Biochemical and Biophysical Research Communications **273,** 5–9 (2000)
doi:10.1006/bbrc.2000.2897, available online at http://www.idealibrary.com on IDEAL®

Activation of NADPH Oxidase in Alzheimer's Disease Brains

Shun Shimohama,* Hiroko Tanino,† Naoko Kawakami,† Naoki Okamura,‡ Hiroomi Kodama,‡ Teruhide Yamaguchi,§ Takao Hayakawa,§ Akihiko Nunomura,¶ Shigeru Chiba,¶ George Perry,‖ Mark A. Smith,‖ and Sadaki Fujimoto†

**Department of Neurology, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan; †Department of Environmental Biochemistry, Kyoto Pharmaceutical University, Kyoto 607-8414, Japan; ‡Department of Physiological Chemistry, Hiroshima University School of Medicine, Hiroshima 734-8551, Japan; §Division of Biological Chemistry, National Institute of Health Sciences, Tokyo 158-8501, Japan; ¶Department of Psychiatry and Neurology, Asahikawa Medical College, Asahikawa 078-8510, Japan; and ‖Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106*

Received May 17, 2000

The present study is the first to show that superoxide (O_2^-) forming NADPH oxidase is activated in Alzheimer's disease (AD) brains by demonstrating the marked translocation of the cytosolic factors p47-*phox* and p67-*phox* to the membrane. In conjunction with a recent *in vitro* study showing that amyloid β activates O_2^- forming NADPH oxidase in microglia, where these *phox* proteins are localized in this study, the present results suggest that, in AD, NADPH oxidase is activated in microglia, resulting in the formation of reactive oxygen species which can be toxic to neighboring neurons in AD.

Key Words: **Alzheimer's disease; NADPH oxidase; p47-*phox;* p67-*phox;* amyloid β; translocation; superoxide; microglia.**

Alzheimer's disease (AD) is clinically characterized by dementia and pathologically a progressive loss of neurons, senile plaques and neurofibrillary tangles. In senile plaques, central deposits of extracellular amyloid β (Aβ) fibrils are surrounded by dystrophic neurites, activated microglia and reactive astrocytes (1). Although the mechanism underlying neuronal loss in AD remains unclear, the excessive formation of Aβ from its precursor protein and its deposition in the brain is commonly thought as a key event in the progressive neuronal damage (2). In fact, studies investigating the neurotoxic effect of Aβ have resulted in the idea that Aβ, through microglial activation and consequent production of toxic and inflammatory mediators such as nitric oxide (NO), cytokines and reactive oxygen species (ROS), is indirectly neurotoxic (3, 4).

Recently, Bianca *et al.* (5) showed *in vitro* that Aβ activates NADPH oxidase in microglia, monocytes, and neutrophils. NADPH oxidase in phagocytic cells is an electron transport system that catalyzes the one-electron reduction of oxygen to O_2^-, a process essential for host defence against invading microorganisms. NADPH oxidase is composed of several proteins, and when the cells are in resting state the oxidase is inactive, and some components are located on the plasma membrane (flavocytochrome *b*), and some components are in the cytosol (p47-*phox,* p67-*phox,* and a low-molecular-weight GTP-binding protein, Rac). The translocation of the cytosolic components on the plasma membrane where, in association with the subunits of flavocytochrome b, results in the activation of NADPH oxidase. Therefore, at least some of the cytosolic components such as p47-*phox* and p67-*phox* (*phox* refers to phagocyte oxidase) are translocated to the membrane when the enzyme is activated (6).

Although Aβ can activate NADPH oxidase resulting in the formation of O_2^- in phagocytic cells *in vitro* (5), it remains to be demonstrated whether or not NADPH oxidase is activated in AD. The present study is the first to demonstrate that the cytosolic factors p47-*phox* and p67-*phox* are markedly translocated to the membrane in AD, and that *phox* proteins are expressed in microglia. These results strongly suggest that, in AD, microglial NADPH oxidase is activated, resulting in the formation of ROS.

