Review

Assessment of oxidative stress and antioxidant property using electron spin resonance (ESR) spectroscopy

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The pathophysiology of hypertension or stroke is associated with an excess of ROS generation in the vascular system, and results in induction of various pathological cascades of cerebrovascular damage. We have demonstrated that electron spin resonance methods using a spin trap or spin probe will be useful for understanding redox status under conditions of oxidative stress in the spontaneously hypertensive rat or stroke-prone spontaneously hypertensive rat brain. We have used electron spin resonance imaging and noninvasive L-band electron spin resonance to characterize the higher degree of brain oxidative stress in the stroke-prone spontaneously hypertensive rat and spontaneously hypertensive rat than in the Wistar-Kyoto rat brain, and the lower extent of oxidative stress in the spontaneously hypertensive rat than in the stroke-prone spontaneously hypertensive rat brain. Indeed, we may be able to confirm propofol medium-chain triglyceride/long-chain triglyceride (MCT/LCT) as neuroprotective anesthesia and crocetin as antioxidant food factor against human stroke after screening for antioxidant properties in stroke models such as stroke-prone spontaneously hypertensive rat. Thus, our electron spin resonance biomedical application suggests that it could be used to assess antioxidant effects on oxidative stress in the brain using spontaneously hypertensive rat and stroke-prone spontaneously hypertensive rat. We hope that further advances in the instrumentation used for electron spin resonance imaging and the development of optimized nontoxic spin probes will make this technology even more promising for novel clinical prediction or noninvasive diagnosis of human stroke. After screening drugs or foods for antioxidant property using *in vitro* or *in vivo* electron spin resonance assessment, it will be possible to find and develop novel drugs or food factors with such properties for the prevention of stroke in the near future.

Key Words: **reactive oxygen species, hypertension, stroke, ESR, oxidative stress, antioxidant property**

It is well known that free radicals, including reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$) or hydroxyl radical ($HO^{\bullet}$), contribute to the development of several diseases by causing oxidative stress.[1] Oxidative stress induced by ROS occurs when the production of ROS exceeds the capacity of the cell to detoxify these potentially injurious oxidants using endogenous antioxidant defense systems; however, where an excessive amount of ROS is produced or defense mechanisms are impaired, oxidative stress leading to events such as lipid peroxidation may occur.[1–3]

One well-accepted example for the mechanism of ROS-induced vascular disease, such as hypertension, is that oxidative stress caused by excess $O_2^{\bullet-}$ scavenging endothelial nitric oxide ($^{\bullet}NO$; a vascular smooth muscle relaxant) could contribute to increased vascular smooth muscle contraction and hence elevated total peripheral resistance.[4–7] These phenomena would also appear to be important in contributing to several pathological conditions in the central nervous system (CNS), including stroke.[8–11] Therefore, ROS-induced oxidative stress has been implicated as a potential contributor to the pathogenesis of the ischemia-reperfusion CNS injury characteristic of hypertension and stroke.

Electron spin resonance (ESR) has been recognized as one of the most powerful techniques for the detection of ROS in the form of free radicals, in biological tissues and cells. It has been developed an ESR-based technique to determine the effects of reactions induced by ROS in biological systems *in vitro*[12,13] and *in vivo*[14–17] with an emphasis on a rodent disease model.[18–22] Regarding *in vitro* ESR applications, spin trapping technique is well known as ESR spin adduct to detect ROS, quantifying spin concentration in experimental systems.[12,13,23–28] Otherwise, for *in vivo* ESR applications, nitroxyl radicals are very useful as exogenous spin probes to measure free radical distribution, oxygen concentration, and redox metabolism in biological systems. It has been suggested that the blood brain barrier (BBB)-permeable molecule 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (MC-PROXYL) is a suitable spin probe for the study of free radical reactions induced by ROS in the brains of small animal models using *in vivo* ESR detection.[18–22,29–32] Using a combination of these ESR methods collectively focused on animal models of disease, recent results show that oxidative stress plays a key role in the vascular injury that occurs in hypertension and stroke. The spontaneously hypertensive rat (SHR) or stroke-prone SHR (SHRSP), models of essential hypertension or stroke, respectively, exhibit several characteristics of the increased oxidative stress induced by ROS.[18,20,33–39] This review will focus on oxidative stress induced by ROS, which have a critical role in the pathogenesis of vascular disease in the brain, in hypertension, or in stroke, using the rodent model. Thus, we demonstrate in this review that results obtained from our laboratory by *in vitro* or *in vivo* ESR techniques could serve as a useful basis for evaluating oxidative stress induced by ROS in the brain of a rodent disease model, such as SHR or SHRSP. Furthermore, ESR techniques may be applicable to the assessment of antioxidant properties of drugs or food factors used for clinical treatment of human ROS-induced conditions following their use for investigation into rodent disease models.

