Published in final edited form as:

Radiat Res. 2012 April ; 177(4): 514–523.

Oxidative Stress Imaging in Live Animals with Techniques Based on Electron Paramagnetic Resonance

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Abstract

Oxidative stress has been the object of considerable biological and biochemical investigation. Quantification has been difficult although the quantitative level of products of biological oxidations in tissues and tissue products has emerged as a widely used technique. The relationship between these products and the amount of oxidative stress is less clear. Imaging oxidative stress with electron paramagnetic resonance related magnetic resonance imaging, while not addressing the specific issue of quantification of initiating events, focuses on the anatomic specific location of the oxidative stress. Moreover, the relative quantification of oxidative stress of one location against another is possible, sharpening our understanding of oxidative stress. This promises to improve our understanding of oxidative stress and its deleterious consequences and enhance our understanding of the effectiveness of interventions to modulate oxidative stress and its consequences.

ASSESSMENT OF OXIDATIVE STRESS IN BIOLOGICAL SYSTEMS

Reactive oxygen species (ROS) are generated constantly in living cells as a byproduct of oxidative metabolism. Their deleterious effects on cell components are determined by the rate of generation of ROS in the cell and the concentration of low-molecular-weight antioxidants together with activities of enzymatic antioxidants. Factors increasing the rate of ROS generation (i.e. ionizing radiation or metal ions) as well as factors decreasing antioxidant capacity lead to oxidative stress, causing enhanced oxidative damage of cellular components. Oxidative stress usually leads to a decrease in antioxidant defense capacities or depletion of reducing ability of exposed tissue. Closely connected to oxidative stress is tissue redox environment, or redox state, understood widely as bioreductive capacity of the system, or, more precisely, as redox buffer capacity (1). Stronger oxidative stress produces greater changes in the redox state. Both terms are convenient operational concepts and still await the complete understanding of spatial and kinetic factors involved.

Oxidative stress is implicated in pathology of many diseases, such as diabetes, intoxication, neurological disorders and ischemia. Estimation of level of the oxidative stress in tissues is useful to determine the mechanisms and the role of ROS in these pathologies as well as the

extent and significance of antioxidant therapies. What is more, both initial redox state and oxidative stress generated might differ considerably among tissues and are influenced by other spatially differentiated factors, such as hypoxia. Therefore, 3D spatial mapping of oxidative stress is particularly informative in investigating the mechanisms of oxidative stress and related pathologies.

To confirm the presence of the oxidative stress, most often indirect detection of the oxidation products is used. Oxidative modifications of various cell components may serve as indicators of the oxidative stress, such as e.g. DNA/RNA damage, oxidative protein products or lipid peroxidation of lipid membrane components (2). In addition, measurement of the antioxidant defense system components, such as catalase, SOD or glutathione can be performed. Direct measurements of reactive oxygen species is possible using several methods, including EPR (3). A method most widely used in cellular studies employs probes that fluoresce when exposed to oxidation, such as dichlorofluorescein (4). These measurements are generally nonspecific and rely on the transformation of a chemical species that is added to the biological system. The transformation takes place in response to the presence of ROS, but they may react with other reducing or oxidizing biological components as well. Other free radicals, such as intermediate probe radicals, as well as ROS can be generated (5). Recently, fluorescent probes responding to more specific oxidative agents such as mitochondrial superoxide have become available (6). In the *in vivo* setting, the context of animal measurements, however, fluorescence at optical frequencies can be measured only from surface tissues no more than a few millimeters deep due to the limited tissue penetration of light. This significant absorption is dependent on various aspects of the tissues (e.g. skin pigmentation) that compromise quantification of the signal. Electron paramagnetic resonance (EPR) imaging, particularly at frequencies of 1 to 1.5 GHz or lower, can overcome these limitations and provides quantitative noninvasive, three-dimensional images of oxidative stress in living animals.

Imaging of the oxidative stress using EPR is based on the monitoring the signal of the paramagnetic redox-sensitive probe in a whole organism or a chosen part of the organism and analyzing the time-dependent decrease of this signal. The most commonly used redox-sensitive spin probes are nitroxides, which interact with many biological redox-active compounds, such as ascorbate, glutathione, flavins, redox enzymes, etc. Administration of specific inhibitors or enhancers of ROS will modify this decay and thus could provide more detailed insight into the redox state of the system. This approach was first studied spectroscopically *in vitro* in a wide variety of settings (7–10), then transferred into *in vivo* studies, and finally applied in imaging of oxidative stress.