MATERIALS AND METHODS

Materials. Mouse monoclonal anti-human p47-*phox* and -human p67-*phox* antibodies were obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-mouse p47-*phox* antibody was generated as described previously (7). Anti-microtubule-

0006-291X/00 $35.00

associated protein 2 (MAP2) was obtained from Sigma Chemical Co. (St. Louis, MO). Anti-glial fibrillary acidic protein (GFAP) was obtained from Dako Co. (Japan), and isolectin B_4 from Sigma Chemical Co. (St. Louis, MO). An enhanced chemiluminescent detection system (ECL kit) from Amersham (Buckinghamshire, England) and Vectastain ABC Elite kit from Vector Laboratories (Burlingame, CA) were used for immunodetection. All other chemicals were of reagent grade and were obtained commercially.

Preparation of human and rat neutrophils and their stimulation. Neutrophils were obtained from normal adult human subjects and male adult Wistar rats and stimulated by phorbol 12-myristate 13-acetate (PMA) as described previously (8). The plasma membrane fraction was used as a positive control for detecting p47-*phox* and p67-*phox* proteins.

Preparation of brain extracts. Brain tissues from the frontal lobe obtained at autopsy from 13 patients diagnosed clinically and histopathologically as having AD (mean age, 79 years; mean postmortem delay, 7 h), and from 12 age-matched non-demented control subjects (mean age, 78 years; mean postmortem delay, 14 h) were used in this study. The neuropathologic assessment of AD was made in accordance with the criteria of the Consortium to Establish a Registry for AD (CERAD) (9), while control brains exhibited negligible microscopic neuropathological changes. The brain tissues were frozen immediately after autopsy, and stored at −80°C until thawing for homogenization and biochemical assays.

Brain tissue samples from the cerebral cortex were homogenized, and centrifuged at 3000*g* for 10 min and the supernatant thus obtained was centrifuged at 105,000*g* for 60 min. Then the supernatant was used as the cytosolic fraction, and the pellet was resuspended with the homogenization buffer, and used as the membranous fraction (10).

Preparation of cell extracts from rat primary cultures of neurons, astrocytes, and microglia. Primary cortical neurons were prepared from 19-day-old rat fetuses as described previously (11). Astroglial primary cultures were prepared from newborn rat pups as described previously (12). Highly enriched microglial primary cultures were prepared from a primary culture of neonatal rat cerebral tissue by the method of Nakajima *et al.* (13). The animals were treated in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals (14). The purity of neurons, astrocytes, and microglia was estimated to be >95% by staining with anti-MAP2, -GFAP, and isolectin B_4 antibodies, respectively (data not shown).

Cell lysates were prepared as described previously (11, 12).

Immunochemical detection. Immunochemical detection was performed using anti-p47-*phox* or -p67-*phox* antibodies (1:2000) according to a method described previously (10, 12). Immunoreactivity was visualized using the ECL method and analyzed by quantitative densitometry using a computed image analysis program (NIH image 1.51). Results are given as mean ± standard error of the mean (SEM). Statistical significance was determined using analysis of variance (ANOVA) and Dunnett's two-tailed test.

RESULTS

Presence of p47-phox and p67-phox Proteins in Human Brain Tissue

The membranous fraction of stimulated human neutrophils was used as a positive control for p47-*phox* and p67-*phox* proteins. Anti-human p67-*phox* antibody (Transduction Laboratories) detected p67-*phox* proteins in not only human samples (brain tissues and neutrophils) but also rat samples (neurons, astrocytes and microglia of primary cultures, and neutrophils), while anti-human p47-*phox* antibody (Transduction Laboratories) detected p47-*phox* proteins in human samples, but not in rat samples. In contrast, anti-mouse p47-*phox* antibody (7) detected p47-*phox* proteins in rat samples, but not in human samples (data not shown). Therefore, anti-human p47-*phox* antibody (Transduction Laboratories) was used for human samples, while anti-mouse p47-*phox* antibody (7) for rat samples.

