発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Koga Y, Hirooka Y, Araki S, et al	High salt diet increases blood pressure via oxidative stress in the brainstem in spontaneously hypertensive rats.	International Society of Hypertension		180	2006
Kishi T, Hirooka Y, Takeshita A, et al	The sympatho-inhibitory effects of statin in the shrsp along with the anti-oxidant effect in the rvlm	International Society of Hypertension		390	2006
Hirooka Y	Central cardiovascular regulation: From hypertension to heart failure	International Society of Hypertension Investigator Initiated Symposium			2006
Nozoe M, Hirooka Y, Koga Y, et al	Increased reactive oxygen species in the nucleus tractus solitarius are involved in the neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats.	Circulation	114	II-169	2006
Sagara Y, Hirooka Y, Nozoe M, et al	Contribution of angiotensin II in the increased reactive oxygen species in rostral ventrolateral medulla and enhanced central sympathetic outflow in stroke-prone spontaneously hypertensive rats.	Circulation	114	II-271	2006

Transworld Research Network
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Central Mechanisms of Cardiovascular Regulation 2007: ISBN: 81-7895-265-3
Editor: Takao Kubo and Tomoyuki Kuwaki

Role of nitric oxide and oxidative stress in the brainstem in cardiovascular regulation

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Abstract

Nitric oxide (NO), a gaseous molecule, in the brain stem plays an important role in cardiovascular regulation. In general, it inhibits sympathetic nerve activity thereby reducing blood pressure. Reactive oxygen species (ROS) counteracts the action of NO and their production is increased in the pathophysiological states that manifest activation of the sympathetic nervous system, such as hypertension and heart failure. The arterial baroreceptor reflex is the major feedback

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control system that acts to stabilize blood pressure. Abnormalities of this reflex are considered to be an underlying mechanism in the cardiovascular diseases such as hypertension and heart failure. There is accumulating evidence, however, that central nervous system mechanisms are involved in the enhanced sympathetic drive that occurs in these disease states. This article reviews studies performed in our laboratory in which a gene transfer technique, in combination with other methods, was used to determine the functional role of NO and ROS in the brain stem in the central control of cardiovascular regulation. We developed a technique to transfer adenovirus vectors encoding specific genes into the nucleus tractus solitarius (NTS) or the rostral ventrolateral medulla (RVLM) of rats in vivo. We applied this technique to hypertensive rats as well as in mice with heart failure to explore the pathophysiological significance of NO and ROS.

Introduction

Nitric oxide (NO) was originally identified as the endothelium-derived relaxing factor and there is now growing body of evidence that abnormality of NO production contributes to the cardiovascular pathophysiology [1]. NO is also present in the central nervous system (CNS) [1,2]. There are three isoforms of NO synthase (NOS). Two are constitutive enzymes, endothelial NOS (eNOS) and neuronal NOS (nNOS). The other one is inducible NOS (NOS) that is induced by inflammatory stimuli and normally its expression is rare. In neurons, nNOS is abundantly present. Many scientists are interested in its action related to long-term potentiation [3]. We have been studied the role of NO in the brain stem in neural control of circulation and found that it really plays an important role in it [4]. These findings are consistent with studies from other laboratories [1, 2], although there is still controversy [5]. Reactive oxygen species, such as superoxide and hydroxyl radicals, counteract action of NO. Thus, the balance between NO and ROS production determines the final physiological effects. It has been shown that ROS production is increased in the pathophysiological states, such as hypertension and heart failure, which deteriorates the progression of the disease status, particularly from the studies in the field of vascular biology. ROS production also occurs in the CNS and related to degenerative neurological diseases and aging. It is not well understood, however, regarding their role in neural control of circulation. Considering NO's action in the CNS, we hypothesized ROS also plays an important role in central cardiovascular regulation. Therefore, I summarize a series of studies regarding role of NO and ROS within the brain stem in neural control of circulation from our laboratories and describe some interesting studies related to recent progress in this topic from other laboratories.

The arterial baroreceptor reflex is the major mechanism for maintaining stable blood pressure [6, 7]. Based on studies in anesthetized animals, the key sites (nuclei) along this pathway and the neurotransmitters involved are located in the brainstem [6-9]. Primary afferent fibers from arterial baroreceptors terminate in the nucleus tractus solitarius (NTS). Baroreceptor signals are conveyed from the NTS via an excitatory pathway to GABAergic neurons in the caudal ventrolateral medulla, which project to spinally projecting neurons in the rostral ventrolateral medulla (RVLM) [6]. The RVLM contains the sympathetic premotor neurons, which then project to the intermediolateral column in the spinal cord where sympathetic preganglionic neurons are located [6]. Finally, postganglionic sympathetic nerves innervate various organs, including the vessels and heart [6]. Therefore, many nuclei are involved in neural regulation of the cardiovascular system. Recent studies in anesthetized animals, however, evidence that a central nervous system mechanism(s) is involved in the abnormal neural control of circulation that occurs in pathophysiological states, such as hypertension and heart failure, in contrast to previous models [7, 8, 10] (Fig. 1). Although the central nervous system is importantly involved in the neural control of circulation, it is technically difficult to examine the role of the central nervous system in cardiovascular regulation in awake animals. A recent development in molecular biology and bioengineering has enabled us to study the role of specific genes and their products