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ESR Spin Trapping/Spin Probe Methods

The only technique that can 'see' free radicals directly and specifically is ESR because it detects the presence of unpaired electrons. However, ESR detects only fairly unreactive radicals, since reactive ones do not accumulate to sufficiently high levels to be measured. One solution to this problem is to add 'spin traps' or 'spin probes', agents that intercept reactive radicals, reacting with them to form a stable radical that can be detected by ESR.[1,12] Thus, ESR has been recognized as one of the most powerful techniques for the detection of ROS in the form of free radicals, in biological tissues and cells. It has been developed an ESR-based technique to determine the effects of ROS-mediated reactions in biological experiments *in vitro*[12,13] and *in vivo*,[14–17] with an emphasis on rodent disease models such as the SHR and SHRSP models.[18–22] Regarding *in vitro* ESR applications, spin trapping technique is a well known technique for detecting and quantifying ROS concentrations in experimental systems. We previously reported various effects of ROS such as $HO^{\bullet}$,[23,28] singlet oxygen ($^1O_2$),[24,40] hydrogen peroxide (H_2O_2),[25] and $O_2^{\bullet-}$[26,27,41] in experimental biological systems using the ESR spin trapping technique.

Nitroxyl radicals are very useful as exogenous spin probes for the measurement of the free radical distribution in biological systems,[14,15,18–22,32,42–44] especially stable radicals such as nitroxyl radical 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) or 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (hydroxy-TEMPO). These are often used as spin probes for *in vivo* ESR, operating in the lower-frequency microwave bands (≤1 GHz), the so-called L-band, where the dielectric losses of biological systems are lower.[16,45] During the last decade, significant advances in L-band and *in vivo* ESR techniques have provided useful information on oxidative stress in biological systems.[44,46–48] The signal decay rate of the nitroxyl radical gives evidence of oxidative stress induced by ROS and changes of redox status in biological systems.[44,46,47,49] We have used L-band ESR to characterize the higher degrees of oxidative stress in the SHRSP and SHR than in the WKY brain, and the lower extent of oxidative stress in the SHR than in the SHRSP brain.[18–22] Significant advances in the field of ESR imaging in recent years have now made it possible to visualize the distribution and metabolism of free radicals, and the degree of tissue oxygenation *in vivo*.[50–52] *In vivo* ESR spectroscopy and ESR imaging are useful for investigation of the redox status of living organisms noninvasively. ESR imaging is particularly useful for determining the *in vivo* spatial distribution of free radicals in animals. It has been suggested that BBB-permeable MC-PROXYL is a suitable spin probe for the study of free radical reactions in the brain by *in vivo* ESR.[18–22,29–32] Since the permeability of the BBB to a compound is dependent on its lipophilicity and molecular weight, the partition coefficients between 1-octanol and water of various nitroxyl compounds have been measured (see Miura *et al.*).[30] The partition coefficient values of carbamoyl-PROXYL (0.87) and hydroxy-TEMPO (4.83) were consistent with those reported by Fuchs *et al.*[53] MC-PROXYL was more lipophilic (partition coefficient = 8.70) than carbamoyl-PROXYL and other spin probes, implying high permeability of the BBB to this compound. Our study also demonstrated that relatively large amounts of MC-PROXYL were distributed to the brain compared with the BBB-impermeable carbamoyl-PROXYL. This was observed using ESR imaging for 3D or ESR-computed tomography (CT) of the head regions of mice (Fig. 1).[18] The results of the several studies mentioned above indicate that the *in vivo* ESR/nitroxyl spin probe techniques employed herein could be a powerful tool to detect free radicals selectively and to monitor free radical reactions *in vivo*. Quantitative ESR analysis using MC-PROXYL has the potential to be useful for understanding redox status under conditions of oxidative stress in the rodent brain.[18–20,32]

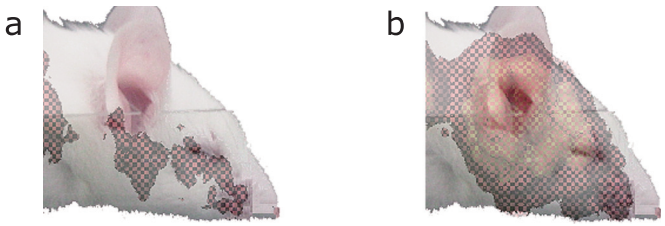

Fig. 1. Typical spatial 3D images obtained 3 min after treatment with carbamoyl-PROXYL (a) and MC-PROXYL (b) in the head region of a live mouse. Reprinted from ref. 18 with permission. To assign the imaged area, representative ESR images were superimposed on a photograph of the studied mouse.

