ORIGINAL ARTICLE

In vivo measurement of superoxide in the cerebral cortex during anoxia-reoxygenation and ischemia-reperfusion

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Abstract. The exact time profile of superoxide generation during anoxia-reoxygenation and ischemia-reperfusion was assessed in the feline cerebral cortex *in vivo* using a chemiluminescence technique with a probe specific for superoxide, 2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (MCLA). MCLA solution was superfused on the cortex throughout the protocol, and MCLA chemiluminescence was measured using a newly developed photon counting system. Reflectance at 398 nm was simultaneously measured to compensate for hemodynamic artifacts resulting from cerebral blood volume changes. In 19 animals, a 90-second anoxia was induced by the inhalation of 100% nitrogen followed by a 40-minute reoxygenation. The chemiluminescence decreased during the period of anoxia ($p < 0.01$) and then exceeded the baseline level at 15 and 20 minutes after reoxygenation ($p < 0.05$). In six animals, superoxide dismutase (SOD) was continuously superfused and anoxia-reoxygenation was performed in the same manner. The chemiluminescence decreased during the period of anoxia ($p < 0.05$) but did not exceed the baseline level during the reoxygenation period, indicating that an increase in superoxide production was the main cause of the chemiluminescence increase. In eight animals, a 15-minute forebrain ischemia was induced by the occlusion of the bilateral common carotid arteries with systemic hypotension (systolic blood pressure less than 50 mmHg) followed by a 30-minute reperfusion. The chemiluminescence decreased during the period of ischemia ($p < 0.01$) and then increased at 20 and 25 minutes after reperfusion ($p < 0.05$). These results indicate that superoxide generation decreases during anoxia and ischemia and then increases within 20 minutes after reoxygenation or reperfusion. (Keio J Med 51 (4): 201–207, December 2002)

Key words: SOD, cat, photon counting, cranial window

Introduction

During cerebral ischemia or cardiac arrest, the oxygen and glucose supplies to the brain are severely reduced, resulting in an energy crisis.[1] Energy depletion causes irreversible tissue damage and thus the early restoration of blood flow is pivotal to reduce the injury; however, injuries are known to progress even after reperfusion.[2] Lipid peroxidation in brain tissue occurs mainly during the post-ischemic reperfusion period. Reactive oxygen species (ROS) are considered important mediators in reperfusion injury.[3]

Among the ROS, superoxide (O_2^-) is the principal reduction product of oxygen. Though relatively inactive itself, its metabolites, peroxynitrite and hydroxyl radical, are highly reactive and can cause tissue injury.[4,5] Many previous reports have shown that O_2^- might play an important role in the progression of tissue injury during cerebral ischemia or reperfusion: brain injury was attenuated by superoxide dismutase (SOD) administration[6] and was suppressed in transgenic mice overproducing SOD,[7–14] while it was more severe in SOD-deficient mice.[15,16] Clarifying the temporal course of O_2^- production is essential to understand the mechanism of ROS-related reperfusion injury. However, the exact timetable of O_2^- production in cerebral ischemia and reperfusion *in vivo* remains unknown.

The short half-life of O_2^- makes it difficult to measure and only a few techniques can be utilized: SOD-inhibitable nitroblue tetrazolium,[17] chemiluminescence (CL),[18,19] electron spin resonance spectroscopy,[20] and cytochrome c-coated platinized carbon electrodes.[21]

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Among them, the CL technique is very reliable, has a better time resolution, and is less invasive. This technique is also suitable for continuous *in vivo* measurements and has been used to assess ROS production.[18,19] However, specific CL probes for the detection of O_2^- have not been used to investigate cerebral ischemia. Furthermore, the presence of hemodynamic artifacts was not excluded in previous studies using CL.

The aim of the present study was to develop a new *in vivo* CL technique for assessing the exact time profile of O_2^- generation in the feline cerebral cortex during anoxia-reoxygenation and ischemia-reperfusion phases.