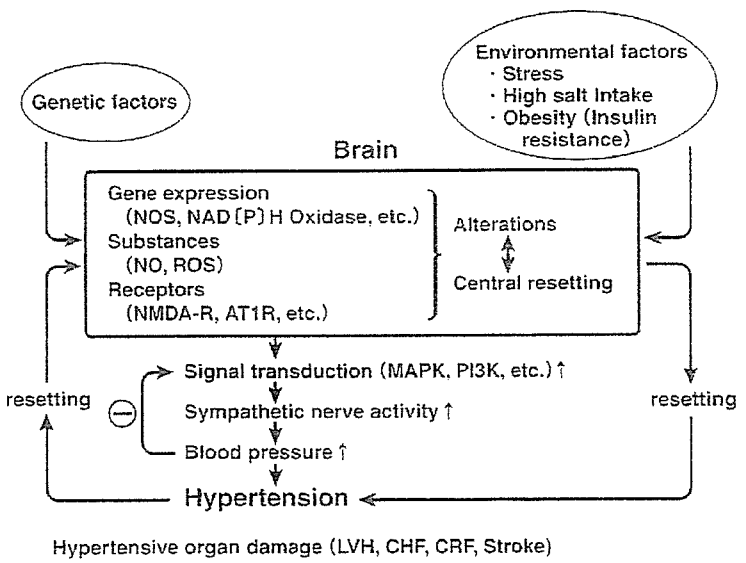

Figure 1. Proposed scheme demonstrating how NO and ROS in the brain lead to hypertension and hypertensive organ damage. NMDA, *N*-methyl-D-asparate receptors; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3 kinase; LVH, left ventricular hypertrophy; CHF, congestive heart failure; CRF, chronic renal failure.

within specific brain nuclei in cardiovascular regulation [11,12]. We developed a technique to transfer gene-encoding adenovirus vectors locally into the NTS or RVLM of rats in vivo [13, 14]. Blood pressure and heart rate were monitored using a radio-telemetry system. We applied this technique to hypertensive rats and mice with heart failure to explore the pathophysiological role of NO and ROS in these cardiovascular disease states [11].

Role of NO in the brain stem in blood pressure regulation

Administration of N^G-monomethyl-L-arginine (L-NMMA), a non-selective NOS blocker, into the NTS produced an increase in renal sympathetic nerve activity and arterial pressure in anesthetized rabbits [15]. Furthermore, perfusion with L-arginine, a precursor of NO synthesis, or sodium nitroprusside, an NO donor, increased neuronal activity in neurons recorded in the NTS of brain stem slices [16]. The effects of NO in the NTS on the depressor response are caused by the facilitatory release of L-glutamate [17]. Intracisternal administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME), which inhibits NOS of medullary sites, increased arterial pressure and renal sympathetic nerve activity in anesthetized rabbits [18]. Activation of the renin-angiotensin system in the NTS is involved in the increased sympathetic nerve activity via angiotensin type 1 (AT1) receptors [19].

We extended the series of studies using a gene transfer technique in conscious rats. To increase in NO production locally in the NTS or the RVLM, we transfected an adnovirus vector encoding eNOS [20, 21]. We used adenovirus vectors encoding either the bacterial ß-galactosidase gene or the bovine eNOS gene. These adenoviral vectors were constructed in the Gene Transfer Vector Core Laboratory at the University of Iowa [22, 23]. Adult male Wistar-Kyoto rats (WKY) were used for gene transfer. The rats were anesthetized with sodium pentobarbital and placed in a stereotaxic frame. Microinjections were performed at six sites bilaterally in the NTS. An adenoviral suspension containing $1x10^8$ plaque forming units (pfu)/mL was injected into each injection site over a 5-min period. The RVLM was also identified by monitoring mean arterial pressure after the injection of a small dose of L-glutamate. Identification of the RVLM was confirmed according to the following criteria: (1) an increase in mean arterial pressure occurred immediately after the injection of L-glutamate, (2) the response plateau occurred within 20 seconds of the injection, and (3) the change in mean arterial pressure was greater than 20 mmHg [21]. An adenoviral suspension containing $1x10^8$ pfu/mL was slowly injected into each site over 15 min. The RVLM transfection was confirmed by immunohistochemical staining for phenylethanolamine-*N*-methyltransferase, an enzyme that catalyzes the final step of epinephrine synthesis and is identified specifically in the C1 neurons in

the RVLM. After the injection, all rats recovered from anesthesia and were kept unrestrained and free to move in their cages.

On day 7 after the gene transfer, animals were deeply anesthetized with an overdose of pentobarbital. Sections of the medulla (50 μm) were evaluated for either ß-galactosidase expression by X-Gal staining or eNOS expression by immunohistochemistry [20]. Western blot analysis for eNOS protein was performed and the expression level time course was examined [20]. Staining for ß-galactosidase was observed locally in the NTS or the RVLM of the section of the medulla, and ß-galactidase activity was also observed on day 7 after the gene transfer. Immunohistocemistry and Western blot analysis revealed a peak in the local expression of eNOS protein in the NTS (Fig. 2) and the RVLM (Fig. 3) on day 7 after the gene transfer.

The production of NO in the NTS or RVLM was measured as the nitrite and nitrate (nitrite/nitrate) level by in vivo microdialysis before and on day 7 after the gene transfer [20, 24]. The basal level of nitrite/nitrate in the NTS or the RVLM was significantly higher in the AdeNOS-transfected rats than in the Adßgal-transfected rats [20, 24].