Besides monitoring the redox state of tissues in an indirect way, EPR also enables the detection of specific free radicals directly, such as superoxide ions in biological systems by using spin trapping (11, 12). However, due to the usual low concentration of ROS in cellular environments, this technique is available as spectroscopy only and can presently be used for imaging only to a limited degree (13–16). When the concentration of the free radical is high enough, e.g. after irradiation, *in vivo* detection of generated hydroxyl radical is possible (17).

NITROXIDES AS REDOX-SENSITIVE PROBES IN BIOLOGICAL SYSTEMS

Redox-sensitive spin probes (nitroxides, aminoxyl radicals) are free radicals that are stable in solution. In the presence of many biological redox-active compounds and enzymes, such as ascorbate, glutathione, superoxide and others, they participate in redox reactions (7, 10, 18–20). Acceptance of an electron (reduction) leads to a hydroxyl-amine and to giving an electron (oxidation) to N-oxo ammonium salts (Fig. 1). In living cells or tissues both

reduction and oxidation reactions can occur, and all three forms can exist (21). Nitroxide/hydroxylamine pairs acts as a cycling antioxidant (22–24), and nitroxide/oxoammonium cation mimics the action of superoxide dismutase (25). These antioxidant properties of nitroxides make them good radioprotectors (26, 27). After administration of nitroxide into the cell or tissue, a decay in paramagnetic signal is observed, mainly due to reduction to hydroxylamines (7, 18). This decay is faster in hypoxic tissues (28–30). Hydroxylamine may be also reoxidized back to the nitroxide, and after *in vivo* administration of EPR-silent hydroxylamine, EPR signal reaches a similar level as after administering its nitroxide counterpart (31). Beside redox reactions, other pharmacokinetical mechanisms of nitroxide decay have to be considered, such as washout from the tissue of interest. For example, it has been shown that in the muscle the rate of nitroxide washout is 1–20 times higher than the rate of reduction (32). Effects such as diffusion through the tissue and recirculation by the blood were negligible (32). Therefore, a careful estimation of all the factors responsible for the decay in a particular model is required before drawing conclusions as to the redox state. A confirmation of the redox-sensitive role for nitroxides came from an MRI study, where pharmacokinetic images of these nitroxides in different tissues in mice were obtained simultaneously, as well as oxidized plus reduced forms measured in tissue extracts by EPR. These results verified that for cell membrane-permeable nitroxides, their metabolism, measured as reduction rate, reflects the intracellular redox status (33).

The discovery of the dependence of nitroxide paramagnetism decay on reduction-oxidation reactions in biological systems, suggested early on the possible application of these compounds to study redox environment (10). The ratio between the EPR-visible nitroxide form and the EPR-silent hydroxylamine form depends on the redox environment (34). Mid-range redox potential of this redox couple [$E/2 = 0.17\text{--}0.42\text{ V}$ compared to Ag/AgCl (19)], and the resulting large extent of redox reactions influencing the nitroxide/hydroxylamine ratio makes them a good marker of cellular redox state.

Oxidative stress causes an increase in the rate of nitroxide bioreduction, whereas the presence of antioxidants leads to a slower rate of nitroxide bioreduction. Therefore, the assessment of redox state using nitroxides might also be used for evaluation of oxidative stress. Usually a comparison of the nitroxide decay is made under oxidative stress conditions, and in the absence of oxidative factors, or alternatively, some scavengers of oxidative species can be applied. These relationships were studied first by EPR spectroscopy *in vivo* (35–37) before being applied to EPR redox state imaging.

RESONANCE TECHNIQUES FOR REDOX STATE IMAGING

There are three basic instrumental approaches to the resonance imaging of redox state *in vivo*: EPR alone, Overhauser enhanced MRI (OMRI) or MRI with nitroxides as contrast agents. Direct EPR imaging with time resolution allowing registering spin probe decay provides 2D or 3D oxidative stress images of tissues such as tumors, heart or brain *in vivo* (38–41). Spatial resolution in redox imaging with EPR is affected by linewidth of nitroxides (typically ~1 gauss) and their relatively short half-life *in vivo*. The half-life of nitroxides in most tissues is of the order of minutes (19), and such a time window allows probing of the tissue redox environment, as the experiments described below confirm. Prolonging the half-life of nitroxides *in vivo* is possible, but it might affect their function as a redox-sensitive probe. This severely limits the sensitivity of the method and spatial resolution of redox mapping using direct EPR imaging of nitroxides.

