in sympathetic vasomotor tone seen in these animal models (Chan et al., 2002, 2005). It follows that it is the transition from oxidative stress to nitrosative stress in the RVLM that underpins the shift from increase to decrease in sympathetic vasomotor tone during the progression towards brain stem death.

5.6.2. Ubiquitin-proteasome system

It is now clear that most proteins in the cytoplasm and nucleus of eukaryotic cells are degraded via the ubiquitin-proteasome system (UPS) (Ciechanover and Schwartz, 1998; Glickman and Ciechanover, 2002). The highly conserved 76 amino acid protein ubiquitin is best known for its role in targeting proteins for degradation by the 26S proteasome via a three-step mechanism. The ubiquitin-activating enzyme, E1, first activates ubiquitin. Following activation, one of several ubiquitin-conjugating enzymes (E2) transfers ubiquitin from E1 to a member of the ubiquitin-protein ligase family (E3), to which the substrate protein is specifically bound. Following conjugation, the protein moiety of the adduct is degraded by the 26S proteasome complex. After the degradative process at the 26S proteasome, the ubiquitin chain is released from the target protein remnant and is disassembled by de-ubiquitinating enzymes. The latter are subdivided into two gene families, ubiquitin carboxyl-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (Wilkinson, 1997). Of the three known mammalian members of the UCH family (Mayer and Wilkinson, 1989; Wilkinson et al., 1989), UCH isozyme L1 (UCH-L1) is among the most abundantly present proteins in the brain (Doran et al., 1983), and is found specifically in central and peripheral neurons (Kajimoto et al., 1992; Wilkinson et al., 1989).

Proteomic analysis (Chou et al., 2011) revealed that members of the UPS, including proteasome subunit alpha type-1, ubiquitin and UCH-L1 are present in the RVLM, and de novo synthesis of UCH-L1 in the RVLM is crucial to the pro-life process during brain stem death (Chang et al., 2004b). Furthermore, ubiquitination and de-ubiqutination in the RVLM remain operational throughout experimental brain death (Wu et al., 2012). Based on an endotoxemia model, augmented polyubiquitination, enhanced proteasomal activities and recycling of ubiquitin through sustained de-ubiquitination in the RVLM play a vital role in brain stem death (Wu et al., 2012).

Of interests is that in addition to the tug-of-war between sumoylation and ubiquitination on NF-kB activation (see Sections 5.5.1 and 5.5.5), there is also a tug-of-war between the synthesis and degradation of NOS II in the RVLM during brain stem death (Wu et al., 2011). The most crucial hint of this double-edge sword action of UPS arises from the observation that there is a drastic difference between upregulation of NOS II mRNA (22 folds) and protein (1.3 fold) in the RVLM during experimental brain stem death. It turns out that by mediating IkB degradation, UPS is on one hand crucially involved in the synthesis of NOS II via transcriptional activation of NF-kB (Karin and Ben-Neriah, 2000; Yaron et al., 1997). On the other hand, UPS is also engaged in the degradation of NOS II at the RVLM, with de-ubiquitination crucially involved in this process. In the final analysis, a crucial determinant in the tug-of-war between maintained and reduced sympathetic vasomotor tone during brain stem death resides in the temporal balance between the continuous degradation of NOS II and the progressively augmented synthesis of this isozyme in the RVLM. Furthermore, recycling of ubiquitin in the RVLM through sustained de-ubiquitination is crucial to uninterrupted degradation of NOS II, which is essential for the maintenance of sympathetic vasomotor tone.

6. Conclusions

There are at least four lessons to be learnt from this review. The first lesson concerns the major difference in clinical outcome between neurogenic hypertension and brain stem death. The former is reversible and controllable with appropriate therapy, but the latter, once confirmed, is irreversible. Such a disparity in outcome is causally related to the differential participation of oxidative stress and nitrosative stress in the RVLM. An increase in sympathetic vasomotor tone because of augmented oxidative stress in the RVLM is responsible for the generation of neurogenic hypertension (Fig. 1). On the other hand, a progression from oxidative stress to nitrosative stress in the RVLM underlines the succession of increase to decrease in sympathetic vasomotor tone during the transition from the pro-life phase to pro-death phase in brain stem death (Fig. 2).