The UA-10 telemetry system (Data Sciences International, St. Paul, MN, USA) was used to measure arterial blood pressure and heart rate. Mean arterial pressure and heart rate were recorded continuously for 10 min daily between 10:00 AM and 11:00 AM using a multichannel amplifier and signal converter. We measured the urinary norepinephrine concentration before and on day 7 after

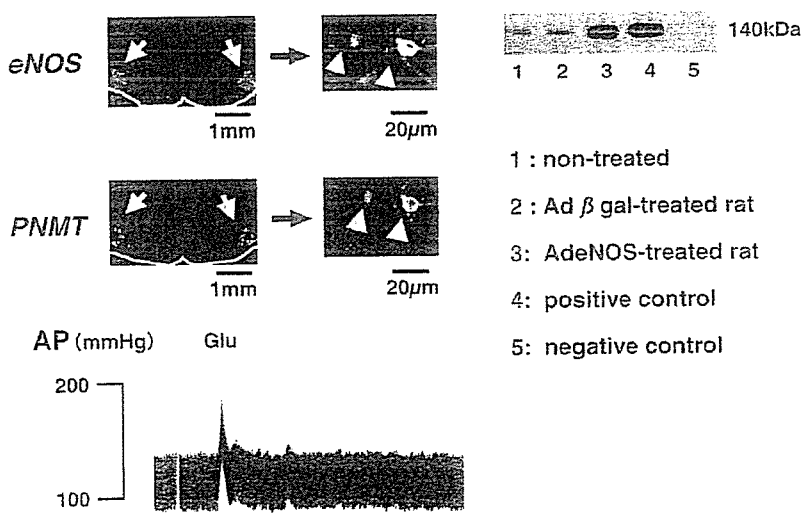

Figure 2. Expression of eNOS protein in the RVLM by immunohistochemistry and Western blot analysis (upper panel). The lower panel shows the pressor site in the RVLM by L-glutamate (Adopted and modified with permission from Kishi, T., et al., 2001).

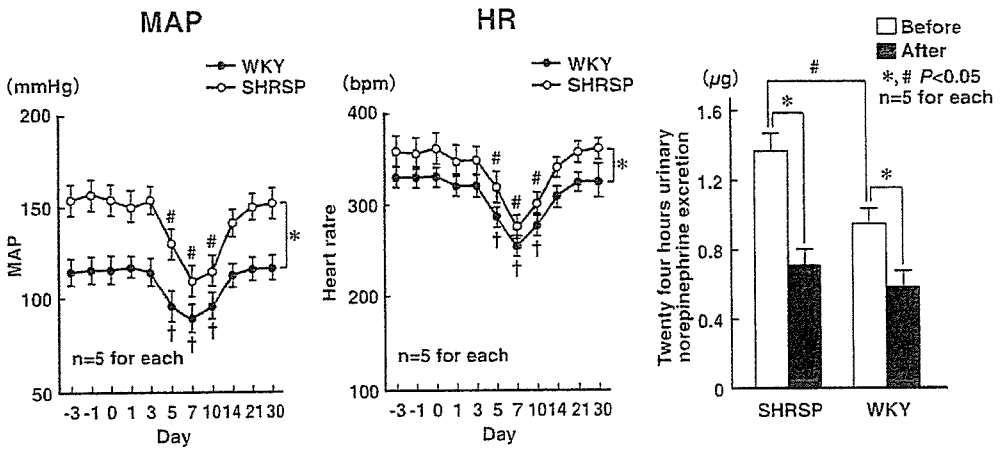

Figure 3. Time courses of mean arterial pressure (MAP) and heart rate (HR) after transfection of AdeNOS into the RVLM in WKY and SHRSP. Urinary norepinephrine excretion before and on day 7 after the gene transfer (Adopted and modified with permission from Kishi, T., et al., 2002).

the gene transfer by high-performance liquid chromatography, and calculated the urinary 24-h norepinephrine excretion.

Blood pressure and heart rate were decreased in rats transfected with AdeNOS into the NTS on days 5 to 10 after the gene transfer. In contrast, these variables did not change in the Adßgal-transfected rats [20]. Urinary norepinephrine excretion on day 7 after eNOS gene transfer was significantly decreased [20], suggesting that the decrease in blood pressure is mediated by the inhibition of sympathetic nerve activity. A similar response was observed in rats transfected with AdeNOS into the RVLM (Fig. 2) [21]. We suggest that these responses induced by the overexpression of eNOS in the NTS or RVLM might be mediated by the enhanced release of glutamate or GABA, respectively [4, 21].

Using an adenovirus vector, a dominant negative eNOS mutant was transfected into the NTS [25, 26]. Expression of a dominant negative eNOS mutant did not affect baseline cardiovascular parameters or baroreflex sensitivity in a rat working heart-brainstem preparation [25]. In conscious rats, however, transfection of a dominant negative eNOS mutant into the NTS decreases heart rate and increases spontaneous baroreflex gain measured using a time-series method [26]. They concluded that endogenous eNOS is constitutively active within the NTS and is involved in setting the baroreflex gain and resting heart rate [26]. The time course of peak changes of these parameters occurred on day 21 after the gene transfer, although complete recovery of these variables after transfection of the gene was not observed. It is not known why the transfected gene expression time courses differed among the studies.