Methods

Animal preparation

All procedures were performed in compliance with the Experimental Animal Guidelines of Keio University School of Medicine and were approved by the Experimental Committee of Keio University. A total of 33 adult male cats, weighing 2.5 to 4.5 kg, were used in this study. The cats were anesthetized with an intraperitoneal injection of 50 mg/kg of α-chloralose (Sigma Chemical Co.) and 500 mg/kg of urethane (Sigma Chemical Co.). The rectal temperature was maintained at 37.0 ± 0.5°C by a warming pad. An incision was made in the neck and the animals were tracheotomized and artificially ventilated (Harvard Respirator, Model 662). A catheter (3 Fr) was placed in the left lingual artery to enable the injection of a small amount of saline; this step was necessary to obtain the cerebrovascular hemodilution curves. The bilateral carotid arteries were isolated and loops made from polyethylene catheter (PE-10) were carefully passed around the arteries for later occlusion and release. Catheters (5 Fr) were inserted into one femoral vein and both arteries: a catheter was inserted into one femoral artery to monitor the arterial blood pressure using an electronic recorder (Rikadenki Kogyo, Model R-66), another catheter was inserted into the other femoral artery to obtain blood gas analysis samples (arterial pH, PaO_2, and $PaCO_2$), and a third catheter was inserted into a femoral vein for blood withdrawal and re-infusion during the ischemia-reperfusion protocol. The head of each animal was fixed in a stereotactic frame (Narishige Co. Ltd.). After the skin on the head was incised and the muscles were removed from the skull, a burr hole was trephined in the left temporoparietal region of the skull and the dura was carefully incised. A cranial window (10 mm in diameter) with inlet and outlet tubes was sealed into the burr hole using dental cement. An infusion pump was connected to the inlet tube with a catheter and artificial cerebrospinal fluid (aCSF) was superfused beneath the cranial window at a rate of 5.0 ml/hour. The composition of the aCSF was as follows: Na, 140 mEq; K, 2.5 mEq; Ca, 1.5 mEq; Mg, 1.0 mEq; Cl, 130 mEq; HCO_3, 14.5 mEq; glucose, 3.3 mEq; and HEPES, 10 mEq (pH 7.35 at 37°C).

MCLA chemiluminescence

2-Methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo-[1,2-α]pyrazin-3-one (MCLA: Tokyo Kasei Co. Ltd.), a Cypridina luciferin analog, is a highly sensitive and specific probe for detecting O_2^-. The light emitted by the probe has a maximum intensity at a wavelength of 465 nm in the presence of O_2^-, which has been widely used to detect O_2^- both *in vivo*[22–25] and *in vitro*.[26–28] Since the probe partially penetrates into the cell membrane,[29] it reflects the O_2^- concentration in both the extracellular and intracellular spaces.

Photon counting system

Optical signals were measured using the system illustrated in Fig. 1. A microscope equipped with a dichroic beam splitter and two photomultiplier tubes (R647-04, Hamamatsu Photonics K.K.) was focused on a cortical region devoid of large vessels. A xenon-arc lamp (Model PS-X500, Japan Spectroscopic Co., Ltd) set at a wavelength of 398-nm was used to light the area with a constant intensity. The 398-nm reflectance and MCLA CL from the cortex were measured simultaneously using the system and the dichroic beam splitter was used to separate the two optical signals. MCLA CL (465 nm) passing through bandpass filter 1 (BPF 1: 465 ± 10 nm) was detected using photomultiplier tube 1 (PMT 1), while the 398-nm reflectance passing through bandpass filter 2 (BPF 2: 398 ± 10 nm) was detected using photomultiplier tube 2 (PMT 2). The signals were transferred to, processed by, and then visually displayed in real time on a personal computer. These measurements were performed in a dark room where the background photon count was less than 50 counts/sec.

Correction of hemodynamic influence

When light passes through a tissue containing blood, its intensity is attenuated by hemoglobin absorption.[30,31] Because the MCLA CL intensity measured *in vivo* is influenced by the changes in cerebral blood volume (CBV), hemodynamic artifacts were corrected to assess the true changes in MCLA CL. This method of hemodynamic artifact correction has been described elsewhere.[32–34]

During the resting state, a small amount of saline (1.0 to 2.0 ml) was injected through the lingual artery inducing hemodilution and causing a transient increase in the apparent MCLA CL and 398-nm reflectance. The

