Xanthine Oxidase is One of the Major Sources of Superoxide Anion Radicals in Blood after Reperfusion in Rats with Forebrain Ischemia–Reperfusion

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Abstract

We recently reported that excessive superoxide anion radical ($O_2^{-}\cdot$) was generated in the jugular vein during reperfusion in rats with forebrain ischemia-reperfusion using a novel electrochemical sensor and excessive $O_2^{-}\cdot$ generation was associated with oxidative stress, early inflammation, and endothelial injury. However, the source of $O_2^{-}\cdot$ was still unclear. Therefore, we used allopurinol, a potent inhibitor of xanthine oxidase (XO), to clarify the source of $O_2^{-}\cdot$ generated in rats with forebrain ischemia-reperfusion. The increased $O_2^{-}\cdot$ current and the quantified partial value of electricity (Q), which was calculated by the integration of the current, were significantly attenuated after reperfusion by pretreatment with allopurinol. Malondialdehyde (MDA) in the brain and plasma, High-mobility group box 1 (HMGB1) in plasma, and intercellular adhesion molecule-1 (ICAM-1) in the brain and plasma were significantly attenuated in rats pretreated with allopurinol with dose-dependency in comparison to those in control rats. There were significant correlations between total Q and MDA, HMGB, or ICAM-1 in the brain and plasma. Allopurinol pretreatment suppressed $O_2^{-}\cdot$ generation in the brain-perfused blood in the jugular vein, and oxidative stress, early inflammation, and endothelial injury in the acute phase of forebrain ischemia-reperfusion. Thus, XO is one of the major sources of $O_2^{-}\cdot$ in blood after reperfusion in rats with forebrain ischemia-reperfusion.

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Abbreviations: ROS, reactive oxygen species; $O_2^{-}\cdot$, superoxide anion radical; XO, xanthine oxidase; NADPH, nicotinamide adenine dinucleotide phosphate; XOR, xanthine oxidoreductase; XDH, xanthine hydrogenase; ATP, adenosine triphosphate; FBI/R, forebrain ischemia/reperfusion; HMGB1, high-mobility group box 1; ΔI, difference in $O_2^{-}\cdot$ current; Q, quantified partial value of electricity; Q_I, Q during ischemia; Q_R, Q after reperfusion; MDA, malondialdehyde; ICAM-1, intercellular adhesion molecule-1; sICAM-1, soluble ICAM-1; PaO_2, arterial oxygen pressure; $PaCO_2$, arterial carbon dioxide pressure; MAP, mean arterial blood pressure; BE, base excess.

1.Introduction

Reactive oxygen species (ROS) play essential roles in biological and physiological homeostasis *in vivo*. However, the excessive production of ROS leads to oxidative stress and tissue injury (Zweier and Taluker, 2006). Among the ROS, the superoxide anion radical ($O_2^{-}\cdot$) is the key radical because many ROS are derived from $O_2^{-}\cdot$ (Guldi and Prato, 2000). Therefore, *in vivo* monitoring of $O_2^{-}\cdot$ is necessary to understand the conditions of oxidative stress in human pathophysiological states.

It is well known that some enzymes are activated during global and focal cerebral ischemia–reperfusion, leading to the production of excessive ROS (Macdonald and Stoodley, 1998; Warner et al., 2004; White et al., 2000). Of these ROS, $O_2^{-}\cdot$ is the key radical causing tissue injury and neural cell death. The possible sources of $O_2^{-}\cdot$ are considered to be xanthine oxidase (XO) (McCord, 1985; Parks and Granger, 1986), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Abramov et al., 2007; Brennan et al., 2009; Chen et al., 2009; Girouard et al., 2009; Jackman et al., 2009), the mitochondrial respiratory chain (Sims and Anderson, 2002), and the metabolic cascade of arachidonic acid (Krause et al., 1988). However, the principal source of $O_2^{-}\cdot$ during the cerebral ischemia–reperfusion remains unclear.

