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In Vivo Real-time Monitoring and Evaluation for Superoxide Anion Radical Generation with an Electrochemical Sensor

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Abstract Recently, we established an *in vivo* method to directly and continuously monitor and evaluate $O_2^{-\cdot}$ using an electrochemical $O_2^{-\cdot}$ sensor. The generated $O_2^{-\cdot}$ is measured as a current and evaluated as a difference in the current from the baseline to the actual reacted $O_2^{-\cdot}$ current (ΔI) and a quantified partial value of electricity (Q), which is calculated by integration of differences between baseline and actual reacted current. To clarify the dynamics of $O_2^{-\cdot}$ *in vivo* and their $O_2^{-\cdot}$-related pathophysiology, the accuracy and efficacy of this method were confirmed in phosphate-buffered saline and human blood and we applied this sensor to rat models of endotoxemia, forebrain ischemia-reperfusion (FBI/R), and heatstroke. This is a novel method for measuring $O_2^{-\cdot}$ *in vivo*, and could be used to monitor and treat the pathophysiology caused by excessive $O_2^{-\cdot}$ generation in animals and humans.

Key words: superoxide anion radical, electrochemical sensor, endotoxemia, cerebral ischemia-reperfusion, heatstroke

Introduction

Reactive oxygen species (ROS) have an essential role in homeostasis *in vivo*. However, the excessive ROS generation leads to oxidative stress and tissue injury.[1-4] Among ROS, the superoxide anion radical ($O_2^{-\cdot}$) is the key radical because it functions as a messenger in signaling pathways and as an effector of the oxidative stress attributable to many toxic ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH·), and peroxynitrite ($ONOO^-$), both intracellularly and extracellularly.[1-5] However, the dynamics of the $O_2^{-\cdot}$ circulating in the blood have been unclear because it was difficult to detect $O_2^{-\cdot}$ *in vivo* due to its instability. Recently, an all-synthetic electrochemical sensor that can detect $O_2^{-\cdot}$ specifically *in vitro* has been developed,[6,7] and we applied this sensor to rat models of endotoxemia,[8,9] forebrain ischemia-reperfusion (FBI/R),[10-16] and heatstroke[17] to clarify the dynamics of $O_2^{-\cdot}$ *in vivo* and their $O_2^{-\cdot}$-related pathophysiology.

Electrochemical sensor detecting superoxide anion radical ($O_2^{-\cdot}$)

This 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. This sensor has a highly catalytic activity for the oxidation of $O_2^{-\cdot}$, and can measure a current generated by the oxidation of $O_2^{-\cdot}$.[6,7,18] In this sensor, the axial coordination of an imidazole ligand to the iron porphyrin center enhances its selectivity for $O_2^{-\cdot}$ by impeding the undesired coordination of H_2O_2, which results from the dismutation of $O_2^{-\cdot}$.[6,7]

In vivo monitoring of $O_2^{-\cdot}$

The sensor can detect $O_2^{-\cdot}$ as a current generated by the oxidation of $O_2^{-\cdot}$.[6,7,18] The sensor is connected to a ROS analysis system, which includes a computer to measure and analyze the $O_2^{-\cdot}$ current.[18] The current data are recorded at two points per second by the ROS analysis system, and a smoothing procedure (a moving method) was applied to the data because the data contained noise and artifacts attributed to heartbeats, mechanical ventilation, and the heating pad used for body temperature control in the *in vivo* experiments. The elevation of the current is related with the $O_2^{-\cdot}$ concentration generated by a reaction between xanthine and xanthine oxidase in saline[6,7] and whole blood of rat and human.[8] In face of *in vivo* monitoring of $O_2^{-\cdot}$, we have applied intravascular measurement.[8-17] The sites of monitoring are in the right atrium in rats subjected to endotoxemia[8,9] and heatstroke,[17] and in the jugular vein in rats subjected to the FBI/R.[10-16] This sensor does not work without any flow, because $O_2^{-\cdot}$ must hit the sensor surface. In addition, if foreign bodies, such as thrombi, adhere to the sensor surface, this sensor will not work. Further, our sensor can detect $O_2^{-\cdot}$ that is generated around the sensor, and has not yet been abolished by antioxidants.