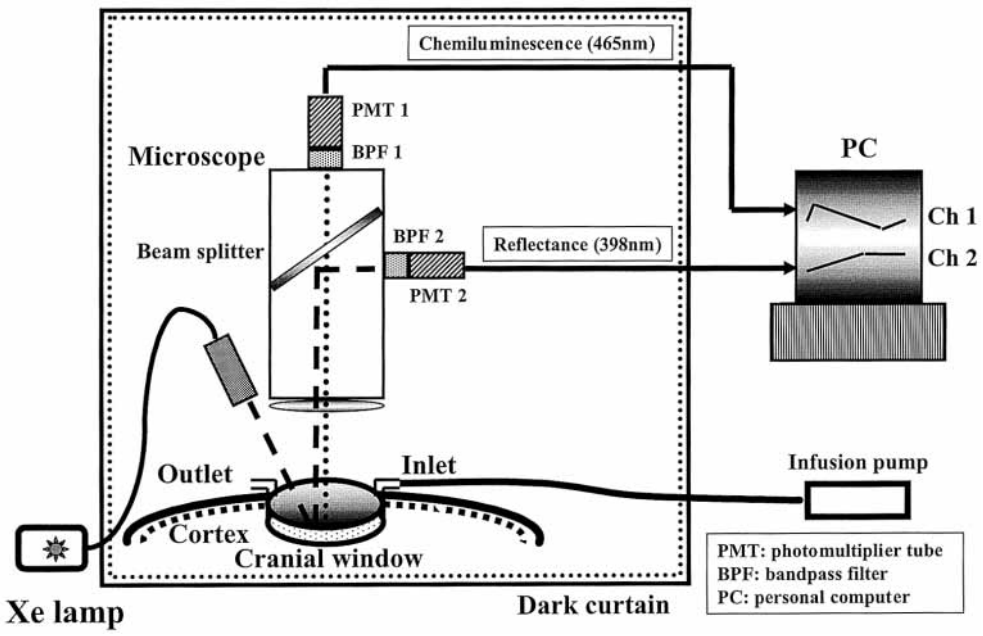

Fig. 1 Photon counting system. A xenon-arc lamp (398 nm) is projected on the cerebral cortex. The 398 nm reflectance (398 nm) and MCLA chemiluminescence (460 nm) are collected with a microscope, are separated with a dichroic beam splitter, and detected by photomultiplier tube 1 (PMT 1) and photomultiplier tube 2 (PMT 2) with bandpass filter 1 (BPF 1: 465 ± 10 nm) and bandpass filter 2 (BPF 2: 398 ± 10 nm), respectively. The signals are then analyzed by a personal computer and monitored on a display. All solutions are superfused from an inlet tube over the cortex and excreted through an outlet tube, using an infusion pump.

resulting dilution curve was utilized to obtain the hemodynamic correction factor (k factor) for each animal. The k factor can be expressed as follows:

$$k = DCL/DR$$

where DCL is the difference between the peak intensity and the basal intensity of the apparent MCLA CL during hemodilution and DR is the difference between the peak intensity and the basal intensity of the 398-nm reflectance during hemodilution. The corrected MCLA CL can, therefore, be expressed as:

$$cCL = CL - k \times R$$

where cCL represents the change in the corrected MCLA CL intensity, CL represents the change in the apparent MCLA CL intensity, and R represents the change in the 398-nm reflectance intensity.

Experimental protocol

MCLA CL and 398-nm reflectance were simultaneously measured throughout the procedure. Before the following experimental protocols were performed, the background photon level was measured under aCSF superfusion. Then, aCSF containing 100 μM of MCLA (MCLA solution) was superfused at a rate of 5.0 ml/hour. The baseline MCLA CL and 398 nm reflectance levels were designated as the points where their intensities became constant, after about 30 minutes of MCLA superfusion in the non-SOD and ischemia-reperfusion groups. On the other hand, the baseline of the SOD-group was designated as the steady state after SOD superfusion. The value of photon counting at the baseline was assigned to 100% CL in the following analysis.

1) Anoxia-reoxygenation (n = 25)

a) Non-SOD group (n = 19)

Anoxia was induced by 100% nitrogen inhalation for 90 seconds and then followed by 40 minutes of reoxygenation with room air.

b) SOD group (n = 6)

MCLA solution containing 1000 units/ml SOD (from Bovine Kidney, Sigma Chemical Co.) was superfused onto the cortical surface at a rate of 5.0 ml/hour throughout the protocol. After 30 minutes of SOD superfusion, when the intensity of both the MCLA CL and 398-nm reflectance had stabilized, anoxia and reoxygenation were induced as in the non-SOD group.

2) Ischemia-reperfusion (n = 8)

A 15-minute period of forebrain ischemia was induced by occluding the bilateral common carotid arteries. In addition, hypotension was induced by the withdrawal of venous blood (50 to 100 ml) from the femoral vein so that the systolic arterial blood pressure was maintained at less than 50 mmHg. The ischemia was then followed by a 30-minute reperfusion period.