To improve spatial resolution, OMRI and MRI are used. The schematic comparison of the three approaches is shown in Fig. 2. All three techniques are based on the sensitivity of nitroxides to the redox state of the imaged tissue, but employing MRI provides more spatial

resolution and anatomical information in addition to the redox state. OMRI is a double resonance technique based on the Overhauser effect. A nitroxide is injected in the animal and the nitroxide electron is irradiated, changing the electron spin population. This stimulates changes in the water proton spin population through the Overhauser effect, enhancing the water proton signal in the sample. Water proton MRI is a readout of the nitroxide concentration and is used to obtain the image. Multiple MRIs obtained in this fashion show the reduction in enhancement in time and provides the high spatial resolution images of the nitroxide signal decay. In contrast, redox state imaging using MRI alone explores the use of nitroxides as T1-contrast agents (42, 43). This technique monitors the change in T1-weighted enhancement induced by the nitroxide in the images. The rate of decrease in this effect depends on the local transformation of nitroxide to hydroxylamine. Excellent reviews have been recently published describing redox imaging of tissues using MRI alone (44, 45); therefore, in this paper, we focus on EPR and OMRI techniques only.

DEVELOPMENT OF EPR REDOX STATE SPECTROSCOPY AND IMAGING

Intense development of imaging techniques in EPR in the 1980s developed in parallel with applications to biological objects. The first 1D² EPR image of a cross section of a celery stalk, obtained at L-band, was published in 1985 (46, 47). This technique was soon extended to a tumor in a living mouse. One-dimensional images (4 projections at 45° angles) of a tumor in a living mouse loaded with nitroxide were collected at L-band (48).

Small biological samples could be visualized at X-band. This approach was applied to study tumor cell spheroids (cellular aggregates of 100–500 µm in diameter). Two-dimensional images consisting of 32–64 projections were acquired within 6–12 min using a 90 G/cm gradient. An approximately 200-µm rim of live cells could be discerned in these images (49). However, studies in the living animals require bigger sample volumes than X-band allows, and further studies were pursued with lower frequencies.³ Nitroxide distributions were shown in a 3D image of a rat tail (50, 51) and 2D whole rat images (52). The same laboratory has also shown sequential 2D images of coronal plane of a rat abdomen, obtained in 5 min, with a spatial resolution of 8 mm (53). Nitroxide 3D distributions in a rat head (54) and 2D images of a whole mouse were performed using spin labeled dextran (55). Spectral-spatial images in living mice were collected using four different nitroxides, and their pharmacokinetics have been compared (56).

Animal organs excised and studied *ex vivo*, especially an isolated beating heart, were a useful model for developing the imaging techniques. Three-dimensional spatial EPR images of a rat heart using nitroxide have shown spatially defined differences in the rate of radical clearance (57). The same paper also presented 3D spectral-spatial images of rabbit aortas, where the aorta wall was clearly seen. This required 64 projections that were taken within 12 min using a 49 G/cm gradient (57). The main limiting factor in obtaining images of better resolution was the stability of nitroxides. For example, the resolution of images of isolated rat hearts obtained with a single line glucose char probe was much better (0.2 mm) than that obtained with nitroxides (1–2 mm) (58, 59). A solution to this problem used polynitroxyl-albumin of prolonged half-life, which allowed 3D imaging of a rat heart with submillimeter resolution. The image contained 144 projections collected in 18 min using a 20 G/cm gradient (60). To avoid artifacts caused by the motion of a beating heart, image acquisition was gated and synchronized with the heartbeat (61, 62). Imaging of larger animal specimens with EPR was accomplished with longitudinally detected ESR/EPR (LODESR) (63, 64).

²The number of spatial dimensions of the EPR image is described as 1, 2 or 3D. Spectral or temporal dimensions are mentioned separately.

³L-band (1–2 GHz), 700/750 MHz and 280/250 MHz.