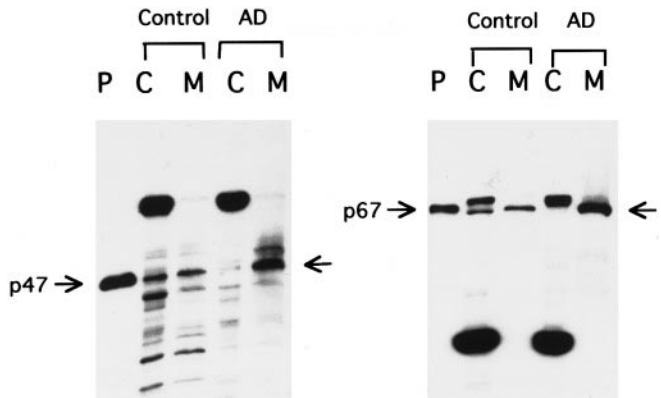

FIG. 1. Presence of p47-*phox* and p67-*phox* in human brain tissue. The cytosolic (10 μg protein/lane) (C) and membranous (10 μg protein/lane) (M) fractions from the frontal cortices of control and AD were used in the immunoblot analysis of p47-phox and p67-phox proteins. The membranous fraction of stimulated human neutrophils was used as a positive control for p47-*phox* and p67-*phox* (P). Antibodies to p47-*phox* and p67-*phox* reacted specifically with the 47- and 67-kDa protein bands, respectively, on immunoblots of the membranous fraction of human neutrophils. Antibodies to p47-*phox* and p67-*phox* detected p47-*phox* and p67-*phox* proteins, respectively, in both the cytosolic and membranous fractions of the control human brains. Several nonspecific protein bands were also identified. In contrast, p47-*phox* and p67-*phox* proteins were predominantly present in the membranous fraction in AD.

On immunoblots, anti-p47-*phox* and -p67-*phox* antibodies detected p47-*phox* and p67-*phox* proteins, respectively, both in the cytosolic and membranous fractions of the control human brain. In contrast, p47-*phox* and p67-*phox* proteins were predominantly present in the membranous fraction of the AD brain (Fig. 1).

Translocation of p47-phox and p67-phox to the Membrane from the Cytosol in AD

Total levels of p47-*phox* and p67-*phox* proteins were significantly increased in AD samples compared with controls. Membrane p47-*phox* and p67-*phox* levels, expressed as a percentage of total p47-*phox* and p67-*phox*, were dramatically and significantly increased in AD, indicating a significant translocation from the cytosol to the membrane in AD (Fig. 2).

Expression of p47-phox and p67-phox in Microglia

Anti-p47-*phox* and -p67-*phox* antibodies reacted with a 47 kDa and a 67 kDa protein band, respectively, on immunoblots of the lysates of primary cultures of