As described above, we examined the effects of AdeNOS in the NTS or RVLM on cardiovascular responses. In a recent study, neuronal NOS (nNOS) gene transfer into the paraventricular hypothalamus was successfully performed [27], although the peak expression of nNOS was observed on day 3 after the gene transfer. Because endogenous nNOS is normally high in the hypothalamus, however, comparison of the expression levels of nNOS before and after gene transfer might be difficult. Thus, throughout our series of studies, we used eNOS instead of nNOS, which is normally abundant in the brain. In addition, the purpose of our series of studies was to increase NO production locally in the NTS or the RVLM for a much longer period in rats transfected with AdeNOS in an awake state. The role of iNOS in the RVLM in pathophysiological conditions such as hypertension, heart failure, and endotoxic shock remains unknown. Although overexpression of iNOS in the RVLM induces pressor and sympathoexcitatory responses, it might be different in a condition such as endotoxemia, in which iNOS is relatively acutely induced in many organs. In such a condition, NO and superoxide produced from iNOS might elicit caspase-dependent apoptotic cell death, thereby causing fatal cardiovascular depression [28]. Therefore, we examined the effect of iNOS overexpression [29] in the RVLM on blood pressure in vivo and found that it increased blood pressure and sympathetic nerve activity [24]. The results of this study suggest that an increase in oxidative stress induces activation of the sympathetic nervous system. Large amounts of NO might consume L-arginine, a precursor, and tetrahydrobiopterine, a co-factor, thereby inducing superoxide production, instead of NO, by uncoupling NOS. Interestingly, Huang et al. recently suggested that high levels of NO inhibit synaptic transmission in rat RVLM neurons by acting on presynaptic N-type Ca^{2+}-channels [30]. They suggested that this action is mediated by peroxynitrite formation and adenosine release [30]. In contrast, however, they also suggested that NO acts presynaptically to increase synaptic transmission on the RVLM neurons via the activation of presynaptic N-type Ca^{2+}-channels and that this presynaptic action of NO is mediated by the cGMP/PKG-coupled signaling pathway [31].

Role of NO in the brain stem in blood pressure regulation of hypertensive rats

Spontaneously hypertensive rats (SHR) or stroke-prone SHR (SHRSP) exhibit increased sympathetic nerve activity during the development of hypertension and there is a positive correlation between blood pressure and sympathetic nerve activity. Some studies suggest that there is a disorder of the L-arginine-NO pathway in SHR [32]. Therefore, we examined whether the effects of increased NO production in the NTS or RVLM of SHR or SHRSP

induced by the overexpression of eNOS on cardiovascular responses are different from those in WKY [33, 34]. We found that the overexpression of eNOS in the NTS elicited a greater depressor response in SHR than in WKY [33]. The depressor response evoked by microinjection of L-glutamate into the NTS did not differ between SHR and WKY, suggesting that the enhanced depressor response induced by the overexpression of eNOS did not result from a different response to inhibitory stimuli from the sympathetic innervation of peripheral vasculatures. Furthermore, intracisternal injection of L-NMMA elicited a greater pressor response in AdeNOS-transfected SHR than in Adßgal-transfected SHR, indicating that the decrease in blood pressure induced by the overexpression of eNOS is mediated by NO. The release of the major neurotransmitter L-glutamate might be altered by NO activity [17] in the brain of SHR, although we did not address this issue in our study.

In support of our findings, a recent study demonstrated that transfection of AdeNOS into the NTS elicits both hypotension and bradycardia in SHR on days 3 to 14 [34]. Within this period, microinjection of the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazole[4,3-alfa]quinoxalin-1-one reverses the depressor response induced by AdeNOS transfection. They also examined both NO and superoxide production in cultured cells before and after AdeNOS transfection, and reported an increase in NO production and a decrease in superoxide production after AdeNOS transfection, suggesting that AdeNOS transfection generates NO and scavenges superoxide [34].

The decrease in blood pressure, heart rate, and urinary norepinephrine excretion induced by the overexpression of eNOS in the RVLM is greater in SHRSP than in WKY (Fig. 3) [35]. Microinjection of bicuculline bilaterally into the RVLM on day 7 after gene transfer produced a gradual increase in blood pressure. In the non-transfected rats, the increase in blood pressure was significantly smaller in SHRSP than in WKY. In the AdeNOS-transfected rats, however, the pressor response induced by microinjection of bicuculline was significantly greater than that in non-transfected rats and did not differ between SHRSP and WKY [34]. Thus, the decrease in blood pressure induced by AdeNOS transfection was mediated by an increase in GABA release, and the increase in NO improved GABA release in AdeNOS-transfected SHRSP. Therefore, dysfunction of the NO pathway and the resulting disinhibition of the RVLM contribute to activate RVLM premotor neurons and increase sympathetic nerve activity in SHRSP. Furthermore, overexpression of eNOS in the RVLM of SHRSP improved the impaired maximum gain of baroreflex control of heart rate [36]. Interestingly, overexpression of eNOS in the NTS did not affect the baroreflex control of heart rate [36]. Thus, we suggest that GABA has a role in improving baroreflex control of heart rate. From the therapeutic aspects, it is interesting that atorvastatin decreases blood pressure via the reduction of sympathetic nerve activity and this effect may be mediated

by an increase in NO production with the upregulation of eNOS expression in the brain [37].

Role of ROS in the brain stem in blood pressure regulation of hypertensive rats

The involvement of reactive oxygen species such as superoxide and hydroxyl radicals is implicated in the pathogenesis of hypertension [38]. The brain contains a high concentration of polyunsaturated fatty acids in its cell membranes. These fatty acids are targets of oxygen-derived free radicals. Thiobarbituric acid-reactive substances (TBARS), end products of lipid peroxidation and an indirect marker of oxidative stress, are increased in the brains of SHRSP compared with those of WKY [39]. In addition, the intensity of electron spin resonance signals taken from the RVLM tissue decreases more rapidly in SHRSP than in WKY [39], suggesting that reactive oxygen species production is greater in the RVLM of SHRSP than in WKY. To confirm the role of reactive oxygen species in the RVLM in SHRSP, we transfected adenovirus vectors encoding the manganese superoxide dismutase (MnSOD) gene (AdMnSOD) bilaterally into the RVLM [39]. Western blot analysis revealed that MnSOD expression was significantly increased in the tissue from the RVLM of the AdMnSOD-transfected SHRSP to the same level as that of WKY on day 10 after the gene transfer. MnSOD activity was also increased in SHRSP on day 10 after the gene transfer. TBARS levels were significantly decreased in the RVLM of AdMnSOD-transfected SHRSP compared with non-treated SHRSP. On day 10 after the gene transfer, mean arterial pressure and heart rate of AdMnSOD-transfected SHRSP were significantly decreased compared with non-treated SHRSP, but not WKY (Fig. 4). Urinary norepinephrine excretion was significantly decreased in AdMnSOD-transfected SHRSP, but not in WKY. Taken together, these results suggest that increases in oxidative stress in the RVLM contribute to the central nervous system mechanisms underlying hypertension in SHRSP.