The second lesson pertains to using the expression “ROS/RNS” as if they represent a singular moiety. By having different cellular sources, regulatory mechanisms on synthesis and degradation, kinetics of chemical reactions, and downstream signaling pathways, ROS and RNS should be regarded as individual chemical entities. It is recognized that ROS and RNS in the RVLM do manifest much more complicated interplays that impact on sympathetic vasomotor tone. Whereas $O_2^{\bullet-}$ and NOS I-generated NO in the RVLM causes sympathoexcitation via activation of glutamatergic neurotransmission, NO derived from NOS II promotes sympathoinhibition by facilitation of GABAergic neurotransmission. ROS-RNS interaction in the form of reaction between $O_2^{\bullet-}$ and NO leads to the formation of peroxynitrite; and uncoupling of NOS I or III results in generation of $O_2^{\bullet-}$ rather than NO. To avoid confusion, these interplays should nevertheless be viewed as an extension of the activities of ROS or RNS as individual moieties.

The third lesson regards the supposition that oxidative stress and nitrosative stress are interchangeable phenomena and are frequently

depicted as the common culprit for cellular damage. In neurogenic hypertension, augmentation of oxidative stress in the RVLM because of impairment of mitochondrial bioenergetics or biogenesis, activation of NADPH oxidase and reduction in antioxidant capacity despite the participation of UCP2, BDNF and Nrf2 results in augmented sympathetic vasomotor tone. In brain stem death, the initial pro-life phase entails production of NOS I-derived NO in the RVLM, with the participation of HSP70, HIF1-α, HO-1, and sumoylation of HIF1-α, that leads to an increase in sympathetic vasomotor tone. This is followed by the delayed pro-death phase that involves the formation of peroxynitrite because of a reaction between $O_2^{\bullet-}$ and NOS II-induced NO, under the positive modulation by NF-κB, PI3K, Akt, or FLJ10540 and negative modulation by PTEN, sumoylation of IκBα, and HSP70, that results in diminution of sympathetic vasomotor tone. The well-defined roles of oxidative stress and nitrosative stress under those two disease conditions clearly denote that they are individual cellular phenomena. We also recognize that interplays between oxidative stress and nitrosative stress do play a role in neurogenic hypertension and brain stem death. The increase in oxidative stress in the RVLM during neurogenic hypertension is accompanied by reduced nitrosative stress primarily because of downregulation of NOS II and and production of $O_2^{\bullet-}$ via NOS uncoupling. It is intriguing to note that, the same cellular scenarios that lead to augmented oxidative stress in the RVLM associated with neurogenic hypertension similarly take place during the pro-life phase of brain stem death and become beneficial by eliciting enhanced sympathetic vasomotor tone. Rather than considering that oxidative stress and nitrosative stress are interchangeable phenomena with unified cellular actions, it is more appropriate to view them individually, paying special attention to their beneficial or detrimental roles under a specific disease or a particular time-window of that disease.

The final lesson concerns therapeutic strategies against oxidative stress and nitrosative stress. Statistics from the World Health Organization (http://www.who.int/gho/ncd/risk_factors/blood_pressure_prevalence_text/en/index.html) indicate that hypertension remains one of the most prevalent cardiovascular diseases worldwide. It follows that a great potential exists to employ antioxidant therapy to keep hypertension in check. One cautionary note is that the narratives in this review are based only on studies on one single brain stem neural substrate that is intimately related to sympathetic vasomotor tone. Despite this focused scope, the picture that emerges with regard to the differential roles of ROS, RNS, oxidative stress and nitrosative stress in neurogenic hypertension and brain stem death is already rather complex. Given the large number of ROS and RNS other than $O_2^{\bullet-}$, NOS I, NOS II and peroxynitrite and the myriad of their associated cellular signaling cascades, along with the multitude of neural substrates that are involved in the generation, maintenance, regulation or modulation of the sympathetic vasomotor tone, a much more complicated modus operandi than that presented in this review is envisaged. Large-scale, randomized, placebo-controlled clinical trials on the use of antioxidant for hypertension have basically failed (Sorriento et al., 2018). To be successful, future antioxidant therapies against neurogenic hypertension must bear in mind this often-ignored complication.

Despite a phenomenon of paramount clinical importance, brain stem death still remains enigmatic to the general populace and even health professionals (Capron, 2001; Drake et al., 2017). It is most unfortunate that this disease condition has never been a popular therapeutic target for drug development. The identification that the progression towards brain stem death entails a shift from oxidative stress to nitrosative stress in the RVLM may perhaps open a new vista for therapeutic intervention to slow down this transition.

Conflict of interest statement

None.

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