Oxidative Stress and ROS in Vascular Disease

Oxidative stress occurs when the production of ROS exceeds the capacity of the cell to detoxify these potentially injurious oxidants using endogenous antioxidant defense systems. Conditions associated with oxidative stress include ischemia/reperfusion, hypercholesterolemia, diabetes, and hypertension. The adhesion of circulating blood cells (leukocytes, platelets) to vascular endothelium is a key element in the pro-inflammatory and prothrombogenic phenotype assumed by the vasculature in these and other disease states that are associated with oxidative stress. There is a growing body of evidence that links the blood cell-endothelial cell interactions in these conditions to the enhanced production of ROS.[54] It is well known that the mechanism of vascular disease, such as hypertension, is due to oxidative stress caused by excess $O_2^{\bullet-}$ scavenging endothelial $^{\bullet}NO$; this can contribute to increased vascular smooth muscle contraction and hence elevated total peripheral resistance.[4–7] We have also demonstrated that the biphasic concentration-dependent regulation of polymorphonuclear leukocyte (PMN)-induced $O_2^{\bullet-}$ generation by $NO^{\bullet}$-derived peroxynitrite ($ONOO^-$) may be of critical importance in regulating processes of inflammation such as ischemia-reperfusion (Fig. 2).[27] These small critical molecules exert opposing effects on vascular tone and chemically react with each other; this invalidates their individual effects and leads to the formation of $ONOO^-$ (Fig. 2). Because of the apparent importance of ROS (and in particular $O_2^{\bullet-}$) in vascular disease, many sources of $O_2^{\bullet-}$ have been investigated, including xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and leakage from mitochondria (Fig. 2).[26,27,55] It has demonstrated that the increased $O_2^{\bullet-}$ production in endothelial cells as well as in vascular smooth muscle cells occurs via a membrane-associated NAD(P)H oxidase (Fig. 2).[56–58] All cell types in the vascular wall produce ROS derived from $O_2^{\bullet-}$-generating protein complexes similar to the NADPH oxidase expressed in PMNs. Specific features of the vascular enzymes include constitutive and inducible activities, substrate specificity, and intracellular $O_2^{\bullet-}$ production. Interestingly, this oxidase is activated upon stimulation with angiotensin II (Fig. 2). This suggests that an activated circulating and/or local renin-angiotensin system, and subsequent increased vascular $O_2^{\bullet-}$ production, are common risk factors for hypertension (Fig. 2) or atherosclerosis.[59–61] Furthermore, our laboratory obtained the first evidence that $HO^{\bullet}$ and singlet oxygen ($^1O_2$) directly induced vascular contraction.[28,40] Taken together, the mechanism of development of hypertension may involve increases in ROS generation in the vascular system (Fig. 2).

Stroke initiates a complex cascade of metabolic events, several of which involve the generation of ROS. The vascular system is the first target of ROS generated in several pathological process,[62,63] and vascular damage is a prominent feature of embolic stroke.[64,65] ROS are formed at an accelerated rate in post-ischemic vascular systems. Endothelial cells and infiltrating leukocytes

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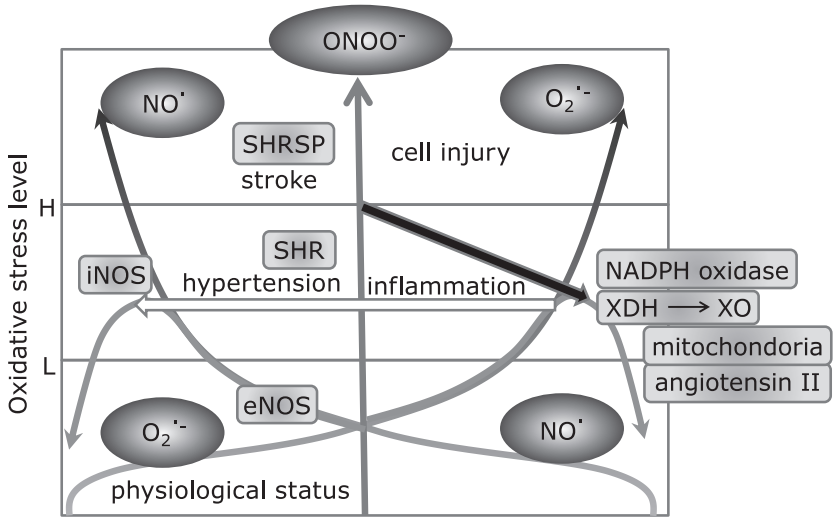

Fig. 2. Schemes relating to mechanisms of hypertension, such as the animal model spontaneously hypertensive rat (SHR), and of stroke, such as the animal model stroke-prone SHR (SHRSP), in terms of aspects of interaction among superoxide ($O_2^{\bullet-}$), nitric oxide ($^{\bullet}NO$), and peroxynitrite ($ONOO^-$). The level of oxidative stress (high, [H] and low, [L]) are indicated by the vertical bar. The relative concentrations of $O_2^{\bullet-}$, $^{\bullet}NO$, and $ONOO^-$ are indicated in each case by the gray arrow. NADPH oxidase = nicotinamide adenine dinucleotide phosphate oxidase, XDH = xanthine dehydrogenase, XO = xanthine oxidase, eNOS = endothelial nitric oxide synthase, iNOS = inducible nitric oxide synthase. This figure shows that the biphasic concentration-dependent regulation of $O_2^{\bullet-}$ generation by $NO^{\bullet}$-derived peroxynitrite ($ONOO^-$) may be of critical importance in regulating processes of inflammation[27] and cell injury such as hypertension/stroke. Because of the apparent importance of ROS (and in particular $O_2^{\bullet-}$) in vascular disease, many sources of $O_2^{\bullet-}$ have been investigated, including XO, NADPH oxidase, and leakage from mitochondria,[26,27,55] and in endothelial cells as well as in vascular smooth muscle cells via a membrane-associated NAD(P)H oxidase,[56–58] which was activated upon stimulation with angiotensin II. The exess $O_2^{\bullet-}$ can be scavenged by $NO^{\bullet}$ synthesized by iNOS leading to the the formation of the potent toxic $ONOO^-$ (indicated white arrow). Under physiological conditions (below low-level of oxidative stress L), $O_2^{\bullet-}$ can be scavenged by $NO^{\bullet}$ synthesized by eNOS with the formation of the $ONOO^-$ due to up-regulated $O_2^{\bullet-}$. The decrease in excess $O_2^{\bullet-}$ generation caused by $NO^{\bullet}$-derived $ONOO^-$ (indicated by black arrow) may be of critical importance in regulating processes during inflammation (up to the high level of oxidative stress H).[27] The mechanism of hypertension may be explained by oxidative stress causing excess $O_2^{\bullet-}$ scavenging which could contribute to increased vascular smooth muscle contraction and hence elevated total peripheral resistance.[4–7] The excess $ONOO^-$ generation resulting from excess $NO^{\bullet}$ generation is of greater importance in states of ROS-induced cell injury such as stroke than in hypertension because a large amount of $O_2^{\bullet-}$ could be generated by XO, NADPH oxidase, and mitochondria, and angiotensin II. Thus, ROS released acutely in large amounts (over the level of oxidative stress H) have been implicated in the cell injury associated with vascular ischemia-reperfusion injury such as stroke.

