Molecular basis for cellular formation of a nitrated cyclic nucleotide, a second messenger for ROS signaling
（活性酸素シグナルのセカンドメッセージ、ニトロ化環状ヌクレオチドの細胞内生成の分子基盤）

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Abstract of the Thesis

Background and Purpose: 8-Nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP) is a novel nitrated derivative of guanosine 3',5'-cyclic monophosphate (cGMP) of which endogenous formation has recently been identified in mammalian cells. 8-Nitro-cGMP can function as a unique electrophilic second messenger that induces antioxidant adaptive response to cells via cGMP adduction to sulfhydryls of redox sensor proteins, the process is called protein S-guanylation. In the first part, I studied chemical and biochemical regulatory mechanisms involved in the formation of 8-nitro-cGMP, with particular focus on the roles of reactive oxygen species (ROS). In the second part of this study, I examined the methodological proof of immunocytochemistry for specific identification of 8-nitro-cGMO in cultured cells.

Methods: In vitro formation of nitrated derivatives was analyzed by means of reverse-phase high performance liquid chromatography equipped with photodiode array detector or with electrochemical detector. Cellular formation of 8-nitro-cGMP in rat glioma C6 cells was analyzed by means of immunocytochemistry with the use of anti-8-nitro-cGMP monoclonal antibody and by means of liquid chromatography-tandem mass spectrometry. Cellular production of ROS was determined by means of fluorescent microspectrometry using chemical probes that become fluorescent upon reaction with ROS.

Results: Chemical analyses demonstrated that peroxynitrite and myeloperoxidase-dependent oxidation of nitrite in the presence of hydrogen peroxide (H₂O₂) were two major pathways for guanine nucleotide nitration. Among guanine nucleotides examined, guanosine 5'-triphosphate was the most sensitive against peroxynitrite-mediated nitration. Immunocytochemical as well as tandem mass spectrometrical analyses revealed that formation of 8-nitro-cGMP in rat glioma C6 cells stimulated with lipopolysaccharide plus pro-inflammatory cytokines was highly dependent on the production of both superoxide and H₂O₂. By using mitochondria-targeted chemical probe MitoSOX™ Red, we found that mitochondria-derived superoxide can act as a direct determinant for 8-nitro-cGMP formation. This is the first demonstration that mitochondria-derived superoxide plays an essential role in biological nitration of guanine nucleotides. Furthermore, we also clarified that mitochondria-derived superoxide production was regulated by NADPH oxidase (Nox2)-generated H₂O₂, suggesting the importance of cross-talk between Nox2-depdent H₂O₂ production and mitochondrial superoxide production. I have also verified that immunocytochemistry is a conventional, powerful, and fairly straightforward method for determining the presence, localization, and relative abundance of 8-nitro-cGMP in cultured cells.

Conclusions: Our data suggest that 8-nitro-cGMP can serve as a unique second messenger that may implicate in regulation of ROS signaling in the presence of NO.