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Modulation of Intracellular Formation of Reactive Oxygen Intermediates in Peritoneal Macrophages and Microglia/Brain Macrophages by Propentofylline

R. B. Banati, P. Schubert, *G. Rothe, J. Gehrmann, †K. Rudolphi, *G. Valet, and G. W. Kreutzberg

*Department of Neuromorphology, Max Planck Institute for Psychiatry, and *Cellular Biochemistry Group, Max Planck Institute for Biochemistry, Martinsried, and †Hoechst AG, Wiesbaden, Germany*

Summary: Ischemia-induced nerve cell death can partly be prevented by propentofylline, a pharmacon structurally related to xanthine derivates that interacts with the neuromodulatory function of endogenous adenosine. To evaluate a possible mechanism of neuroprotection by propentofylline, we studied its effect on the cellular production of reactive oxygen intermediates in microglial cells, which under pathological conditions can differentiate into brain macrophages, in comparison to peritoneal macrophages. Using a flow cytometric assay, we determined the intracellular formation of reactive oxygen intermediates by measuring the oxidation of the membrane-permeable and nonfluorescent dihydrorhodamine 123 to the cationic and intracellularly trapped, green fluorescent rhodamine 123 in single viable cells. Propentofylline at the therapeutic concentration of 50 μM completely inhibited the Ca^{2+}-dependent Con A-induced increase in the production of reactive oxygen intermediates in peritoneal macrophages. In isolated and cultured microglial cells, which have a high spontaneous respiratory burst activity, the spontaneous production of reactive oxygen intermediates was reduced by ~30%. A phorbol 12-myristate 13-acetate–induced rise in the respiratory burst activity could not be inhibited by propentofylline in either cell type. An increased generation of reactive oxygen intermediates is thought to contribute to nerve cell death after brain ischemia, edema, and neurodegenerative diseases like Alzheimer's disease. These pathological conditions are all accompanied by an activation of microglial cells. We therefore suggest that the neuroprotective properties of propentofylline might in part be due to a modulation of the microglial production of potentially harmful reactive oxygen intermediates. **Key Words:** Adenosine—Cytotoxicity—Free radicals—Ischemia—Neuroprotection—Respiratory burst.

Activated phagocytes can produce large amounts of reactive oxygen intermediates that result from a process during which NADPH oxidase reduces O_2 to the superoxide anion (O_2^-), which subsequently dismutates together with H^+ to H_2O_2 and O_2. H_2O_2 is then reduced to hypochlorous acid by myeloperoxidase (Bellavite, 1988). The cascade of metabolic steps, known as "respiratory burst," yields by far more oxidants than other intracellular sources, i.e., mitochondria or microsomes, and is a typical feature of phagocytic cells.

Macrophage-mediated tissue damage (Halliwell and Gutteridge, 1985; Konat and Wiggins, 1985; Halliwell et al., 1988) in the central nervous system during inflammatory responses, in demyelinating diseases, and after peripheral nerve injury, direct brain trauma, and ischemia (Graeber et al., 1988; McGeer et al., 1988*a,b;* Gendelman et al., 1989; Haga et al., 1989; Konno et al., 1989; Morioka et al., 1991; Gehrmann et al., 1992) is accompanied by an increased production of free radicals (Chan and Fishman, 1980; Kogure et al., 1982; Raichle, 1983), which seem also important in Alzheimer's disease (Dellacourte et al., 1988; Zemlan et al., 1989; Benati et al., 1993).

In the present study we describe an inhibitory effect of the xanthine derivate propentofylline (HWA 285; Hoechst A.G., Werk Albert) on the free

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Address correspondence and reprint requests to Dr. R. B. Banati at Max Planck Institute for Psychiatry, Am Klopferspitz 18a, D-8033 Martinsried, Germany.

Abbreviations used: DHR, dihydrorhodamine 123; DMF, *N,N*-dimethylformamide; HBS, Hanks' buffered saline; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMA, phorbol 12-myristate 13-acetate.

radical formation during the respiratory burst of stimulated peritoneal macrophages and cultured microglial cells, the source of endogenous brain macrophages (Banati et al., 1991). The flow cytometric assay used determines the respiratory burst activity by measuring the intracellular oxidation of the membrane-permeable and nonfluorescent dihydrorhodamine 123 (DHR) to the cationic and intracellularly trapped, green fluorescent rhodamine 123 in single viable cells (Rothe et al., 1988). DHR is oxidized to rhodamine 123 in the simultaneous presence of H_2O_2 and peroxidase but not by O_2^- or H_2O_2 alone, as shown in cell-free assays (Rothe and Valet, 1990). The oxidation of DHR in phagocytes specifically measures the NADPH oxidase–dependent intracellular accumulation of reactive oxygen intermediates, i.e., those metabolites that are formed during the respiratory burst. This method further allows the exclusion of dead cells by simultaneous counterstaining with propidium iodide.