It is also suggested that interaction between NO production and ROS alters central sympathetic outflow [40, 41]. In support of our findings, a recent study suggests that increased superoxide anions in the RVLM contribute to hypertension in SHR via interactions with NO [42]. They reported a reduction in MnSOD expression and activity in the RVLM [42]. Microinjection of manganese (III)-tetrakis-(4-benzoic acid) porphyrin (MnTBAP), novel superoxide dismutase mimetic, bilaterally into the RVLM induced a greater reduction in blood pressure, heart rate, and sympathetic nerve activity in SHR than in WKY. Microinjecion of MnTBAP on day 10 after transfection of AdeNOS into the RVLM further decreased blood pressure to the level of that in WKY, suggesting that overexpression of eNOS in the RVLM is not sufficient

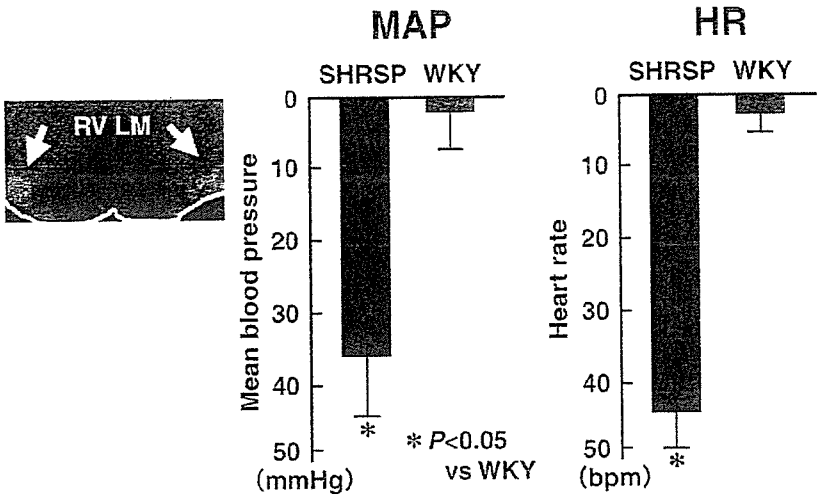

Figure 4. Immunohistochemistry of AdMnSOD expression in the RVLM and changes in mean arterial pressure (MAP) and heart rate (HR) after AdMnSOD gene transfer into the RVLM of SHRSP and WKY (Adopted and modified from Kishi, T., et al., 2004).

to remove excess superoxide anions. Another important point in their findings is that Cu/ZnSOD expression and activity were similar between SHR and WKY, although MnSOD expression and activity were reduced in SHR compared with WKY, suggesting that the scavenging enzymatic system is different between the two strains.

Changes in blood pressure, heart rate, and drinking behavior elicited by injection of angiotensin II in the brain are abolished by prior treatment with AdMnSOD or AdCu/ZnSOD [43]. Furthermore, a requirement for Rac1-dependent NAD(P)H oxidase in these cardiovascular and dipsogenic actions of angiotensin II in the brain was demonstrated using adenovirus-mediated expression of dominant-negative Rac1 [44]. Thus, the sources of reactive oxygen species in the brain in hypertension should be examined. A recent study suggest that downregulation of gene expression and enzyme activity of the antioxidant Cu/ZnSOD, MnSOD, or catalase may underlie the augmented levels of superoxide and hydrogen peroxide in the RVLM thereby leading to oxidative stress and hypertension in SHR [45]. It is important to examine how oxidative stress increases neuronal activity in the RVLM at the cellular levels. It remains determined, however, some studies suggest that p38 mitogen-activated protein kinase is activated by superoxide [46], inhibition of potassium channels or the increase in intracellular Ca^{2+} concentration are involved [47, 48]. Apparently, further studies needed to clarify this question. It is interesting to examine whether orally administered antihypertensive drugs reduce oxidative stress in the brain. We found that amlodipine reduces ROS production in the brain stem associated with sympatho-inhibitory effect [49].

Role of NO and ROS in regulation of the sympathetic nervous system in heart failure

Chronic heart failure is characterized by enhanced sympathetic drive and this further deteriorates the disease state in patients with heart failure [10]. Decreased NO production within the brain is one of the mechanisms underlying enhanced sympathetic outflow in an animal model of heart failure [50-52]. In fact, in situ hybridization and Western blot analysis demonstrate reduced nNOS expression in rats with myocardial infarction [52]. Therefore, we transfected AdeNOS into the NTS to increase local NO production in the NTS of mice with heart failure. Heart failure was induced in the mice by ligating the left coronary artery, thereby producing a myocardial infarction [53]. Western blotting analysis and immunohistochemistry staining for nNOS revealed reduced nNOS expression in the NTS in mice with heart failure. AdeNOS transfection into the NTS in mice with heart failure reduced urinary norepinephrine excretion, suggesting that increased NO production induced by overexpression of eNOS in the NTS attenuates the enhanced sympathetic drive in this model.

nNOS gene transfer with an adenovirus as a vector into the RVLM improves baroreflex control of heart rate and renal sympathetic nerve activity in rats with chronic heart failure [54]. This observation suggests that reduced NO production in the brain stem contributes to impaired baroreflex function in heart failure. Furthermore, intracerebroventricular injection of AdCu/ZnSOD reduces sympathoexcitation in mice with myocardial infarction [55]. In addition, a recent study demonstrates that symvastatin normalizes autonomic function in rabbits with pacing-induced heart failure by inhibiting central superoxide production [56]. NO bioavailability might be influenced by the generation of reactive oxygen species in the brain, thereby modifying sympathetic nerve activity.