Table 1 Physiological Parameters and k Value

	Anoxia/Reoxygenation		Ischemia/ Reperfusion (n = 8)
	Non-SOD group (n = 19)	SOD group (n = 6)	
Body weight (kg)	3.5 ± 0.7	3.5 ± 1.0	3.3 ± 0.8
Systolic blood pressure (mm Hg)	173 ± 17	172 ± 10	170 ± 19
Diastolic blood pressure (mm Hg)	121 ± 15	124 ± 11	113 ± 18
pH	7.375 ± 0.085	7.381 ± 0.055	N.A.
PaO_2 (mm Hg)	131 ± 17	136 ± 24	N.A.
$PaCO_2$ (mm Hg)	29 ± 6	25 ± 5	N.A.
k value	0.27 ± 0.19	0.32 ± 0.22	0.34 ± 0.23

N.A.: not available.

Data analysis

All values are expressed as the mean ± S.D. The corrected CL data were statistically compared with the baseline levels obtained before the induction of anoxia or ischemia. For the statistical analysis, we carried out a Fisher's protected least significant difference test. The level of statistical significance was defined as $p < 0.05$.

Results

Physiological parameters

Physiological parameters under the resting conditions are given in Table 1. Before anoxia or ischemia was induced, the physiological parameters of all animals were within the normal ranges. There was no statistical significance between the SOD and the non-SOD groups.

k factor

The k factor for the non-SOD, SOD, and ischemia-reperfusion groups were 0.27 ± 0.19, 0.32 ± 0.22, and 0.34 ± 0.23, respectively. There was no statistical significance among the three groups (Table 1).

Optical signals in the basal level

Because photon counts reached a steady state after 30 minutes of MCLA superfusion, we defined this condition as the basal level of the optical signals. The MCLA CL and 398-nm reflectance intensities were 1111 ± 543 counts/sec and 1241 ± 553 counts/sec, respectively (n = 33).

1) Anoxia/reoxygenation (n = 25)
a) Non-SOD group (n = 19)

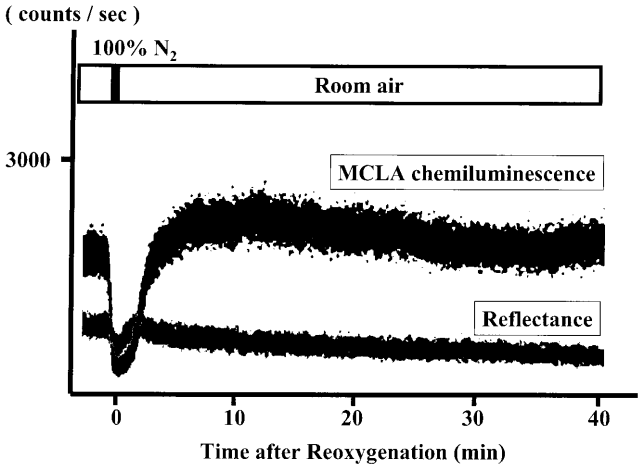

Fig. 2 Representative recording of optical signals obtained during the anoxia and reoxygenation phases in non-SOD group (see text).

Fig. 2 shows representative MCLA CL and 398-nm reflectance recordings. Several seconds after the initiation of 100% nitrogen inhalation, the observed MCLA CL began to decrease markedly. Thirty seconds after reoxygenation, the MCLA CL started to increase sharply, overshooting the baseline level within 3 minutes and remaining high throughout the remainder of the protocol. The 398-nm reflectance also decreased during the period of anoxia (indicating an increase in CBV). Several seconds after reoxygenation, the 398-nm reflectance began to increase and returned to the baseline level within a few minutes. In contrast to the MCLA CL recordings, the 398-nm reflectance recordings gradually decreased thereafter until the end of the protocol.