MATERIAL AND METHODS

Cell culture

Cultures of newborn rat brain were prepared as described previously (Giulian and Baker, 1986; Frei et al., 1987). Peritoneal macrophages were obtained from 12-week-old male white Wistar rats.

For flow cytometric measurement, cells were suspended (3–4 × 10^6 cells/ml) in Hanks' buffered saline (HBS; Sigma Chemie, Deisenhofen, Germany) supplemented with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 5 m*M*, pH 7.35; Serva Feinbiochemica, Heidelberg, Germany) (HBS-HEPES) and stored at 4°C for a maximum of 2 h.

Flow cytometric measurement of respiratory burst

DHR was obtained from Molecular Probes (Eugene, OR, U.S.A.) and dissolved to a 1 m*M* stock solution in *N,N*-dimethylformamide (DMF; Merck, Darmstadt, Germany).

The cellular suspensions of peritoneal macrophages and microglial cells (10 μl) were each further diluted with 1 ml HBS-HEPES and stained for 5 min at 37°C with 10 μl of a 100 μ*M* DHR solution in HBS (1 m*M* stock solution in DMF). The DHR-loaded cells were incubated with propentofylline (1 *M* stock solution in HBS-HEPES) at 10 and 50 μ*M* for 15, 25, 35, 45, and 60 min with and without Con A (100 μg/ml; Sigma Chemie) or phorbol 12-myristate 13-acetate (PMA; 100 n*M;* Sigma Chemie) stimulation. The DNA of dead cells was counterstained with 10 μl of 3 m*M* propidium iodide (Serva Feinbiochemica) solution in HBS 3 min before the flow cytometric measurement. To exclude effects from a possible release of endogenous adenosine, control experiments with incubation medium containing adenosine deaminase (200 U/mg, 5 μg/ml; Sigma Chemie) were performed.

The forward scatter and side scatter and two fluorescences of at least 10,000 cells/sample were measured simultaneously on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). Rhodamine 123 green fluorescence (515–545 nm) and propidium iodide red fluorescence (>650 nm) were measured with the light from an argon laser of 488-nm excitation wavelength.

Statistical analysis

The differences in respiratory burst activities caused by Con A stimulation and treatment with propentofylline were tested for significance by unpaired *t* test (Statistics, K. J. McConway, Blackwell Scientific Software, Oxford, U.K.). The *t* test was performed for each time point including the data of at least four independent experiments. Each single fluorescence value of each experiment was based on the measurement of at least 10,000 cells. Before each experiment the flow cytometer was calibrated with standardized yellow–green fluorescent microspheres of 4.3-μm diameter (Polysciences, St. Goar, Germany), thus ensuring the comparability of the fluorescence values from different experimental series.

RESULTS

Stimulation of peritoneal macrophages with Con A (100 μg/ml) from 25 min onward resulted in a significant (*t* test: $p < 0.001$) increase of the production of reactive oxygen intermediates as measured by higher green fluorescence following the increased oxidation of DHR to rhodamine 123 (Fig. 1a). When peritoneal macrophages were treated with Con A in the presence of 50 μ*M* propentofylline, the stimulatory effect of Con A was completely blocked. The inhibitory effect of propentofylline (50 μ*M*) on the respiratory burst activity became apparent after 15 min. The respiratory burst activities of Con A-stimulated cells under 50 μ*M* propentofylline treatment versus untreated Con A-stimulated peritoneal macrophages were lower with high significance (*t* test: $p < 0.001$).

Cultured microglial cells are characterized by a high spontaneous respiratory burst activity as described earlier. Stimulation with Con A, unlike PMA (Banati et al., 1991), does not result in any further increase of the production of reactive oxygen intermediates. In contrast to peritoneal macrophages, microglial cells showed a decline in the production of reactive oxygen intermediates over time, but continued to produce considerably more free radicals than these at any time. Our flow cytometric method analyzes only the intracellular accumulation of oxidized DHR trapped in living cells and by counterstaining with propidium iodide excludes dead cells. Therefore, the reduction of respiratory burst activity with time is due to cell death preferentially of cells with high respiratory burst activity, which would otherwise have contributed to the average fluorescence value of the total measured cell population of >10,000 cells/sample. Assessment of cellular viability by propidium iodide counterstain-