Conclusions

In conclusion, the series of our study strongly suggest that NO and ROS in brain stem play an important role in central cardiovascular control. In particular, abnormality of these molecules production in the brain stem contributes to neural mechanism(s) of hypertension and heart failure. Therefore, these molecules in the brain may be new targets for the treatment of such disease status.

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4 活性酸素種産生系 ミトコンドリア電子伝達系

はじめに

ヒトは，1日2,000～2,500 kcalものエネルギーを，主としてミトコンドリア呼吸鎖によって得ている。このため，細胞内小器官としてのミトコンドリアは，大量の酸素を消費しており，同時に，常に微量（数%）の活性酸素種（ROS）を産生している。本稿では，ミトコンドリアの電子伝達系の成り立ち，活性酸素産生のメカニズムについて概説する。

ミトコンドリア電子伝達系の成り立ち

ミトコンドリアをもつ真核生物は，酸素呼吸によりグルコースをCO_2とH_2Oにまで完全酸化することにより，効率よく大量のATPを産生する。ミトコンドリア内膜に存在する，ミトコンドリア電子伝達系では，TCA回路で生じたNADHやコハク酸から電子を受け取り，最終的には酸素分子を還元する。電子伝達系には，約70種の蛋白質が関与し，複合体Ⅰから Ⅳ，ATPシンターゼおよびANPトランスロケーターなどを構成している。（図1）電子伝達の過程で放出された自由エネルギーを化学エネルギーに変換してATPを産生するメカニズムは，電子伝達（酸化）によって得られたエネルギーがプロトン濃度勾配によって生じる内膜の電気化学ポテンシャルに変換され，プロトンポンプと共役したATP合成酵素によってATPが合成される，いわゆる化学浸透圧説[1]として知られる。ミトコンドリア電子伝達系では，

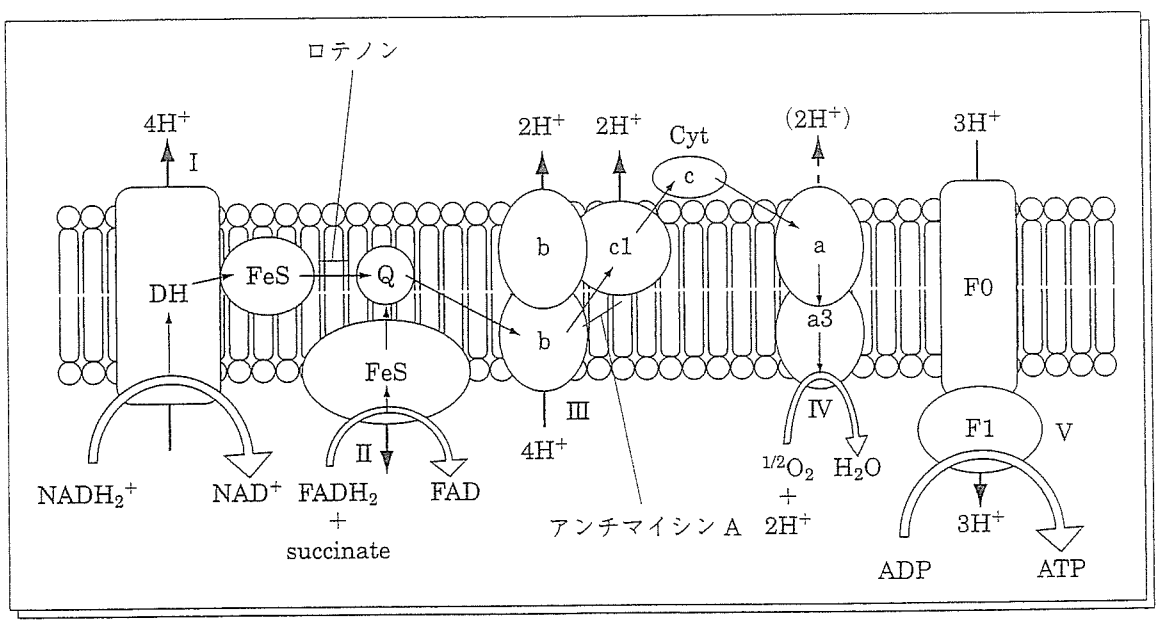

図1　ミトコンドリア電子伝達系と電子の流れ

電子伝達に伴い複合体Ⅰ，Ⅲ，Ⅳがプロトンをマトリックスから膜間腔に汲み出し，内膜に電気化学ポテンシャルを形成する。この電気化学ポテンシャルを利用して，F0F1-ATPase（複合体Ⅴ）がATPを合成している（図1）。複合体Ⅰ（NADH-ユビキノン酸化還元酵素），複合体Ⅲ（ユビキノール-チトクロームc酸化還元酵素），複合体Ⅳ（チトクロームc酸化酵素）はミトコンドリアDNAと核DNA両方でコードされ，複合体Ⅱ（コハク酸-ユビキノン酸化還元酵素）は，核DNAのみでその構成因子がコードされている。