Evaluation for generated $O_2^{-\cdot}$

To evaluate the generated $O_2^{-\cdot}$, we have applied ΔI and a quantified partial value of electricity (Q).[8] The ΔI refers to the difference in the current from the baseline to the actual reacted $O_2^{-\cdot}$ current.[8] The baseline current was defined as the stable state before an invasive intervention in the *in vivo* experiments. The Q is attributed to the generation of $O_2^{-\cdot}$ and is calculated by the integration of the differences between the baseline and the actual reacted $O_2^{-\cdot}$ current for a certain time period.[8] There exists a linear relationship between the Q and the $O_2^{-\cdot}$ concentration generated by a reaction between xanthine and xanthine oxidase in saline[6,7] and whole blood of rat and human.[8] Therefore, the Q reflects the amount of $O_2^{-\cdot}$ generated for a certain period. However, the Q cannot reflect total amount of $O_2^{-\cdot}$ generated in the whole body, because the $O_2^{-\cdot}$ sensor can detect only $O_2^{-\cdot}$ which hit the surface of the sensor.

Endotoxemia

In the endotoxemic rats which were administrated 3 μg/g of lipopolysaccharide (LPS) intravenously, the $O_2^{-\cdot}$ current was measured in the right atrium continuously.[8,9] The ΔI of $O_2^{-\cdot}$ began to increase at 1 hour after LPS administration and continued to increase until 6 hours, while there was no elevation of ΔI in sham-treated rats.[8] The Q for 6 hours also increased significantly in endotoxemic rats, in comparison to those in sham-treated rats.[8] Further, the elevation of the Q was related with the elevation of plasma malondialdehyde (MDA) level which was a marker of lipid peroxidation, the elevation of some cytokines, i.e. tumor necrosis factor-α, interleukin-6, and high mobility group box-1 (HMGB1), and the elevation of soluble intercellular adhesion molecule-1 (sICAM-1) which was a marker of endothelial injury.[8,9] Ulinastatin, a human urinary trypsin inhibitor (UTI), could attenuate the $O_2^{-\cdot}$ generation in endotoxemic rats and could suppress the plasma lipid peroxidation, the production of inflammatory cytokines, and the endothelial injury in eodotoxemic rats.[9]

Forebrain ischemia-reperfusion (FBI/R)

In the rats subjected to FBI/R, the $O_2^{-\cdot}$ current was measured in the jugular vein continuously.[10-16] The ΔI showed marked increase immediately after reperfusion and continued for more than 120 min after FBI/R.[10-16] The Q during ischemia and reperfusion also increased significantly in FBI/R rats, in comparison to those in sham-treated rats.[10,15] The elevation of the Q was related with the elevation of MDA, HMGB1, and ICAM-1 in brain and plasma.[10-16] In the FBI/R pathophysiology, we reported that xanthine oxidase was one of the major source of $O_2^{-\cdot}$ in blood by using allopurinol, an inhibitor of xanthine oxidase.[11] Further, the elevation of the $O_2^{-\cdot}$ generation was suppressed by moderate hypothermia,[13] normobaric hyperoxia,[15] administration of physostigmine which was one of cholinergic agonists,[14] and UTI administration,[16] while hyperglycemia enhanced the $O_2^{-\cdot}$ generation after FBI/R.[12]

Heatstroke

In the heatstroke rats, the $O_2^{-\cdot}$ current was measured in the right atrium continuously.[17] Heatstroke was made by elevation of core temperature to 40.0℃ by 1.0℃/10 min and the onset of heatstroke was defined as the moment when the MAP dropped by 10 mmHg from the peak level.[17] During the induction of heatstroke, the ΔI began to increase at 38 ℃ of core temperature and continued to rise gradually until the onset of heatstroke.[17] After the onset of heatstroke, the ΔI stayed at plateau at 40℃ of core temperature and was suppressed with temperature-dependency.[17] The Q during the induction and after heatstroke also increased significantly in heatstroke rats in comparison to those in sham-treated rats. Moderate hypothermia could suppress the $O_2^{-\cdot}$ generation in heatstroke rats.[17] Furthermore, the elevation of the Q was related with liver injury and the elevation of MDA, HMGB1, and ICAM-1 in liver and plasma.[17]

Conclusion

In conclusion, this is the unique method to directly and continuously monitor and evaluate $O_2^{-\cdot}$ generated *in vivo*. It should be applicable to monitor $O_2^{-\cdot}$ both in animals and humans at the bedside in near future. Excessive $O_2^{-\cdot}$ generation itself will become an enormously important target for the treatment of pathophysiological states in humans and it will be possible to treat patients monitoring and accessing $O_2^{-\cdot}$ generation by the $O_2^{-\cdot}$ sensor and the ROS analysis system.

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Conflict of Interest

The authors state no conflict of interest.

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