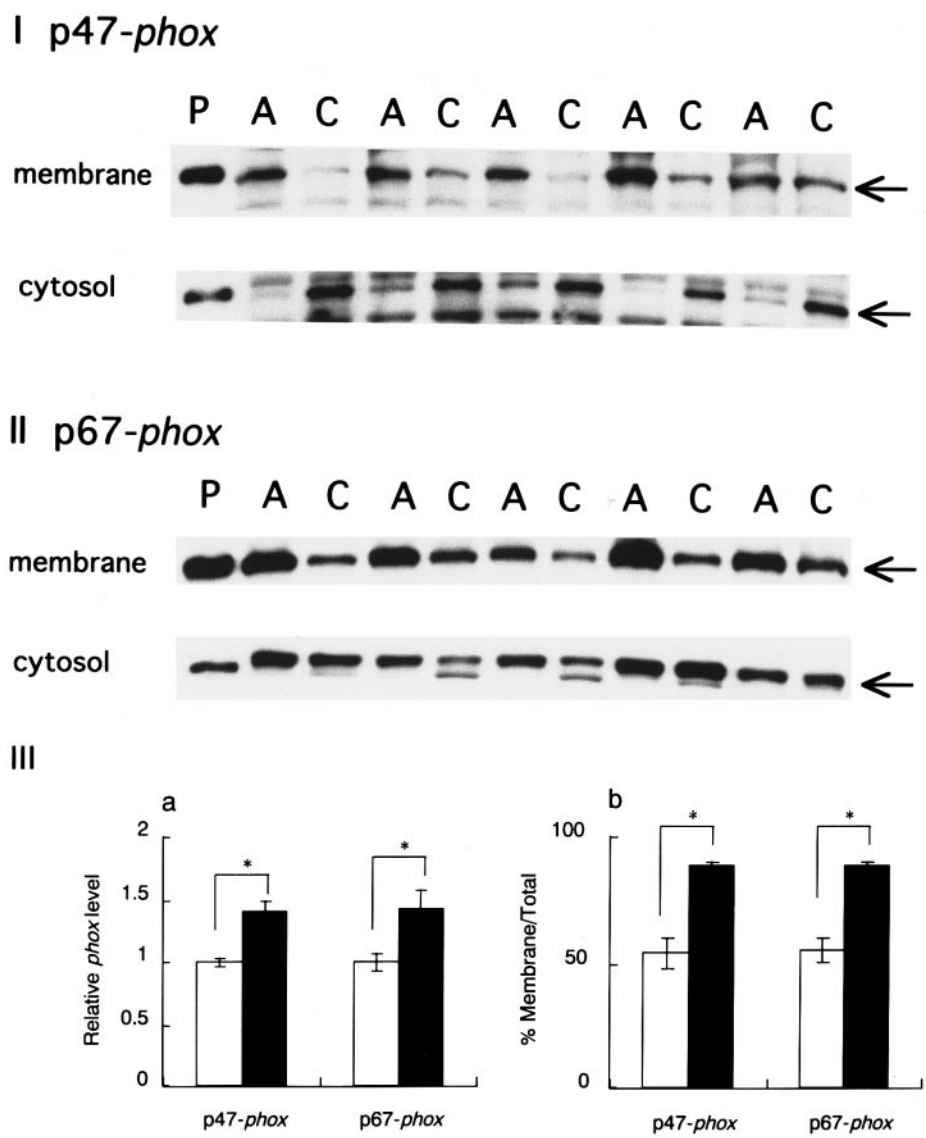

FIG. 2. Translocation of p47-*phox* and p67-*phox* proteins from the cytosol to the membrane in AD. The cytosolic (10 μg protein/lane) and membranous (10 μg protein/lane) fractions from the frontal cortices of 12 control and 13 AD were used in the immunoblot analysis of p47-*phox* and p67-*phox* proteins. The membranous fraction of stimulated human neutrophils was used as a positive control (P). The immunoblots of 5 control (C) and 5 AD (A) cases for p47-*phox* and p67-*phox* proteins are shown in (I) and (II), respectively. To calculate the total p47-*phox* and p67-*phox* levels, the membranous and cytosolic p47-*phox* and p67-*phox* levels were determined by densitometric scanning of immunoblots. The protein concentrations in each fraction were expressed relative to control samples, which were set to 1.0 (III-a). Membranous p47-*phox* and p67-*phox*, expressed as a percentage of total p47-*phox* and p67-*phox*, is shown in (III-b). Note the marked translocation from the cytosol to the membrane in AD. The white bars represent control cases, whereas the black bars represent AD cases. Error bars indicate SEM. Significance: $*P < 0.01$ vs control levels.

microglia and of the membranous fraction of stimulated rat neutrophils. The p47-*phox* and p67-*phox* proteins were not detected in the lysates of primary cultures of either neurons or astrocytes (Fig. 3).

DISCUSSION

The present study showed for the first time that NADPH oxidase is activated in AD brains by demonstrating the significant translocation of the cytosolic factors p47-*phox* and p67-*phox* to the membrane in AD brains. Because both antibodies to p47-*phox* and p67-*phox* used in the present study recognized several nonspecific proteins besides their specific p47-*phox* and p67-*phox* proteins, respectively, it seemed inappropriate to use these antibodies perform immunocytochemically. Therefore, in the present study we prepared separate rat primary cultures of neurons, astrocytes, and microglia and confirmed that these *phox* proteins are limited in expression to microglia.