XO is an interconvertible form of xanthine oxidoreductase (XOR); the other form is xanthine hydrogenase (XDH). In normal tissue, XO exists as XDH. During ischemia, XDH is

cleaved by a calcium-dependent protease that converts it to XO (Parks and Granger, 1986). At the same time, cellular adenosine triphosphate (ATP) is catabolized to hypoxanthine, which accumulates in ischemic tissue. After reperfusion, XO oxidizes hypoxanthine to xanthine, and further oxidizes xanthine to produce uric acid, $O_2^{-}\cdot$, and hydrogen peroxide (Granger et al., 1981; McCord, 1985; Parks and Granger, 1986). On this basis, several studies have investigated the neuroprotective effects of XO inhibitors, most commonly allopurinol, in the pathophysiology of global and focal cerebral ischemia–reperfusion (Itoh et al., 1986; Martz et al., 1989; Lindsay et al., 1991; Van Bel et al., 1998; Akdemir et al., 2001). However, there is no consensus regarding whether XO is the main source of $O_2^{-}\cdot$ in cerebral ischemia–reperfusion, because no method has been available to directly and continuously measure the generation of $O_2^{-}\cdot$ *in vivo*.

Recently, we developed an *in vivo* real-time quantitative $O_2^{-}\cdot$ analysis system with an all-synthetic electrochemical $O_2^{-}\cdot$ sensor (Yuasa and Oyaizu, 2005; Yuasa et al., 2005a,b; Aki et al., 2009; Fujita et al., 2009). The current measured by the sensor correlates strongly with the hitting frequency of $O_2^{-}\cdot$ on the surface of the sensor in the circulating blood (Yuasa et al., 2005a; Fujita et al., 2009). Using this method, we have shown that $O_2^{-}\cdot$ is increased in the jugular veins of rats with forebrain ischemia–reperfusion (FBI/R) during the ischemia and reperfusion periods (Aki et al., 2009). The generation of $O_2^{-}\cdot$ also correlates with both brain and plasma high-mobility group box 1 (HMGB1), which is a key cytokine during early inflammation in the pathophysiology of cerebral

ischemia–reperfusion (Kim et al., 2006, 2008; Qiu et al., 2008; Aki et al., 2009). Furthermore, excessive production of $O_2^{-}\cdot$ after ischemia-reperfusion was associated with early inflammation, oxidative stress, and endothelial activation in the brain and plasma (Aki et al., 2009).

In this study, we investigated whether XO is the source of $O_2^{-}\cdot$ in the rat model of FBI/R using our novel electrochemical $O_2^{-}\cdot$ sensor and by administering allopurinol, a potent XO inhibitor.

2. Result

2.1. Plasma $O_2^{-}\cdot$ Current during Forebrain Ischemia–Reperfusion in Rats

The baseline $O_2^{-}\cdot$ current was recorded before ischemia and the differences (ΔI) were recorded during the preischemia, ischemia, and reperfusion periods (Figure 1). During the ischemia period, ΔI of $O_2^{-}\cdot$ increased gradually in all groups. Just after reperfusion, a further elevation of ΔI was observed in the control group. In the low-allopurinol group and the high-allopurinol group, ΔI of $O_2^{-}\cdot$ was significantly attenuated after reperfusion compared with that in the control group ($P < 0.05$ for the control group vs. the low-allopurinol group in the periods 2–6, 36–52, 60–78, and 108–112 min after reperfusion; $P < 0.01$ for the control group vs. the low-allopurinol group at 8–34, 54–58, 80–106, and 114–120 min after reperfusion; $P < 0.01$ for the control group vs. the high-allopurinol group 2–120 min after reperfusion). The $O_2^{-}\cdot$ current tended to be more attenuated in the high-allopurinol group than in the low-allopurinol group.

2.2. Quantified Partial Value of Electricity (Q) of $O_2^{-}\cdot$ during Ischemia and after Reperfusion

Figure 2 shows the values for Q during ischemia (Q_I) and after reperfusion (Q_R). There was no significant difference in the Q_I, although Q_I in the low-allopurinol and high-allopurinol groups tended to be attenuated compared with that in the control group (Fig. 2A). Q_R in the low-allopurinol and high-allopurinol groups was significantly attenuated compared with that in the control group ($P < 0.01$; Fig. 2B). Q_R tended to be more attenuated in the high-allopurinol group than in the low-allopurinol group.

2.3. Malondialdehyde (MDA) Levels in the Forebrain Tissue and Plasma

Figure 3A presents the MDA levels in the forebrain tissue 120 min after reperfusion in the three groups. The brain MDA in the low-allopurinol and high-allopurinol groups was significantly lower than that in the control group ($P < 0.01$). The brain MDA in the high-allopurinol group tended to be lower than that in the low-allopurinol group, but they were not significantly different. Figure 3B presents the plasma MDA levels 120 min after reperfusion in all groups. The plasma MDA level in the low-allopurinol group was significantly lower than that in the control group ($P < 0.05$). The plasma MDA in the high-allopurinol group was significantly lower than that in the low-allopurinol group and in the control group ($P < 0.01$ for the high-allopurinol group vs. both the control group and the low-allopurinol group). The correlation coefficient was calculated for total Q, which is the sum of Q_I and Q_R, and brain MDA ($r = 0.7134$, $P < 0.01$; Fig. 3C) and for total Q and plasma MDA ($r = 0.6271$, $P < 0.01$; Fig. 3D).