contribute to this ROS production. We have already confirmed the generation of ROS, mainly $O_2^{\bullet-}$, via the NADPH oxidase-derived oxidant burst of activated PMNs,[27] or the xanthine-xanthine oxidase system (Fig. 2).[26] Furthermore, there is evidence that ROS may play a critical role in vascular reactivity in cerebral arteries; $O_2^{\bullet-}$ may then escape into the extracellular space via an anion channel,[63] after which $O_2^{\bullet-}$ rapidly dismutates to H_2O_2 through catalytic reaction with superoxide dismutase. Potent ROS, $HO^{\bullet}$, can be formed by the Haber-Weiss reaction from $O_2^{\bullet-}$ and H_2O_2. However, this reaction is considered too slow to compete with the dismutation reaction.[66] $HO^{\bullet}$ can be generated more efficiently via the Haber-Weiss reaction, if ferrous iron is present, via a reaction known as the biological Fenton reaction. Exposure of cellular components of vascular smooth muscle to exogenous ROS can lead to cellular dysfunction and necrosis. Thus, ROS released acutely in large amounts have been traditionally implicated in the cell death associated with vascular ischemia-reperfusion injury. Therefore, ROS are crucial factors in the pathogenesis of vascular incompetence following vascular damage, such as that due to stroke.

Activation of the NADPH oxidase-derived oxidant burst of PMNs is of more critical importance in stroke than in hypertension because a large amount of $O_2^{\bullet-}$ could be generated by NADPH oxidase expressed in PMNs (Fig. 2). This excess $O_2^{\bullet-}$ generation would be associated with the increasing formation of other ROS. Further PMN-derived $O_2^{\bullet-}$ can be scavenged by $NO^{\bullet}$, with the formation of the potent toxic $ONOO^-$ (Fig. 2).[27] Thus, it should be noted that severe hypertension and cerebrovascular diseases induced by excess ROS generation can lead to stroke. Therefore, we need to know the differences in the level of oxidative stress induced by ROS in hypertension and stroke. It is likely that this information will be useful in understanding mechanisms of hypertension or stroke in relation to oxidative stress induced by ROS.

Evaluation of Oxidative Stress in SHR and SHRSP Brain Using ESR

The development of genetic models for research on hypertension and stroke (SHR and SHRSP) has contributed to the ability to predict and prevent these conditions.[67–70] Cerebral ischemia and reperfusion cause delayed neuronal death in rodents such as Mongolian gerbils and SHRSP, both of which have been used as experimental stroke models.[39] The SHR, a model of essential hypertension, has several characteristics of increased oxidative stress (Fig. 2).[71,72] There is significant *in vitro* evidence indicating that $O_2^{\bullet-}$ contributes to increased systemic vascular tone in SHR.[34,73] It has also been shown by *in vivo* fluorescence microscopy that blood vessels of SHR generate excessive amounts of $O_2^{\bullet-}$.[33] Severe hypertension and cerebrovascular diseases develop in SHRSP.[36] ROS generation occurs after reperfusion and this free radical-induced oxidative stress can greatly damage neurons in the SHRSP.[74] Oxidative stress induced by excessive generation of $O_2^{\bullet-}$ in SHRSP tissues leads to increased hypertensive responses, which may be relevant to the pathophysiology of stroke (Fig. 2).[18,20,35,75] This would therefore be an important mechanism of oxidative stress for production of excessive ROS, mainly $O_2^{\bullet-}$, in animal models such as SHR or SHRSP (Fig. 2).