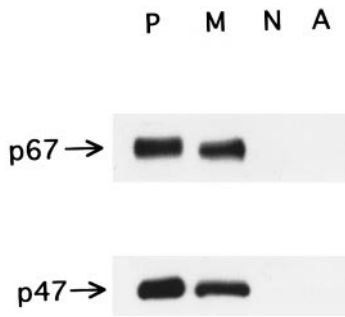

FIG. 3. Expression of p47-*phox* and p67-*phox* in microglia. The membranous fraction of stimulated rat neutrophils was used as a positive control (P). Antibodies to p47-*phox* and p67-*phox* reacted with the 47- and 67-kDa protein bands, respectively, on immunoblots of the lysates of primary cultures of microglia (M) and of a positive control (P). The p47-*phox* and p67-*phox* proteins were not detected in the lysates of primary cultures of either neurons (N) or astrocytes (A).

One of the most striking cellular responses induced by the pathological feature of AD is indisputably elaborated by microglia (4, 15, 16). That the response of microglia to Aβ might include stimulation of hydrogen peroxide (H_2O_2) production through activation of NADPH oxidase was demonstrated *in vitro* for rat microglia as well as in neutorophils and monocytes exposed to Aβ (5). NADPH oxidase activation is an essential component of the human cellular immune response of phagocytes and is also involved in nonspecific tissue damage associated with a variety of inflammatory diseases. It is known that various reactions participate in the signal transduction for NADPH oxidase activation. Cytokines such as interleukin-1 and tumor necrosis factor-α, which are induced in AD (17, 18) and have triggering and priming effect on NADPH oxidase (6), may contribute to NADPH oxidase activation in addition to Aβ. Activation of NADPH oxidase results in production of O_2^-. This is then rapidly converted to secondary toxic ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and hypochlorous acid (HOCl) or reactive nitrogen species (RNS) such as peroxynitrite, which can efficiently kill microorganisms. NADPH oxidase is a highly regulated membrane-bound enzyme complex that is composed of a number of cytosolic and membrane-bound proteins. p47-*phox* and p67-*phox*, two of the components of NADPH oxidase, are normally located in the cytosol in resting cells and their translocation from the cytosol to the membrane is required for O_2^- generation *in vitro* and *in vivo* (6). The present study clearly indicated the marked translocation of p47-*phox* and p67-*phox* to the membrane, strongly suggesting that NADPH oxidase is activated in AD brains. Therefore, these results suggest that, in AD, NADPH oxidase is activated. To confirm this, it is preferred to determine NADPH oxidase activity in brain tissues. However, the active form of NADPH oxidase has been found to be quite unstable (19). Therefore, it seems to be impractical to assay the activity of NADPH oxidase in human brain tissues. Formation of ROS and RNS from NADPH oxidase may be responsible for the prominent oxidative damage to neurons in AD. That O_2^- and possibly NO are produced by cells distinct from neurons, e.g. microglia, suggests an important role for diffusible reactive species such as H_2O_2, NO and peroxynitrite (3).

The present study is consistent with the notion that microglia display phagocytic activity and may be a key instigator of neuronal injury associated with Aβ deposition (4). Therapeutic measures that reduce the microglial response could therefore be useful to impede the pathological processes in AD. Although the exact role of p47-*phox* and p67-*phox* in NADPH oxidase assembly is still unknown, p47-*phox* and p67-*phox* are required for O_2^- generation. Therefore, agents that suppress the translocation of p47-*phox* and p67-*phox* may be useful in curbing neuronal injury in AD.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and grants from the Ministry of Welfare of Japan, the Inamori Foundation, and the Smoking Research Foundation and the National Institute of Health. We thank Drs. T. Tsuji, T. Imura, T. Kihara and Ms. M. Nishioka for technical assistance.