ミトコンドリア電子伝達系からのスーパーオキシド産生と脂質過酸化反応

正常な状態でも，この電子伝達系は消費する酸素の数パーセントをスーパーオキシドとして産生するが，さらに，電子伝達系の異常によって電子伝達系複合体の機能低下が存在すると，さらなるスーパーオキシド産生をきたす。主な産生部位は，呼吸鎖の複合体Ⅰと複合体Ⅲであり，複合体Ⅰは，NADHを基質とし，ロテノンで阻害される。複合体Ⅲは，アンチマイシンAで阻害されると，スーパーオキシドが産生される（図2, 3）。スーパーオキシドの産生部位は，ユビセミキノンで，三重項酸素に一電子が付加されて，スーパーオキシドが産生されると考えられる。

一方で，脂質過酸化とは，リン脂質などに含有されている側鎖に，分子状酸素が付加さ

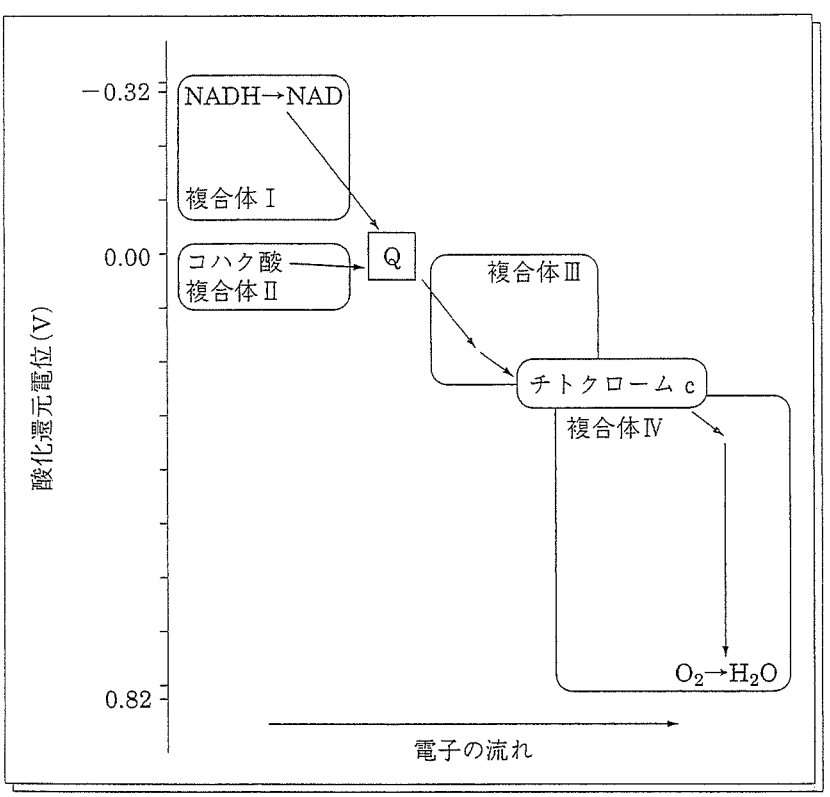

図2　ミトコンドリア電子伝達系と酸化還元電位

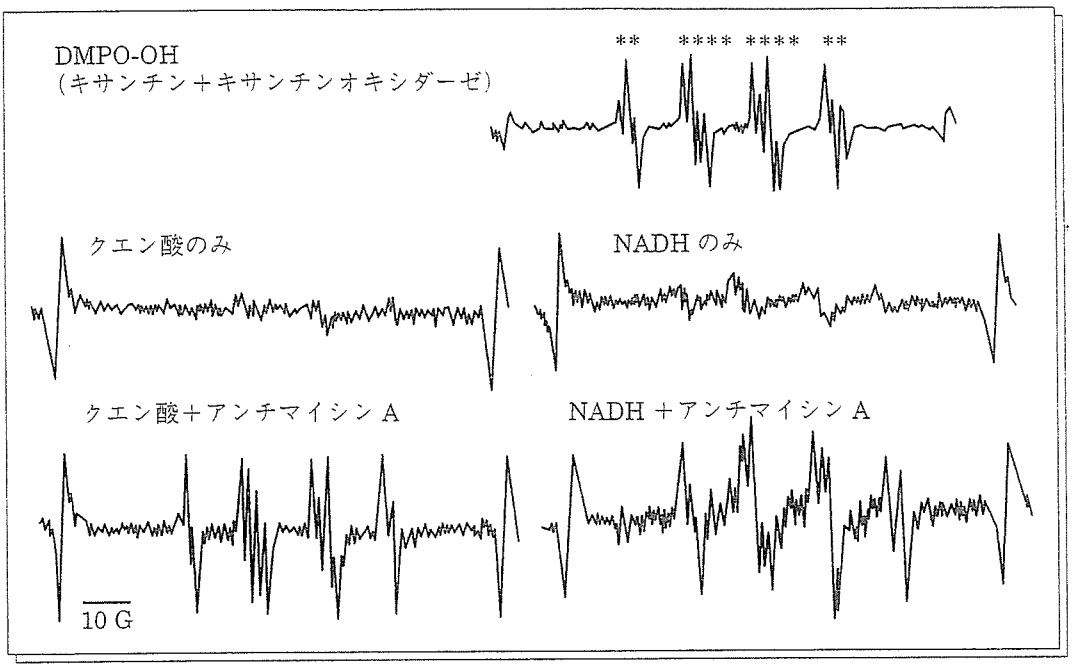

図3　DMPO-OH の ESR シグナル
ミトコンドリア亜粒子に基質（NADH またはクエン酸）を加え，アンチマイシン A によって複合体Ⅲを阻害すると，スーパーオキシドが産生される。