2.4. HMGB1 Levels in the Forebrain Tissue and Plasma

Figure 4A presents the HMGB1 levels in the forebrain cytoplasm 120 min after reperfusion in the three groups. The brain HMGB1 in the low-allopurinol and high-allopurinol groups tended to be lower than that in the control group, but not statistically significantly. Figure 4B presents the plasma HMGB1 levels 120 min after reperfusion in all groups. The plasma HMGB1 in the low-allopurinol and high-allopurinol groups was significantly lower than that in the control group ($P < 0.05$ for the control group vs. the low-allopurinol group, $P < 0.01$ for the control group vs. the high-allopurinol group). The plasma HMGB1 in the high-allopurinol group tended to be lower than that in the low-allopurinol group, but the difference was not significant. The correlation coefficient was calculated for total Q and brain HMGB1 ($r = 0.5738$, $P < 0.01$; Fig. 4C) and for total Q and plasma HMGB1 ($r = 0.6907$, $P < 0.01$; Fig. 4D).

2.5. Intercellular Adhesion Molecule-1 (ICAM-1) Levels in the Brain Tissue and Plasma

ICAM-1 in the forebrain tissue 120 min after reperfusion is shown in Fig. 5A. Brain ICAM-1 in the high-allopurinol group was significantly lower than those in the control group ($P < 0.05$). There was no significant difference in brain ICAM-1 between the control group and the low-allopurinol group. Plasma soluble ICAM-1 (sICAM-1) 120 min after reperfusion is shown in Fig. 5B. Plasma sICAM-1 in the low-allopurinol group and high-allopurinol group were significantly lower than that in the control group ($P < 0.01$). The correlation coefficient was calculated for total Q and brain ICAM-1 ($r = 0.5795$, $P < 0.01$; Fig. 5C) and for total Q and plasma

sICAM-1 (r = 0.8183, $P < 0.01$; Fig. 5D).

2.6. Physiological Parameters

Table 1 shows the physiological parameters for the rats during the experiments. There were no significant differences in body weight (data not shown) or mean arterial blood pressure during the preischemia and reperfusion periods in all groups. Arterial oxygen pressures (PaO_2) and arterial carbon dioxide pressure ($PaCO_2$) were well controlled and did not differ among the three groups throughout the experiments. Metabolic acidosis was observed with the elevation of plasma lactate after reperfusion in all three groups.

3. Discussion

In the rat with FBI/R, we have shown that $O_2^{-}\cdot$ generation is elevated in the jugular vein after reperfusion with our real-time quantitative $O_2^{-}\cdot$ analysis system (Aki et al., 2009). This fact was confirmed in the present study. The possible sources of $O_2^{-}\cdot$ in the pathophysiology of focal and global cerebral ischemia–reperfusion include XO (McCord, 1985; Parks and Granger, 1986), NADPH oxidase (Abramov et al., 2007; Brennan et al., 2009; Chen et al., 2009; Girouard et al., 2009; Jackman et al., 2009), the mitochondrial respiratory chain (Sims and Anderson, 2002), and the metabolic cascade of arachidonic acid (Krause et al., 1988), but where this $O_2^{-}\cdot$ comes from has been unclear. In this study, we demonstrated using allopurinol, a potent inhibitor of XO, that XO is the

important source of $O_2^{-}\cdot$ after reperfusion in the pathophysiology of global cerebral ischemia–reperfusion.