In vivo L-band ESR and L-band ESR imaging methods have

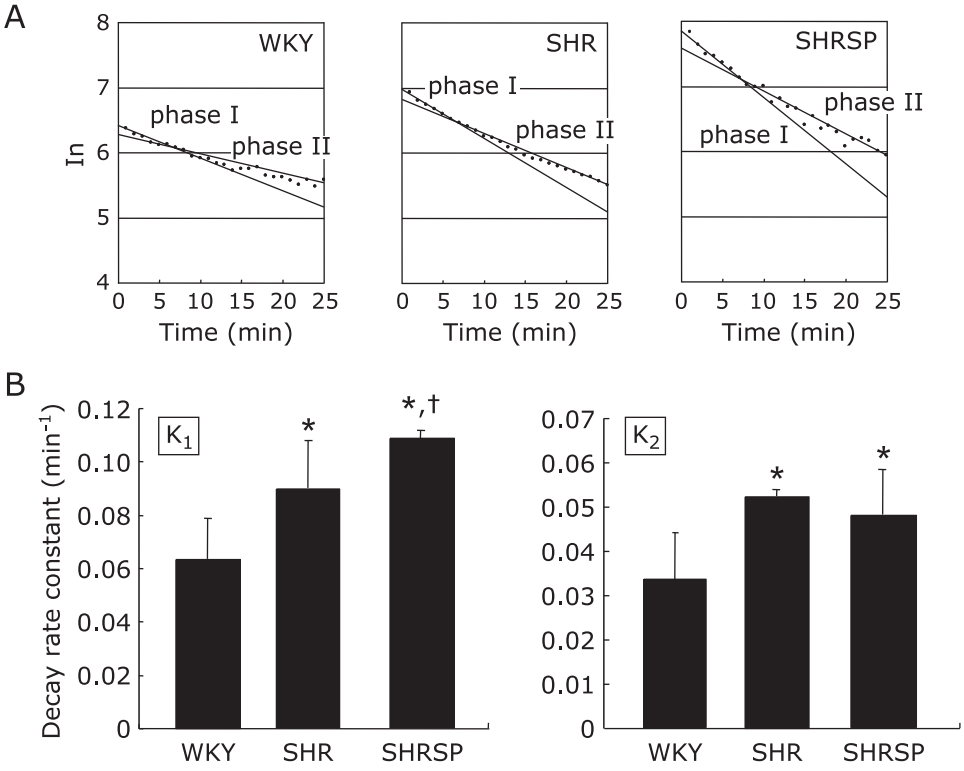

Fig. 3. L-band ESR signal decay rate constant of MC-PROXYL in the head region of live WKY, SHR, and SHRSP after *i.v.* injection of MC-PROXYL. Reprinted from ref. 20 with permission. Rats were treated with MC-PROXYL (140 mmol/l, 10 ml/kg) via the tail vein, and ESR spectra were measured at the head region of live rats. A, The logarithmic signal intensity of the second peak of the ESR spectrum of the nitroxyl spin probes was plotted against time. Linearity was observed in phase I and phase II of the corresponding semilogarithmic plots. B, K_1, and K_2 indicate the decay rate constant (min^{-1}) in phase I and phase II, as shown in A. Each column represents the mean ± SD (n = 3–6). *p<0.05 vs the corresponding value for WKY. †p<0.05 vs the corresponding value for SHR.

also been employed in brains from normotensive rats (Wistar-Kyoto rat (WKY)), SHR, and SHRSP using MC-PROXYL. We obtained pharmacokinetic profiles of MC-PROXYL ESR signals in the SHR and SHRSP brain. These can be divided into phase I and phase II, according to a two-compartment model of distribution, using *in vivo* L-band ESR (Fig. 3A).[18,20] Nitroxyl spin probes are administered systemically and are eliminated principally by a combination of excretion via the kidney and reduction to the hydroxyl amines.[18,20,76] The decay of the nitroxyl spin probe, MC-PROXYL, which is reflected in ESR signal intensity in the head region, appears to follow the usual pharmacokinetic pattern for excretion of drugs: those that tend to stay in the vascular system are excreted very rapidly (phase I) due to reduction by ascorbate, while lipophilic nitroxyl spin probes, such as MC-PROXYL, show much slower excretion via the kidney (phase II) (Fig. 3A).[76] The decay rate constants for MC-PROXYL in phase I (K_1) and phase II (K_2) in the brain were faster in SHR and SHRSP than in WKY. Furthermore, the phase I decay rates (K_1) in SHRSP were more rapid than in SHR, while no significant difference was observed in phase II decay rates (K_2) between SHRSP and SHR (Fig. 3B).[20] These results suggest that the higher oxidative stress in SHRSP brain occurred in the cerebral vascular system rather than in the tissue responsible for degrading the MC-PROXYL ESR signal in the brain. Taken together, our data indicated that the level of oxidative stress in the cerebrovascular system in rodent brain was in the following descending order: SHRSP>SHR>WKY (Fig. 3 and 6), which is the same descending order as for blood pressure in these animals. It has already been demonstrated that no differences exist between SHR and WKY with respect to blood flow in the heart, brain, lungs, spleen, and adrenal.[77] In spite of the large differences in arterial pressure, blood flow in the brain did not differ significantly between WKY and SHRSP.[78] Thus, the pathophysiology of hypertension and therefore stroke could involve increases in the level of oxidative stress by ROS generation in the cerebrovascular system (Fig. 3 and 6).