EPR REDOX STATE IMAGING—STUDIES IN VIVO

Spatial Nitroxide Distribution Imaging

Several studies demonstrated the feasibility of redox state imaging using nitroxides in different organs. For example, a 2D image of rat cross section in the kidney region using OMRI technique was shown (65). Two-dimensional images of the whole body of a mouse were collected at S-band (2–4 GHz) every 6 min. This allowed for analysis of the signal decay from different organs. MRI has been used for identification of anatomical structure (66). Another study from the same group demonstrated 1D spatial imaging in human skin using a surface coil. Nitroxide (^{15}N -PDT) was applied topically, and then the rate of penetration into the skin was estimated with a single gradient perpendicular to the skin. Two distinct bands of nitroxide distribution along the depth of the skin were observed (67, 68). The surface coil approach was also used to generate 2D images of a slice of rat kidney. The images were taken every 2 min, and differences in the reduction of the signal from parenchyma and pelvis were detected (69).

Development of the imaging techniques was a stimulus that accelerated spin probe design. A new ester derivative for brain studies was introduced that was stable and reached high levels in the brain (70, 71). 3-Hydroxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl was also applied to brain imaging as a BBB-permeable spin probe. Two-dimensional slices of images of rat brain showing the distribution of the nitroxide were presented (72). Aceto-methoxy esters that are both persistent and concentrate in the intracellular environment have recently been developed (73).

Redox Imaging of Tumors using EPR

Demonstrating differences in the redox state of tissue requires measurement of nitroxide reduction rate, i.e. time resolution of the imaging technique of the order of minutes. Such a functional approach has been successful in many experimental setups.

Two-dimensional sequential images of RIF-1 tumors taken every 2 min confirmed a more rapid reduction of nitroxide in tumor tissue than in muscle (74, 75). In the same tumor type, heterogeneity in redox maps was observed as well as a reduction of the rate with decrease in GSH or an increase in oxygenation. Spatial resolution was $0.2 \times 0.2 \times 5$ mm (38, 76, 77). An example of redox mapping of tumors in animals breathing air and carbogen is shown in Fig. 3. Similarly, nitroxide reduction rate images from tumors on day 5, 7, 12 and 14 of tumor growth show heterogeneity in redox state and changes with time. In animals with depleted thiols, the reduction rate was 24 and 36% slower in normal tissue and tumor, respectively (78).

A differentiation of normal and tumor tissue was shown in a model of chemically induced gastric cancer in rat. The induction was correlated with generation of a high level of oxidative stress. Three-dimensional images showed a faster rate of reduction nitroxide in the tumor in comparison with normal gastric mucosa. These images were obtained using a surface coil that was introduced into the peritoneal cavity through a small incision (39).

Pulsed EPRI was used to obtain the spatial distribution of ^{15}N -PDT nitroxide in a murine tumor. The signal decayed rapidly below the noise level, preventing kinetics measurements, but a 3D nitroxide spatial distribution map was obtained and an oxygen map was calculated from the linewidths of the nitroxide (79).

Nontumor Oxidative Stress EPR Imaging

Because oxidative stress leads to a faster reduction rate of nitroxides, time-resolved nitroxide imaging allows visualization of oxidative stress *in vivo*. This approach has been employed in many types of tissues in the context of numerous important pathologies, such as diabetes, ischemia-reperfusion or intoxication.

Two-dimensional images of living mice with carbon-tetrachloride damaged livers have shown much a slower reduction rate in the liver region than in untreated mice (80). An interesting study was performed using 1D imaging of nitroxide reduction in the skin of a living rat. Diverse bioreduction rates of Tempo were shown in different layers of the skin with an image resolution about 0.1 mm. The reduction rate was decreased after UVB irradiation (67, 81).

Oxidative stress was also imaged in the diabetic mouse kidney. The nitroxide reduction rate was much faster in diabetic mice than in the control animals. After treatment with an angiotensin II type 1 receptor blocker, the reduction returned to control levels, confirming the antioxidant properties of this drug (82).

In the heart, myocardial ischemia is a known oxidative stress factor. An infarct region in the rat heart was detected using 3D nitroxide imaging. Faster nitroxide reduction was observed in the risk region (40, 83).