Allopurinol pretreatment suppressed the $O_2^{-}\cdot$ generation measured with the $O_2^{-}\cdot$ sensor in the jugular vein (Fig. 1 and 2). XO is widely distributed throughout various organs, including the brain, endothelium, and plasma. In brain tissue, ischemia leads to energy failure and causes the uncontrolled release of glutamate into the extracellular space (Lipton, 1999). High concentrations of extracellular glutamate open the Ca^{2+} channels, with the consequent accumulation of intracellular Ca^{2+}. Ca^{2+} activates the XO responsible for the generation of $O_2^{-}\cdot$, and neuronal cell death then occurs (Parks and Granger, 1986; Choi and Rothman, 1990). A variety of pathophysiological conditions, including hepatic ischemia–reperfusion and hemorrhagic shock, are reported to induce the systemic release of XO (Yokoyama et al., 1990; Pacher et al., 2006). In the pathophysiology of cerebral ischemia–reperfusion, the systemic release of XO might be induced by reperfusion. In the intravascular environment, the circulating XO is bound to glycosaminoglycans on the surfaces of endothelial cells, from where it continues to produce $O_2^{-}\cdot$ (Pacher et al., 2006). Endothelial XO activity has also been reported to be regulated by H_2O_2 and calcium, and by NADPH oxidase (McNally et al., 2003, 2005). It has been suggested that there is a positive feedback loop in the $O_2^{-}\cdot$ generation associated with XO (McNally et al., 2005). Allopurinol itself has ROS scavenging effects (Das et al., 1987; Moorhouse et al., 1987), so that allopurinol might suppress the positive

feedback loop of $O_2^{-}\cdot$ generation due to both inhibition of XO and the ROS scavenging effect.

The $O_2^{-}\cdot$ current was significantly attenuated after reperfusion in the low-allopurinol and high-allopurinol groups compared with that in the control group (Fig. 1). The $O_2^{-}\cdot$ current in the low-allopurinol group was not increased after the reperfusion and the current in the high-allopurinol group was attenuated to the baseline level after the reperfusion (Fig. 1). Q_R was also significantly attenuated in the low-allopurinol and high-allopurinol groups compared with that in the control group (Fig. 2). The attenuation of $O_2^{-}\cdot$ generation by allopurinol was also dose dependent, although Q_R in the high-allopurinol group was not statistically significantly different from that in the low-allopurinol group (Fig. 2). These facts indicate that XO is one of the main sources of excessive $O_2^{-}\cdot$ in blood after the reperfusion in rats with forebrain ischemia-reperfusion.

NADPH oxidase was thought to be another major source of $O_2^{-}\cdot$ in the cerebral ischemia pethophysiology (Abramov et al., 2007; Brennan et al., 2009; Chen et al., 2009; Girouard et al., 2009; Jackman et al., 2009). It was reported that NADPH oxidase expressed in neurons, microglia, and astrocytes constitutively (Bedard and Krause, 2007). Abramov et al. (2007) suggested three distinct mechanisms of $O_2^{-}\cdot$ generation in neurons, which included initial burst of ROS from mitochondria, second phase of ROS generation associated with XO, and third phase of ROS generation associated with NADPH oxidase. Furthermore, Fan et al. (2007) reported systemic hypotension itself activates neutrophils and increased $O_2^{-}\cdot$ generation via activation of NADPH

oxidase pathway. In the present study, Q_I in the low-allopurinol and high-allopurinol groups was not significantly different from that in the control group, probably because of the variance of the control group. The gradual elevation in the $O_2^{-}\cdot$ current and Q_I during the forebrain ischemia period might reflect the activity of NADPH oxidase in the brain and blood.

Lipid peroxidation is one of the major consequences of free-radical-mediated injury to the brain (Cherubini et al., 2005). The brain tissue MDA levels reflect the oxidative damage to brain lipids in this study. It has been reported that the plasma MDA level correlates well with infarct size, the severity of stroke, and stroke outcome (Gariballa et al., 2002; Polidori et al., 2002). The forebrain tissue MDA levels and plasma MDA levels in the low-allopurinol and high-allopurinol groups were significantly lower than those in the control group (Fig. 3A and 3B). These facts are consistent with the attenuation of the $O_2^{-}\cdot$ current and Q. Allopurinol itself has ROS scavenging effects (Das et al., 1987; Moorhouse et al., 1987) and can cross blood brain barrier (Palmer et al., 1993). These mechanisms in addition to XO inhibition acted on the suppression of brain and plasma MDA. The correlation coefficient was calculated for total Q and forebrain tissue MDA (Fig. 3C) and for total Q and plasma MDA (Fig. 3D). Total Q correlated much better with brain MDA than with plasma MDA ($r = 0.7134$ and 0.6271, respectively). These data indicate that allopurinol acts better in the brain than in the blood, and prevents cerebral injury by suppressing excessive production of $O_2^{-}\cdot$. This fact was consistent with the report of Palmer et al. (1993) which reported

that allopurinol was distributed in blood more than in the brain, and was more remarkable in both HMGB1 and ICAM-1.