Direct Assessment of Antioxidant Property of Drugs or Food Factors Using *in vitro* or *in vivo* ESR

The most convincing evidence that oxidative stress induced by ROS generation is involved in brain disease such as stroke is the repeated observation that compounds that inhibit lipid peroxidation, or scavenge ROS, can prevent post-traumatic pathophysiological changes and promote functional recovery and survival in experimental studies. Nevertheless, the significance of ROS and lipid peroxidation ultimately depends on whether it can be demonstrated that early application of an effective ROS scavenger can promote survival and neurological recovery after CNS injury and stroke in humans.[8] It is well known that ROS scavengers may decrease edema and tissue damage in stroke.[79,80] Thus, the neuroprotective properties of anti-stroke agents could be associated with their antioxidant properties, indicating ROS scavenging activity. However, there is very little direct evidence of their antioxidant properties, specifically due to a lack of techniques for detecting ROS and for the assessment of oxidative stress levels *in vivo*, particularly in a suitable stroke animal model. Therefore, we need further studies using ESR techniques, both *in vitro* and *in vivo*, aimed at characterizing antioxidant properties of neuroprotective agents.

Propofol anesthesia has been associated with lower intracranial pressure and cerebral swelling than volatile anesthesia in brain tumor patients undergoing craniotomy.[81] The potential neuro-

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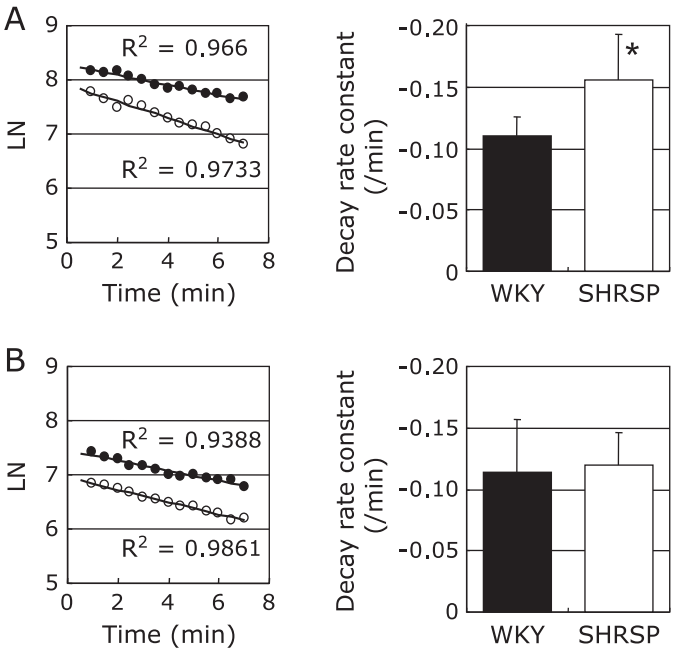

Fig. 4. Effects of propofol MCT/LCT on SHRSP-induced oxidative stress in the brain. Reprinted from ref. 21 with permission. Rats were anesthetized with pentobarbital (30 mg/kg, *i.v.*) (A) and propofol MCT/LCT (20 mg/kg, *i.v.*) (B). L-band ESR was used to determine the signal decay of MC-PROXYL in the head region of live WKYs and SHRSPs after *i.v.* injection of MC-PROXYL. Rats were treated with MC-PROXYL (140 mM, 5 ml/kg) via the tail vein, and ESR spectra were measured within the head region of live rats. The logarithmic signal intensity of the second peak within the ESR spectrum of the nitroxyl spin probes was plotted against time. Inset: Regression line fitted to a typical plot of L-band ESR data. Data are presented as mean ± SD of WKYs ($n = 5$) or SHRSPs ($n = 5$) with pentobarbital and with propofol MCT/LCT. *Significance $p<0.05$ from the corresponding control value of the WKYs.