Free Radical Scavengers Clarify the Mechanisms of Oxidative Stress

More in-depth information about the mechanism of oxidative stress and related pathologies can be obtained by applying various free radical or ROS scavengers. Coadministration of specific radical scavengers with nitroxide has served to elucidate the role of specific components of oxidative stress (84). For example, in a diesel exhaust particle (DEP) inhalation model, intratracheal nitroxide reduction was significantly enhanced by DEP in thoracic region (85). The enhanced signal decay of the nitroxide was completely suppressed when radical scavengers were administered along with nitroxides such as dimethylthiourea, dimethylsulfoxide and mannitol. An iron chelator, desferrioxamine also suppressed the enhanced decay. Simultaneous administration of SOD accelerated the enhanced signal decay, while administration of catalase suppressed the acceleration, suggesting that intratracheal exposure to DEP produced hydroxyl radical in the lung through an iron-catalyzed reaction.

Another study employing the same approach to elucidate oxidative mechanisms was performed in a gastric ulcer model in rats (86). In the stomachs of NH₄OH-treated rats, nitroxide reduction was faster than that in the stomachs of saline-treated rats after intragastric administration. Coadministration of the hydroxyl radical scavengers mannitol or catalase suppressed the enhanced reduction of nitroxide in a dose-dependent manner.

Even though both of the above studies were performed spectroscopically, they illustrate well the potential scope of the oxidative stress EPRI for studying the mechanisms involved in oxidative stress.

Most *in vivo* redox state images have been acquired in mice and rats. Most images in rats have been in portions of rats, although Overhauser MRI experiments are carried out with radiofrequencies high enough to visualize human size subjects (87).

Redox Imaging of the Brain

Extensive redox imaging studies were conducted in the rat brain. Early studies focused on the distribution of nitroxides in the rat head (88–90). To obtain high levels of nitroxide in the

brain, a continuous infusion was used (91). In time, new blood-brain barrier-permeable nitroxides were developed, and their rates of reduction were compared in the rat cerebral cortex, striatum and hippocampus area (92).

A differential response of hippocampus and cerebral cortex to kainic-acid induced seizure was detected using EPR redox imaging (89). The ability of the intrahippocampus to reduce the nitroxide radical was impaired in the epileptic rats and was intact in the cerebral cortex. These findings were confirmed using an acyl-protected hydroxyl-amine, which was oxidized to form a nitroxide (93). The results showed that the oxidative stress in the hippocampus and striatum was enhanced and not changed relative to that in the control animals in the cerebral cortex.

In another study from the same research center, rats were treated with neuroleptics, which are known to cause oxidative stress in the brain (94). EPR imaging using nitroxides demonstrated that the reducing abilities of the striatum and cerebral cortex decreased in the rats treated with neuroleptics.

Ischemia in the murine brain was found to increase with nitroxide half-life (95). Likewise, in neonatal rats with ischemic-reperfusion injury, the reducing ability of cortex and striatum was diminished, and treatment with an antioxidant agent caused the reduction to return to control level (96). The effects of neonatal stress and acute stress on the redox state of the adult rat brain were also studied using EPR redox imaging. It turned out that the intracerebral reducing ability was significantly increased in rats exposed to neonatal stress and significantly depleted in the same rats that had been subjected to acute stress (41).

REDOX IMAGING USING OMRI

OMRI is a dynamic nuclear polarization technique for imaging free radicals based on MRI (97). It uses the Overhauser effect, in which saturation of electron spin resonance of a free radical causes a polarized nuclear spin state (98).

By applying EPR RF to the two-spin system, which contains electron spin from nitroxide and proton spin of water, relative populations of the four states are modified through dipole-dipole interaction for a nitroxide radical solution (99, 100). This EPR saturation thus results in enhanced proton nuclear magnetic resonance of water protons in the solution compared with thermal equilibrium without EPR RF. In this way, it is possible to image nitroxide content indirectly from changes in proton intensity. The advantage of OMRI approach in free radical imaging is in its high spatial resolution.

Figure 4 shows typical OMRI images after intravenous administration of a blood-brain-permeable nitroxide (101). The spatial resolution of the redox-sensitive nitroxide was 0.5 mm. After 24 h reperfusion after 1 h ischemia, the nitroxide reduction in rat brain was imaged. The images showed that OMRI image intensity decreased significantly slower over time in the ischemic hemisphere than the contralateral hemisphere, although amounts of the nitroxide initially in both hemispheres were the same.