HMGB1, originally identified as a nuclear DNA-binding protein, has recently been characterized as a key cytokine that causes inflammatory responses in various organ systems (Lotze and Tracey, 2005; Yang et al., 2005). Some investigators have reported that HMGB1 is released from neurons early after ischemic injury and acts as a novel mediator linking acute brain damage and subsequent inflammatory processes (Dirnagl et al., 1999; Kim et al., 2006, 2008; Qiu et al., 2008). We also reported recently that HMGB1 was associated with jugular venous $O_2^{-}\cdot$ generation (Aki et al., 2009). In this study, brain HMGB1 in the low-allopurinol and high-allopurinol groups tended to be attenuated compared with that in the control group (Fig. 4A). Plasma HMGB1 levels in the low-allopurinol and high-allopurinol groups were significantly lower than that in the control group (Fig. 4B). These results indicate that allopurinol attenuates the inflammatory response in the early phase of reperfusion via the attenuation of HMGB1 which was associated with the suppression of $O_2^{-}\cdot$ generation. The correlation coefficient was calculated for total Q and forebrain tissue HMGB1 levels (Fig. 4C) and for total Q and plasma HMGB1 levels (Fig. 4D). These results support our previous data and suggest the existence of an $O_2^{-}\cdot$–mediated HMGB1 loop in the pathophysiology of cerebral ischemia–reperfusion (Aki et al., 2009).

ICAM-1 is one of the most widely recognized adhesion molecules. The mRNA of

ICAM-1 in endothelial cells was reported to be upregulated by HMGB1 (Qiu et al., 2008). The upregulation of ICAM-1 expression in endothelial cells should enhance inflammation, recruiting immune cells to the ischemic lesion and exacerbating tissue injury. In the present study, the expression of brain ICAM-1 was attenuated significantly in the high-allopurinol group compared with those in the control group and the low-allopurinol group (Fig. 5A), and plasma ICAM-1 was attenuated in the low-allopurinol group and the high-allopurinol group compared with that in the control group (Fig. 5B). These results suggested that high-dose allopurinol suppressed the ICAM-1 expression and its peel-off in the brain vascular endothelium and low-dose allopurinol could prevent only the peel-off of ICAM-1.

In this study, we confirmed that $O_2^{-}\cdot$ values, including ΔI and Q, might be predictive indicators of oxidative stress and early inflammation. These results also indicate that XO is the important source of excessive $O_2^{-}\cdot$ after the reperfusion and that XO inhibitors might be useful therapeutic tools of the treatment of cerebral ischemia–reperfusion, because allopurinol inhibited excessive $O_2^{-}\cdot$ generation, lipid peroxidation, early inflammation, and endothelial injury in the present study.

There were some limitations to this study. First, our in vivo real-time quantitative $O_2^{-}\cdot$ analysis system cannot directly measure $O_2^{-}\cdot$ generation in brain tissue. Second, we investigated $O_2^{-}\cdot$ generation for only 120 min after reperfusion, and much longer measurements using our system

with the novel electrochemical $O_2^{-}\cdot$ sensor should be made.

In conclusion, XO is one of the major sources of $O_2^{-}\cdot$ generated in blood after reperfusion by FBI/R. This in vivo real-time quantitative $O_2^{-}\cdot$ analysis system might be useful at the bedside in the near future.

4. Experimental Procedures

4.1. Animals

The study protocol was approved by the Animal Experiment Committee of Yamaguchi University and all rats were handled according to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Twenty-one male, pathogen-free Wistar rats, weighing 250–300 g, were randomly assigned to one of three experimental groups: a control group (n = 7, saline), a low-allopurinol group (n = 7, 100 μg/g allopurinol), and a high-allopurinol group (n = 7, 200 μg/g allopurinol). The low-allopurinol group received 100 μg/g allopurinol dissolved in 0.3 mL of saline and the high-allopurinol group received 200 μg/g allopurinol dissolved in 0.3 mL of saline, both given intraperitoneally 24 h and 1 h before forebrain ischemia was established. The control group received an equivalent amount of saline over the same time course.