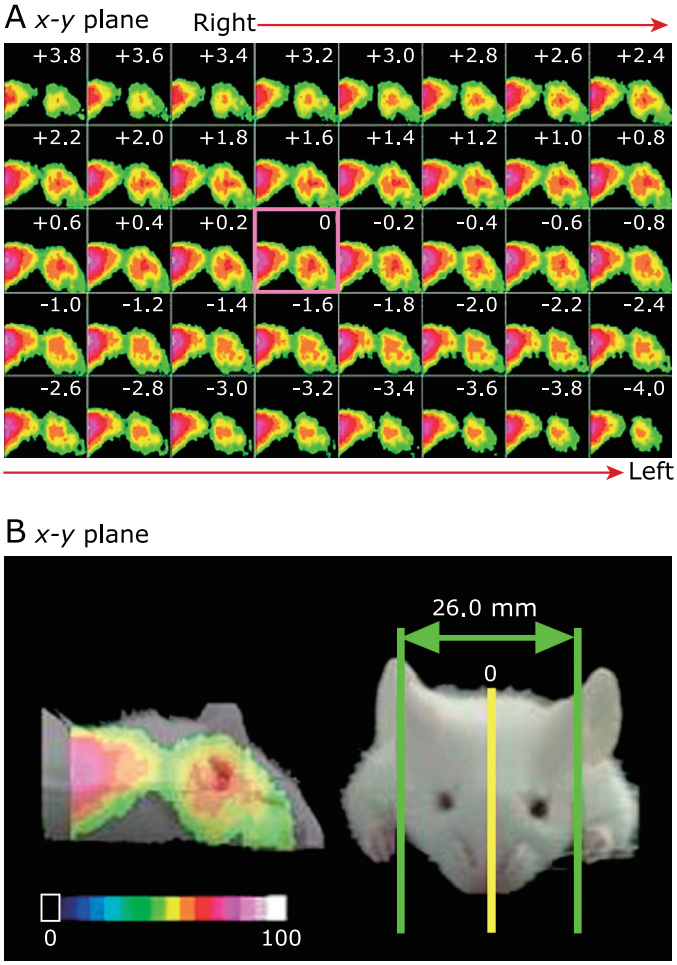

Fig. 5. A typical slice pattern of 3D ESR-CT images (*x-y* plane) obtained at 3 min after the treatment with MC-PROXYL in the head region of living mouse. Reprinted from ref. 19 with permission. To assign the anatomic position, representative ESR-CT images of MC-PROXYL in slices on the *x-y* plane (0, the midsagittal section) derived from the corresponding images in panel B are a superimposed picture for the studied mouse. As indicated by the attached color scale (16 colors; white and 100 being the maximum ESR signal), ESR images were reproduced in 16 colors and signals lower than 10% of the maximal signal intensity detected in all slices were regarded as noise.

protective effect of propofol may be mediated by its antioxidant properties, which have been shown to play a role in apoptosis, ischemia-reperfusion injury and inflammatory-induced neuronal damage,[81,82] due to reduction of lipid peroxidation via the generation of ROS.[81,83,84] Our present results provide the first direct evidence of the antioxidant properties of propofol and a vehicle such as medium-chain triglyceride/long-chain triglyceride (MCT/LCT) using *in vitro* ESR spin tapping technique. Interestingly, we found that the effects of the vehicle are most probably due to scavenging $HO^{\bullet}$ from the biological Fenton's reaction in tissues such as the brain.[21]

Secondly, we confirmed that oxidative stress in the brain of SHRSPs was higher than that in WKYs anesthetized with pentobarbital (Fig. 4A).[21] When propofol MCT/LCT was used as an anesthetic, rather than pentobarbital, the level of oxidative stress in SHRSP then became similar to that seen in WKY (Fig. 4B), suggesting that propofol MCT/LCT reduced SHRSP-induced oxidative stress in the brain.[21] Consequently, propofol MCT/LCT could be particularly useful in adjusting levels of anesthesia in cases of ROS-induced brain disease.

Crocetin is a natural carotenoid compound found in the stigmas of saffron (*Crocus sativus* L.) and the fruits of *Gardenia jasminoides* Ellis. This yellow compound has been used as an important spice and natural food colorant in various parts of the world.[85] In addition, saffron and gardenia fruits have been used as traditional medicine and crocetin is one of the major active compounds of these herbal medicines. In the present study, we used the ESR technique to investigate the ROS scavenging effect of food factors such as crocetin and the decay rate constant of MC-PROXYL in the isolated brain of the SHRSPs.[22] The results showed that crocetin to SHRSPs was capable of reducing ROS-mediated oxidative stress in the brain due to a direct antioxidant effect,[22] indicating that ESR technique could be useful for the assessment of the antioxidant property of food factors such as crocetin.

A better knowledge of clinical and laboratory markers of functional outcome would result in a more individualized and possibly improved approach to management of patients with oxidative stress-induced diseases such as hypertension or stroke. It is well accepted that infarct size as detected in neuroimaging studies constitutes a strong predictor of clinical outcome. However, CT scan or brain magnetic resonance imaging (MRI) information concerning the extent of a cerebral infarction is usually available too late after clinical onset to be of help in the decision-making phase. The predictive value of neuroimaging is then limited to the long-term phase of stroke. As a result, prediction of stroke outcome basically relies on clinical findings. As previously mentioned, we have now developed an *in vivo* ESR imaging system