Fig. 3 shows the percent changes of the corrected MCLA CL to the baseline level during the anoxia and reoxygenation phases. The corrected MCLA CL decreased significantly at the end of the anoxia phase (75.1 ± 23.9%: $p < 0.01$), and increased significantly at

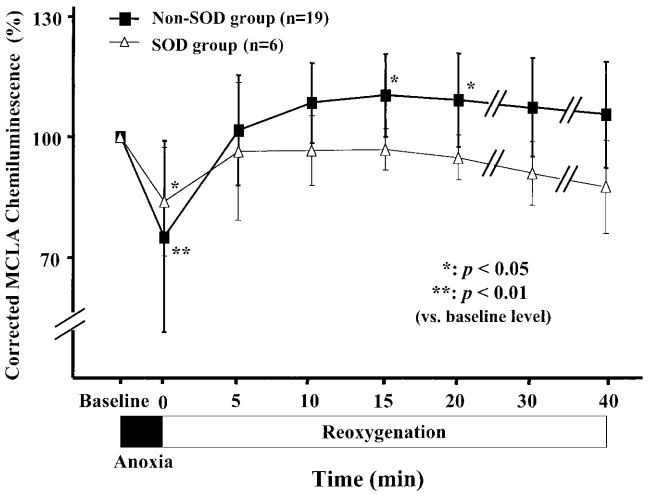

Fig. 3 Changes in MCLA chemiluminescence during the anoxia and reoxygenation phase. The percent changes in the corrected MCLA chemiluminescence compared to the baseline level decreased significantly at the end of the anoxic phase in both the non-SOD group ($75.1 \pm 23.9\%$; $p < 0.01$) and the SOD group ($83.9 \pm 13.4\%$; $p < 0.05$). After reoxygenation, the corrected MCLA chemiluminescence increased significantly at 15 and 20 minutes in the non-SOD group ($110.3 \pm 10.3\%$; $p < 0.05$ and $109.1 \pm 11.7\%$; $p < 0.05$, respectively), but did not increase in the SOD group. *: $p < 0.05$, **: $p < 0.01$ to the corrected MCLA chemiluminescence levels at the baseline level (Fisher's protected least significant difference test).

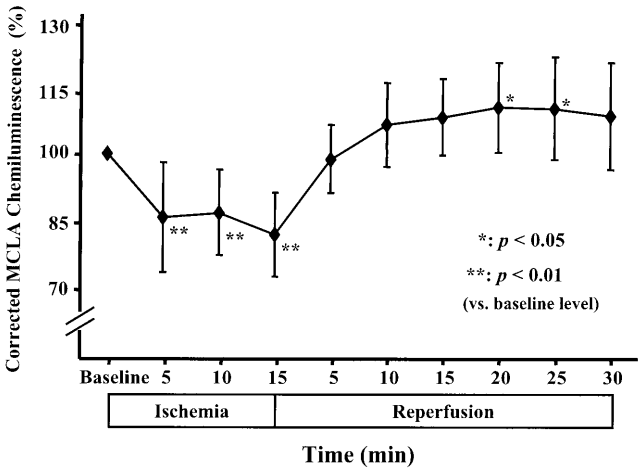

Fig. 4 Changes in MCLA chemiluminescence during the ischemia and reperfusion phase. The percent changes in the corrected MCLA chemiluminescence decreased significantly during the ischemia phase ($87.0 \pm 14.0\%$, $87.9 \pm 10.4\%$, and $82.8 \pm 9.8\%$ after 5, 10, and 15 minutes of ischemia; $p < 0.01$) and then increased to surpass the baseline level at 20 and 25 minutes after reperfusion ($113.9 \pm 8.5\%$ and $113.4 \pm 8.4\%$, respectively; $p < 0.05$). *: $p < 0.05$, **: $p < 0.01$ to the corrected MCLA chemiluminescence levels at the baseline level (Fisher's protected least significant difference test).

15 and 20 minutes after reoxygenation ($110.3 \pm 10.3\%$ and $109.1 \pm 11.7\%$, respectively: $p < 0.05$).

b) SOD group ($n = 6$)

When MCLA solution containing SOD was superfused instead of MCLA solution without SOD, optical signals began to decrease and stabilized within 30 minutes; MCLA decreased from 1086 ± 464 counts/sec in the basal level to 468 ± 170 counts/sec after SOD superfusion and the 398-nm reflectance decreased from 1517 ± 673 counts/sec to 1284 ± 522 counts/sec. Thus, the corrected MCLA CL became $51.1 \pm 9.0\%$ of the basal level after SOD superfusion. The anoxia-reoxygenation protocol was then carried out. Percent change in corrected MCLA intensity was calculated by comparing the signal intensity after the anoxia to that before anoxia. As shown in Fig. 3, the percent changes of the corrected MCLA CL level decreased significantly at the end of the anoxia phase ($83.9 \pm 13.4\%$: $p < 0.05$). The corrected MCLA CL level almost recovered during reoxygenation but did not exceed the baseline level.