4.2. Animal Preparation

Transient forebrain ischemia was established as in our previous study (Aki et al., 2009). In brief, under isoflurane anesthesia (3% during surgery) and mechanical ventilation (SN-480-7

respirator, Shimano Manufacturing Co. Ltd., Tokyo, Japan) through a tracheotomy tube, an arterial catheter was inserted to measure blood pressure and to take a blood sample from the left femoral artery. A venous catheter was inserted into the right atrium through the right external jugular vein to both administer drugs and remove blood to induce a state of hypotension. The distal side of the right jugular vein was ligated. The $O_2^{-}\cdot$ sensor was inserted from the left anterior facial vein to the external jugular vein. The distal side of the left anterior facial vein and the proximal branches of the external jugular vein were ligated and the left posterior external jugular vein and the cephalic vein were ligated under a surgical microscope to reduce the blood flow from the face and neck. The isoflurane concentration was reduced to 0.7% with 40% oxygen and the physiological parameters were stabilized for at least 20 min.

4.3. Induction of Forebrain Ischemia-Reperfusion

After the baseline measurements of the $O_2^{-}\cdot$ current were made, forebrain ischemia was established by the occlusion of the bilateral common carotid arteries, and blood was shed to reduce the mean arterial blood pressure to 40–45 mmHg for 10 min. Forebrain ischemia was confirmed by the complete suppression of electroencephalographic activity. Reperfusion was achieved by releasing the bilateral carotid artery occlusion and returning the shed blood. The generation of $O_2^{-}\cdot$ was evaluated as the difference between the current and the baseline current detected with the $O_2^{-}\cdot$ sensor during the preischemia, ischemia, and reperfusion periods, as described previously (Aki et al.,

2009). PaO_2, $PaCO_2$, pH, and the base excess of the arterial blood, the direct arterial blood pressure, and the pharyngeal temperature were also measured. The pharyngeal temperature was maintained at 37.0°C throughout the experiment. At 120 min after reperfusion, the blood was sampled and replaced with ice-cold saline. After the rats had been sacrificed, the brains were removed, frozen in liquid nitrogen, and stored at –80 °C until MDA, HMGB1, and ICAM-1 analyses. The blood was centrifuged at 900 × g for 10 min at 4 °C and the plasma was stored at –80 ° until the analyses.

4.4. Plasma $O_2^{-}\cdot$ Measurement and Evaluation

The $O_2^{-}\cdot$ generated was measured as a current. The $O_2^{-}\cdot$ current was measured with a ROS analysis system using an electrochemical $O_2^{-}\cdot$ sensor (Actiive Co. Ltd., Noda, Japan), as described in our previous study (Yuasa and Oyaizu, 2005; Aki et al., 2009; Fujita et al., 2009). This $O_2^{-}\cdot$ sensor has a carbon working electrode coated with a polymeric iron porphyrin complex, bromo-iron(III)(*5,10,15,20-tetra*(*3*-thienyl)porphyrin) ligated two *1*-methylimidazole as an axial ligand ([$Fe(im)_2$(tpp)]Br), which mimics cytochrome c, and a stainless-steel counter electrode (Yuasa et al., 2005a, b). This sensor has a highly catalytic activity for the oxidation of $O_2^{-}\cdot$, and there exists a linear relationship between the current and the $O_2^{-}\cdot$ concentration in phosphate buffered saline and human blood (Yuasa et al., 2005a; Fujita et al., 2009). The sensor has been shown to be sensitive and specific for extracellular $O_2^{-}\cdot$ and do not respond to nitric oxide or H_2O_2 (Fujita et al., 2009).

The current data were recorded at two points per second and smoothing procedures (i.e.,

moving methods) were applied in the data analysis, because the data contained noise and artifacts. The current data were presented as ΔI, which refers to the difference in the current from baseline to the actual measured current**,** described as our previous study (Aki et al., 2009).

The measured $O_2^{-}\cdot$ current was evaluated as Q, which correlates with the amount of $O_2^{-}\cdot$ generated. The baseline current was defined as the stable state before the onset of ischemia. The differences between the baseline current and the actual $O_2^{-}\cdot$ measured current were integrated during the ischemic period as Q_I and during the reperfusion period as Q_R, described as our previous study (Aki et al., 2009). Total Q was calculated by the sum of Q_I and Q_R, which reflected the amount of $O_2^{-}\cdot$ generated throughout forebrain ischemia-reperfusion.

4.5. MDA Analysis

The brain tissue of the left frontal lobe was homogenized in ice-cold 50 mM Tris-HCI buffer (pH 7.4) with 5 mM butylated hydroxytoluene (in acetonitrile) using a Polytron PT-MR3100 homogenizer (Kinematica, Littau, Switzerland). MDA levels in the forebrain homogenate and the plasma 120 min after reperfusion were analyzed with the Bioxytech® MDA-586™ Kit (OxisResearch, Foster, CA, USA). The protein concentration in the forebrain homogenate was determined as described by Bradford (Bradford, 1976). The final results are presented as pmol MDA/mg protein in the forebrain homogenate and as μM in the plasma.