Imaging studies using OMRI/nitroxides demonstrated that redox status can be imaged with high resolution in living animals, both in the pharmacodynamics of nitroxides and analysis of disease models: diverse distribution and bioreduction of nitroxides in living mice (102, 103), heterogeneous redox capacity in tumor bearing mice (104), and decreased brain antioxidant levels and mitochondrial dysfunction in an ischemia-reperfusion mouse model (101). In combination with conventional MRI, a detailed redox map was obtained and used to examine the oxidative mechanisms involved.

A very interesting approach was reported by Utsumi *et al.* using OMRI and different isotope-labeled nitroxides that could be used for redox molecular imaging (105). Nitroxides with either lipophilic and lipophobic characteristics were labeled with ^{14}N and ^{15}N isotopes and administered to the mouse either intragastrically or intravenously. Both nitroxide distributions in a mouse were imaged with OMRI by excitation of the specific magnetic resonance absorption of each isotopes, resulting in simultaneous separate images of the two nitroxides.

Along with organic synthesis of nitroxides for location-and reaction-specific detection, the OMRI technique appears to be promising for imaging redox related diseases models and the therapeutic effects of redox-related drugs to the diseases.

POTENTIAL PROBLEMS WITH REDOX IMAGING

A number of potential problems have been identified and associated with redox imaging that may interfere with the proper interpretation of these images. These include transport of the nitroxide from the local region, which would confound the interpretation of the diminution of the nitroxide signal as being due to local bioreduction. This concern has largely been allayed by the clever set of experiments comparing an ^{15}N nitroxide whose distribution is largely extracellular and an ^{14}N nitroxide with both the intracellular and extracellular distribution described above (105). These studies allow measurements distinguishing intra- and extracellular processes. The conclusions from experiments comparing both sets of measurements were essentially consistent, allaying concerns about the difficulty with nitroxide transport.

Concern about the effect of broadening of the spectral line by oxygen is a relatively small effect, particularly for nitroxides with natural abundance hydrogen. Hydrogen nuclei cause a peak-to-peak unresolved broadening that is independent of oxygen. This is nearly an order of magnitude larger component of the spectral line width than that caused by oxygen change (106). Similar concerns with self broadening and consequent signal height reduction are otherwise relatively small (107). Finally, these concerns can be alleviated if necessary with spectroscopic imaging with integration over the spectral dimension of the image (108). Other local effects can affect this component (109) but they are, again, small and not likely to substantially affect the bioreduction measurement, where the size of the signal diminishes by factors of three to ten.

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OXIDATIVE STRESS IMAGING

FIG. 1.
Reactions of nitroxides in biological systems.

FIG. 2.

Comparison of oxidative stress measurement using nitroxides as a redox-sensitive probe between EPR, OMRI and MRI. Redox state of the tissue is reflected by the ratio of paramagnetic form of the nitroxide and its reduced non-paramagnetic form, hydroxylamine. All three techniques are based on monitoring the kinetics of nitroxide decay in the tissue. EPR signal measures the absorption of microwave energy by nitroxide, directly reflecting the amount of paramagnetic form present in the sample. OMRI detects changes in the proton relaxation induced by excited nitroxide spins. MRI also measures the proton relaxation, but nitroxide spins act here as contrast agents.

FIG. 3.

Redox mapping of tumor. After tail vein infusion of 3-CP, a series of 2D images of the nitroxide from tumors of air- or carbogen-breathing mice were measured using the L-band EPR imaging method. The image data were acquired using a magnetic-field gradient of 150 mT/m at 16 orientations in the 2D plane. Two-dimensional spatial mapping of nitroxide distribution, pseudo-first-order rate constant, and frequency plot of the nitroxide decay constant in the tumor tissue of air-breathing (a1–a3) and carbogen-breathing (b1–b3) mice are shown in the figure. From Ilangoan *et al.* (77) with permission.

FIG. 4.

Nitroxide reduction in rat brain. Panel A: Time-dependent OMRI image of methoxycarbonyl-PROXYL in the head region and image showing the decay rates. Panel B: Semi-logarithmic plot of image intensities of the contralateral hemisphere (○) and ischemic hemisphere (●) images. Panel C: The signal decay rates of the contralateral hemisphere (open bar) and ischemic hemisphere (closed bar). Panel D: Amount of total methoxycarbonyl-PROXYL in the contralateral hemisphere (open bar) and ischemic hemisphere (closed bar) determined using X-band EPR. Each value represents the mean $\pm$ SD of four rats. * $P < 0.05$ compared with the contralateral hemisphere. From Yamato *et al.* (101) with permission.