REFERENCES

1. Katzman, R. (1986) Alzheimer's disease. *N. Engl. J. Med.* **314,** 964–973.
2. Selkoe, D. J. (1994) Cell biology of the amyloid beta-protein precursor and the mechanism of Alzheimer's disease. *Annu. Rev. Cell Biol.* **10,** 373–403.
3. Smith, M. A., Richey-Harris, P. L., Sayre, L. M., Beckman, J. S., and Perry, G. (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci.* **17,** 2653–2657.
4. Kalaria, R. N. (1999) Microglia and Alzheimer's disease. *Curr. Opin. Hematol.* **6,** 15–24.
5. Bianca, V. D., Dusi, S., Bianchini, E., Pra, I. D., and Rossi, F. (1999) β-Amyloid activates the O_2^- forming NADPH oxidase in microglia, monocytes, and neutrophils. *J. Biol. Chem.* **274,** 15493–15499.
6. DeLeo, F. R., and Quinn, M. T. (1996) Assembly of the phagocyte NADPH oxidase: Molecular interaction of oxidase proteins. *J. Leukocyte Biol.* **60,** 677–691.
7. Yamaguchi, M., Saeki, S., Yamane, H., Okamura, N., and Ishibashi, S. (1995) Hyperphosphorylated p47-phox lost the ability to activate NADPH oxidase in guinea pig neutrophils. *Biochem. Biophys. Res. Commun.* **216,** 203–208.
8. Kawakami, N., Takemasa, H., Yamaguchi, T., Hayakawa, T., Shimohama, S., and Fujimoto, S. (1998) Indication of a protein kinase C-independent pathway for NADPH oxidase activation in human neutrophils. *Arch. Biochem. Biophys.* **349,** 89–94.
9. Mirra, S. S., Heyman, A., Mckeel, D., Sumi, S. M., Crain, B. J., Brownlee, L. M., Vogel, F. S., Hughes, J. P., van Belle, G., and Berg, L. (1991) The consortium to establish a registry for Alzheimer's disease (CERAD). II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* **41,** 479–486.

10. Shimohama, S., Fujimoto, S., Matsushima, H., Takenawa, T., Taniguchi, T., Perry, G., Whitehouse, P. J., and Kimura, J. (1995) Alteration of phospholipase C-δ protein level and specific activity in Alzheimer's disease. *J. Neurochem.* **64,** 2629–2634.
11. Shimohama, S., Ogawa, N., Tamura, Y., Akaike, A., Tsukahara, T., Iwata, H., and Kimura, J. (1993) Protective effect of nerve growth factor against glutamate-induced neurotoxicity in cultured cortical neurons. *Brain Res.* **632,** 296–302.
12. Imura, T., Shimohama, S., Sato, M., Nishikawa, H., Madono, K., Akaike, A., and Kimura, J. (1999) Differential expression of small heat shock proteins in reactive astrocytes after focal ischemia: Possible role of beta adrenergic receptor. *J. Neurosci.* **19,** 9768–9779.
13. Nakajima, K., and Kohsaka, S. (1993) Characterization of brain microglia and the biological significance in the central nervous system. *Adv. Neurol.* **60,** 734–743.
14. Bayne, K. (1996) Revised guide for the care and use of laboratory animals available: American Physiological Society. *Physiologist* **39,** 199–208.
15. McGeer, P. L., and McGeer, E. G. (1995) The inflammatory response system of brain: Implications for therapy of Alzheimer's and other neurodegenerative diseases. *Brain Res. Rev.* **21,** 195–218.
16. Rogers, J., Webster, S., Lue, L. F., Brachova, L., Civin, W. H., Emmerling, M., Shivers, B., Walker, D., and McGeer, P. (1996) Inflammation and Alzheimer's disease pathogenesis. *Neurobiol. Aging* **17,** 681–686.
17. Tarkowski, E., Blennow, K., Wallin, A., and Tarkowski, A. (1999) Intracerebral production of tumor necrosis factor-alpha, a local neuroprotective agent, in Alzheimer's disease and vascular dementia. *J. Clin. Immunol.* **19,** 223–230.
18. Licastro, F., Pedrini, S., Caputo, L., Annoni, G., Davis, L. J., Ferri, C., Casadei, V., and Grimaldi, L. M. (2000) Increased plasma levels of interleukin-1, interleukin-6 and alpha-1-antichymotrypsin in patients with Alzheimer's disease: Peripheral inflammation or signals from the brain? *J. Neuroimmunol.* **103,** 97–102.
19. Market, M., Glass, G. A., and Babior, B. M. (1985) Respiratory burst oxidase from human neutrophils: Purification and some properties. *Proc. Natl. Acad. Sci. USA* **82,** 3144–3148.