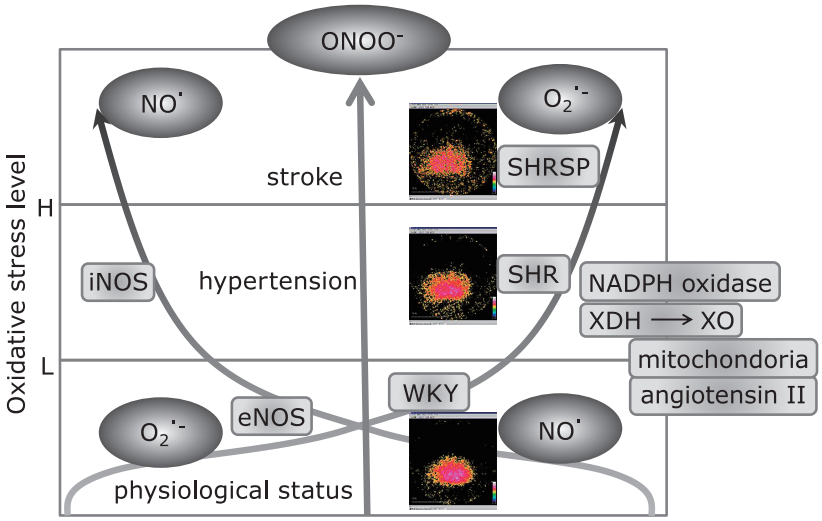

Fig. 6. Scheme of the proposed relationship between oxidative stress measured by ESR imaging and the severity of vascular disease in rodent disease models, spontaneously hypertensive rat (SHR) and stroke-prone SHR (SHRSP), in terms of aspects of interaction among superoxide ($O_2^{\bullet-}$), nitric oxide (•NO), and peroxynitrite ($ONOO^-$). The level of oxidative stress (high, [H] and low, [L]) are indicated by the vertical bar. The relative concentrations of $O_2^{\bullet-}$, •NO, and $ONOO^-$ are indicated in each case by the gray arrows. The relative concentrations of $O_2^{\bullet-}$, •NO, and $ONOO^-$ are indicated in each case by the gray arrow. The inset in Fig. 6 shows 2D ESR projection images (*y-z* plane) of MC-PROXYL distribution in isolated brains of WKY, SHR, and SHRSP. ESR was measured at 11 min after *i.v.* treatment with MC-PROXYL (isolated 30 s after the treatment). As indicated by the attached color scale (16 colors; white and 100 being the maximum ESR signal), ESR images were reproduced in 16 colors and signals lower than 10% of the maximal signal intensity detected in all slices were regarded as noise. Reprinted from ref. 20 with permission. NADPH oxidase = nicotinamide adenine dinucleotide phosphate oxidase, XDH = xanthine dehydrogenase, XO = xanthine oxidase, eNOS = endothelial nitric oxide synthase, iNOS = inducible nitric oxide synthase, WKY = Wistar-Kyoto rat. This figure shows that the level of oxidative stress in the cerebrovascular system in rodent brain was in the following order: SHRSP>SHR>WKY according to the severity of vascular disease: stroke>hypertension>normotension. Insert; the results of ESR image also indicate that the level of oxidative stress in the cerebrovascular system in rodent brain was in the following order: SHRSP>SHR>WKY brain. They occurred in the cerebral vascular system rather than in the tissue responsible for degrading the ESR images of MC-PROXYL in the isolated brain after 11 min. *i.v.* injection via tail vein.[20]

with high-quality ESR-CT images or reconstructed 3D images by using MC-PROXYL in a rodent model (Fig. 5).[19] It should be noted that such a clinical indicator of acute stroke provides little information in relation to the prevention of stroke. Further advances in the instrumentation used for ESR imaging and in the development of optimized nontoxic spin probes will make ESR technology one of the most novel diagnostic tools for acute stroke or clinical predictors for prevention of stroke in the future. We have already indicated that our ESR assessment can characterize the degree of oxidative stress in the SHR model of hypertension and the SHRSP model of stroke using by ESR imaging system (Fig. 6).[20] It is likely that the level of oxidative stress using ESR assessment may give useful information on pathological developments in the progression from hypertension to stroke.

Another limitation is the lack of measures of antioxidants and antioxidant status within recently reported studies. In fact, very few studies measured any aspect of antioxidant. Future analysis of these measures would enable deeper interpretation of the findings. Since we have evaluated the degree of oxidative stress in the brain of SHR and SHRSP as mentioned above, this means that we have now effectively established novel *in vivo* ESR methods for assessment of antioxidant properties of drugs such as propofol MCT/LCT[21] or food factors such as crocetin[22] using SHR or SHRSP.

Conclusion

In conclusion, we have demonstrated that ESR methods using spin trap or spin probe will be useful for understanding redox status under conditions of oxidative stress in the SHR or SHRSP brain. We have used ESR imaging and noninvasive L-band ESR to characterize the higher degrees of oxidative stress in the SHRSP or SHR than in the WKY brain, and the lower extent of such stress in the SHR than in the SHRSP brain. Indeed, we may be able to confirm propofol MCT/LCT as neuroprotective anesthesia or crocetin as an antioxidant food factor against human stroke after screening for antioxidant properties in stroke models such as SHRSP. Thus, our ESR biomedical application suggests that it could be used to assess antioxidant effects on oxidative stress in the brain using the SHR and SHRSP animal models of disease. We hope that further advances in the instrumentation used for ESR imaging and the development of optimized nontoxic spin probes will make this technology even more promising for novel clinical prediction or noninvasive diagnosis of human stroke. After screening drugs or food factors for antioxidant property using *in vitro* or *in vivo* ESR assessment, it will be possible to find and develop novel drugs or food factors with such properties for the prevention of stroke in the near future.

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Abbreviations

BBB blood brain barrier

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carbamoyl-PROXYL	3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl
CNS	central nervous system
CT	computed tomography
ESR	electron spin resonance
$HO^{\bullet}$	hydroxyl radical
hydroxy-TEMPO	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl
MC-PROXYL	3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl
MCT/LCT	medium-chain triglyceride/long-chain triglyceride
NADPH	nicotinamide adenine dinucleotide phosphate
$^{\bullet}NO$	nitric oxide
$O_2^{\bullet-}$	superoxide
$ONOO^-$	peroxynitrite
PMN	polymorphonuclear leukocyte
ROS	reactive oxygen species
SHR	spontaneously hypertensive rat
SHRSP	stroke-prone SHR
SOD	superoxide dismutase
WKY	Wistar-Kyoto rat

Conflict of Interest

No potential conflicts of interest were disclosed.

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