れる反応である。脂質 LH に，活性酸素などのイニシエーターが作用し，水素引き抜き反応が生じ，生じた脂質ラジカル L· はただちに周囲に存在する酸素と反応し，ペルオキシラジカル LOO· となる。このペルオキシラジカルは，他の脂質から水素を引き抜くことでより安定な過酸化脂質 LOOH となり，このような水素引き抜き反応が連鎖的に生じると考えられている。

ミトコンドリア電子伝達系の異常とミトコンドリア病

ミトコンドリア電子伝達系は，前述のように，核でコードされたサブユニットとミトコンドリア DNA でコードされたサブユニットによって構成されている。ミトコンドリア DNA は，前述のような電子伝達系の蛋白のうち，13 種をコードしている。したがって，ミトコンドリア電子伝達系に関する，いずれかの部分の遺伝子異常によって，ミトコンドリア病は，発症しうることになる。たとえば，特定の tRNA 遺伝子の A8322G 変異によるものは，MERRF （myoclonic epilepsy, myopathy, and ragged red fiber）と呼ばれ，複合体Ⅰおよび Ⅳの減少を認める。Kearns-Sayre 症候群では，欠失型突然変異型ミトコンドリア DNA の蓄積が原因となって生じる。これらミトコンドリア遺伝子疾患の発症は，ミトコンドリア DNA の突然変異の蓄積によることが知られており，心筋症を含め，脳症や家族性糖尿病など，多彩な病態を示す。現時点では，後天性の心疾患に突然変異型ミトコンドリア DNA の蓄積に関する検討は報告されていないが，老化個体や一部の糖尿病でごく少量の突然変異型 DNA の検出がなされたことから，ミトコンドリア遺伝子疾患としての心筋症以外にも，ミトコンドリア DNA の突然変異の蓄積が，慢性疾患の

病態に関与している可能性も示唆されている[2]。

ミトコンドリア DNA と酸化障害

近年，酸化ストレスが細胞の老化やアポトーシスの原因の１つであり，心血管病の成因，増悪因子として重要であることが明らかとなってきている[3]。ミトコンドリアの電子伝達系は，酸化還元反応を繰り返し，最後に酸素分子を還元する反応系であり，酸化ストレスの主要な産生源であると考えられる。最近では，心筋のみならず血管やインスリン抵抗性においてもミトコンドリア由来の酸化ストレスが重要な役割を果たしていることが報告され[4,5]，今後電子伝達系の活性制御が，心血管病の予防と治療の鍵となるであろう。

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（井手　友美）

15 酸化ストレスマーカーの測定
電子スピン共鳴（ESR）法

はじめに

近年，脳梗塞や心血管病，糖尿病，癌などの疾患に活性酸素やフリーラジカルの関与が示唆されている。これらの酸化ストレスが疾患の発症あるいは進展にどのように関与しているのかを明らかにすることは，抗酸化の観点からみた疾患の予防や治療，または新規治療薬の開発に役立つと考えられる。そのためには，生体内で，どの時期に，どのフリーラジカル種が，どこで，どの程度生成し，疾患形成に関係しているのかを明らかにすることは非常に重要である。

電子スピン共鳴（electron spin resonance; ESR）法は核スピン共鳴（nuclear magnetic resonance; NMR）法と同じく電磁気学的分光法の１つで，フリーラジカルを特異的に検出する分光法であり，生体計測用 ESR も市販されている。しかし，生体内で生成するフリーラジカルは非常に微量で短寿命であるため，ESR 装置の感度では直接計測することが困難である。ESR 装置を用いて生体内で生成するフリーラジカルを検出する手法に，スピントラップ法とニトロキシルプローブ法がある。前者は，主に個体から臓器ホモジネートや組織凍結切片，細胞画分などの生体試料を調製し，試料中にスピントラップ剤を添加した後に ESR 計測を行い，その ESR スペクトル波形からフリーラジカル種を同定する手法である。一方，後者は生体計測に利用され，比較的安定なニトロキシルラジカルを生体に投与し，生体計測 ESR 装置で無侵襲的に ESR スペクトルを計測し，その波形の変化の程度，およびその変化に対する各種フリーラジカル消去剤，フリーラジカル産生酵素阻害薬の効果を調べることで，生体内のフリーラジカル動態を解析する手法である。本稿では，ESR の原理について簡単に説明し，スピントラップ法とニトロキシルプローブ法について解説し，その応用例について紹介する。

ESR

活性酸素や活性窒素の多くはフリーラジカルである。フリーラジカルは電子スピンを有するために常磁性（静磁場中で磁性を示すこと）であり，静磁場中でエネルギー状態が分裂する（ゼーマン分裂）。分裂したエネルギー差に相当する電磁波を照射すると，図１のように，よりエネルギー準位の低い基底状態にある電子スピン（β スピン）が電磁波のエネルギーを吸収して，より準位の高い励起状態の電子スピン（α スピン）に遷移する。この現象を電子スピン共鳴（ESR）と呼び，ESR 装置はこのエネルギーの吸収を検出する。ESR スペクトルから g 値や超微細結合定数，線幅などのパラメータが求められ，フリーラジカル自身の運動性や周囲のラジカル，酸素分子など常磁性物質との相互作用についての情報が得られる。

スピントラップ法

スピントラップ法は，不安定なフリーラジカル種を常磁性でないスピントラップ剤と反応させることにより形成した常磁性のスピン