4.6. HMGB1 Analysis

The cytoplasmic fraction of brain tissue was prepared as our previous report (Aki et al., 2009). In brief, the brain tissue of the left frontal lobe was gently homogenized in 10 mM N-2-hydroxyethlpiperazine-N'-ethanesulfonic acid/10 mM KCl buffer with 0.08% NP-40, 0.1 mM ethylenediamine tetraacetic acid, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, and the soluble fraction derived from the cytoplasm was kept at –80°C until further testing. The HMGB1 levels in the cytoplasm of brain and the plasma 120 min after reperfusion were analyzed with an HMGB1 ELISA Kit II™ (Shino-test Corporation, Kanagawa, Japan). The final results are presented as ng HMGB1/mg protein in the forebrain cytoplasm and as ng/mL in the plasma.

4.7. ICAM-1 Analysis

The ICAM-1 levels in the brain homogenate and the plasma (sICAM-1) 120 min after reperfusion were analyzed with the Quantikine® Rat sICAM-1 (CD54) Immunoassay™ kit (R&D Systems, Inc., Minneapolis, MN, USA). The final results are presented as pg ICAM-1/mg protein in the brain homogenate and as ng/mL in the plasma.

4.8. Arterial Blood Gas and Lactate Analyses

Arterial blood gas and lactate analyses were performed with the ABL™ System 555 (Radiometer Medical A/S, Copenhagen, Denmark) during the preischemic and reperfusion periods.

4.7. Statistical Analyses of the Data

The data were analyzed using the SPSS 10.0 statistical software package (SPSS Inc.,

Chicago, IL, USA). The statistical significance of ΔI and the physiological parameters was determined with two-way analysis of variance (ANOVA). The statistical significance of Q_I, Q_R, MDA, HMGB1, and ICAM-1 was determined with one-way ANOVA. When the results of ANOVA were significant, the Bonferroni post hoc test was applied to determine specific group differences. The statistical analysis of the correlation between total Q and MDA, HMGB1, or ICAM-1 was performed using Pearson's correlation coefficient. All data are expressed as the means ± standard deviations (SD) of seven measurements. A value of $P < 0.05$ was considered statistically significant.

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Figure Legends

Figure 1. Actual measured current of the superoxide anion radical ($O_2^{-}\cdot$) in the rats with forebrain ischemia/reperfusion.

The vertical axis indicates the change in the $O_2^{-}\cdot$ current (ΔI), which refers to the difference in the current from baseline to the actual measured current. Each value is the mean ± SD of seven measurements. *$P < 0.05$, the control group vs. the low-allopurinol group. †$P < 0.01$, the control group vs. the low-allopurinol group or the high-allopurinol group.

Figure 2. Differences in the quantified partial value of electricity (Q) derived from the generation of superoxide anion radicals ($O_2^{-}\cdot$) in rats with forebrain ischemia/reperfusion.

A. Q during ischemia (Q_I). The generation of $O_2^{-}\cdot$ was evaluated as Q, which was calculated by the integration of the differences between the $O_2^{-}\cdot$ current at baseline (initial stable state) and the measured current. Each value is the mean ± SD of seven measurements.

B. Q after reperfusion (Q_R). Each value is the mean ± SD of seven measurements. **$P < 0.01$, the control group vs. the low-allopurinol or high-allopurinol group.

Figure 3. Malondialdehyde (MDA) concentrations in forebrain tissue and plasma 120 min after

reperfusion

A. MDA in the forebrain tissue. Each value is the mean ± SD of seven measurements. **$P < 0.01$, the control group vs. the low-allopurinol group or the high-allopurinol group.

B. MDA in the plasma. Each value is the mean ± SD of seven measurements. §$P < 0.05$, the low-allopurinol group vs. the control group; and $P < 0.01$, the low-allopurinol vs. the high-allopurinol group. ††$P < 0.01$, the high-allopurinol group vs. the control group or low-allopurinol group.

C. The relationship between total Q and brain MDA. Total Q is the sum of Q_I and Q_R.

D. The relationship between total Q and plasma MDA.

Figure 4. High-mobility group box 1 (HMGB1) concentrations in the forebrain cytoplasm and plasma 120 min after reperfusion.