2) Ischemia/reperfusion ($n = 8$)

As shown in Fig. 4, the percent changes of the corrected MCLA CL decreased significantly during the ischemia phase ($87.0 \pm 14.0\%$, $87.9 \pm 10.4\%$, and $82.8 \pm 9.8\%$ after 5, 10, and 15 minutes of ischemia; $p < 0.01$) and then increased to surpass the baseline level at 20 and 25 minutes after reperfusion ($113.9 \pm 8.5\%$ and $113.4 \pm 8.4\%$, respectively; $p < 0.05$).

Discussion

We have developed a novel photon counting system for the simultaneous assessment of MCLA CL and 398-nm reflectance in the feline cortex during anoxia and reoxygenation. After correcting for hemodynamic influences by measuring the 398-nm reflectance, CL changes in the cortex were assessed. This study can be summarized as follows: 1) O_2^- production was assessed by a CL method with excellent time resolution and minimal invasiveness using MCLA, an O_2^- sensitive and specific CL probe and 2) hemodynamic influences were taken into account to precisely assess O_2^- production.

Because detected light intensity decreases by the inverse square of the distance between the objective lens and the tissue, movement of the cortical surface may influence the light intensity in this experiment. However, we evaluated the motion artifact by observing the cortex covered with a cranial window through a microscope in a preliminary experiment. The surface image, in fact, never went out of focus during the procedure. Furthermore, we used an objective lens with a long working distance (50 mm) in order to minimize the motion artifact if present. Therefore, we believe that the movement of the cortical surface is negligible in our experiment. Thus, the exact *in vivo* time profile of

O_2^- production in the feline cortex during anoxia-reoxygenation and ischemia-reperfusion was successfully assessed.

Since gender difference in cerebral blood flow has been reported,[35–38] we used only male animals.

SOD superfusion reduced the corrected MCLA CL to half of the basal level, indicating that superoxide is spontaneously produced in the steady state before the insult of anoxia/ischemia. Furthermore, because the membrane permeability of SOD is low, some part of the MCLA CL that was not suppressed by the SOD superfusion may arise from the intracellular space. A small part of basal MCLA CL may also arise from autooxidation due to oxygen itself.

The MCLA CL increased within 20 minutes of reoxygenation and reperfusion. On the other hand, the increase in MCLA CL after reoxygenation was not demonstrated when SOD was superfused throughout the protocol. The SOD-induced suppression of the enhancement in CL during reoxygenation indicates that enhanced O_2^- production was the main cause of the increase in CL. Considering the low membrane permeability of SOD, this result also indicates that the enhancement in O_2^- production is likely to occur mainly in the extracellular space. Some investigators have studied the site of O_2^- production during reperfusion in a transient cerebral ischemia model using electron microscopic histochemistry and Karnovsky's Mn^{2+}/diaminobenzidine (DAB) technique.[39,40] Kontos *et al.* reported that O_2^- reaction products were produced during reperfusion primarily in the extracellular space around the blood vessels and that the products were eliminated by pretreatment with SOD superfusion.[39] Mori *et al.* reported that O_2^- reaction products were detected within the arteries, capillaries, and venular lumens during reperfusion.[40] Furthermore, the extracellular space around blood vessels has been reported to be a possible site of ROS production in various organs. Suematsu *et al.* studied the site of ROS production *in vivo*; they found that ROS production occurred in rat mesenteric microvascular beds through an endothelium-granulocyte interaction using the CL technique[41] and in hypoperfused rat liver utilizing using fluorescence technique.[42] These findings are compatible with our results obtained using the CL technique.

We found that O_2^- production decreased during anoxia and severe forebrain ischemia. The supply of O_2 to the brain tissue is reduced during anoxia or severe forebrain ischemia, which may limit O_2^- production. Furthermore, Marklund *et al.* reported that the half-life of O_2^- becomes shorter as the pH level decreases.[43] Brain pH has also been reported to decrease during severe ischemia[44–46] and cardiac arrest,[47] which may cause the reduction in O_2^- concentration during anoxia or severe forebrain ischemia.

In conclusion, we have established a new CL technique for the *in vivo* assessment of O_2^- generation in the feline cortex. O_2^- generation decreased during a 90-second period of anoxia and severe transient forebrain ischemia and increased within 20 minutes of reoxygenation and reperfusion. The increase in O_2^- production during reoxygenation or reperfusion may occur mainly in the extracellular space.

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