A. HMGB1 in the forebrain cytoplasm. Each value is the mean ± SD of seven measurements.

B. HMGB1 in the plasma. Each value is the mean ± SD of seven measurements. * $P < 0.05$, the control group vs. the low-allopurinol group. ** $P < 0.01$, the control group vs. the high-allopurinol group.

C. The relationship between total Q and brain HMGB1. Total Q is the sum of Q_I and Q_R.

D. The relationship between total Q and plasma HMGB1.

Figure 5. Intercellular adhesion molecule 1 (ICAM-1) in the forebrain tissue and plasma 120 min after reperfusion.

A. ICAM-1 in the forebrain tissue. Each value is the mean ± SD of seven measurements. *P < 0.05 the high-allopurinol group vs. the control group.

B. Soluble ICAM-1 (sICAM-1) in the plasma. Each value is the mean ± SD of seven measurements. **P < 0.01 the control group vs. the low-allopurinol group or the high-allopurinol group.

C. Relationship between total Q and brain ICAM-1. Total Q is the sum of Q_I and Q_R.

D. Relationship between total Q and plasma soluble ICAM-1 (sICAM-1).

Table 1. Physiological parameters.

		After Reperfusion				
	Pre-Ischemia	10 min	30 min	60 min	90 min	120 min
MAP (mmHg)						
control	137 ± 9	142 ± 3	144 ± 7	123 ± 17	101 ± 3	83 ± 8
low-allopurinol	142 ± 4	131 ± 14	143 ± 7	115 ± 7	97 ± 10	80 ± 4
high-allopurinol	153 ± 14	143 ± 11	151 ± 12	119 ± 9	95 ± 5	80 ± 8
PaO_2 (Torr)						
control	98 ± 3	106 ± 11	92 ± 7	86 ± 10	82 ± 11	82 ± 12
low-allopurinol	107 ± 11	107 ± 9	93 ± 10	85 ± 7	81 ± 7	78 ± 8
high-allopurinol	117 ± 18	122 ± 17	103 ± 15	101 ± 18	90 ± 12	93 ± 11
$PaCO_2$ (Torr)						
control	41.8 ± 4.3	45.5 ± 2.6	45.1 ± 3.0	41.6 ± 2.4	41.0 ± 3.0	40.1 ± 1.4
low-allopurinol	38.4 ± 2.1	48.9 ± 3.4	46.2 ± 3.9	42.7 ± 1.7	43.6 ± 2.0	40.6 ± 1.7
high-allopurinol	39.7 ± 2.1	45.0 ± 2.1	44.9 ± 2.3	42.6 ± 2.0	41.3 ± 2.4	39.5 ± 1.6
pH						
control	7.37 ± 0.04	7.04 ± 0.01	7.15 ± 0.03	7.18 ± 0.01	7.15 ± 0.02	7.20 ± 0.02
low-allopurinol	7.40 ± 0.02	7.04 ± 0.03	7.15 ± 0.04	7.18 ± 0.07	7.17 ± 0.06	7.21 ± 0.04

high-allopurinol	7.37 ± 0.04	7.05 ± 0.03	7.17 ± 0.02	7.20 ± 0.02	7.21 ± 0.03	7.23 ± 0.03
BE (mmol/L)						
control	−0.78 ± 2.5	−19.2 ± 0.6	−14 ± 3.2	−12.8 ± 0.5	−15.0 ± 1.3	−13.1 ± 0.9
low-allopurinol	0.5 ± 0.6	−17.7 ± 1.9	−11.8 ± 4.1	−10.2 ± 1.8*	−9.8 ± 1.8*	−10.6 ± 2.2*
high-allopurinol	0.6 ± 1.3	−17.8 ± 2.4	−12.6 ± 2.5	−9.0 ± 1.4*	−10.0 ± 1.3*	−11.3 ± 2.0
Lactate (mmol/L)						
control	1.6 ± 0.8	11.9 ± 1.2	7.2 ± 1.6	7.1 ± 2.8	7.0 ± 3.2	6.7 ± 3.2
low-allopurinol	1.4 ± 0.3	10.9 ± 1.8	6.1 ± 1.3	6.0 ± 1.3	5.5 ± 1.0	4.8 ± 0.9
high-allopurinol	2.3 ± 0.7	10.5 ± 1.4	6.3 ± 0.9	5.5 ± 1.1	4.5 ± 0.9	4.4 ± 1.2

All values are given as means ± standard deviations. MAP, mean arterial blood pressure; BE, base excess. *$P < 0.05$, the control group vs. the low-allopurinol group or high-allopurinol group.