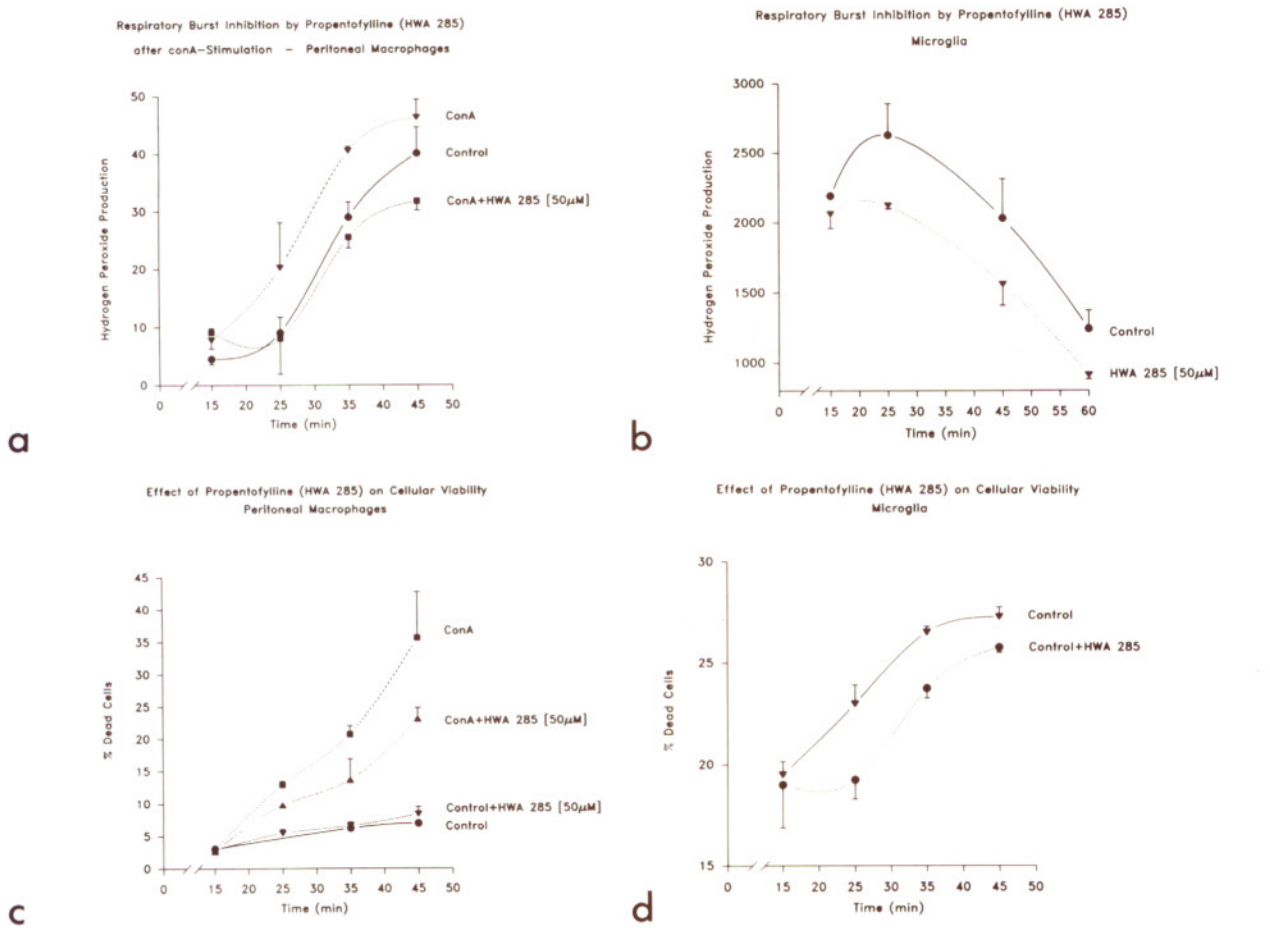

FIG. 1. The production of reactive oxygen intermediates was measured by the oxidation of the membrane-permeable, nonfluorescent dihydrorhodamine 123 (DHR) to the positively charged, membrane-impermeable, and green fluorescent rhodamine 123. The data are presented in standardized fluorescence units (*y*-axis). To allow accurate comparison of the fluorescence values from different experimental series, the fluorescence units were defined by calibrating the flow cytometer with standardized yellow–green fluorescent microspheres of 4.3-μm diameter. Each time point (*x*-axis) contains the data of at least four independent experiments. In each experiment the fluorescence of at least 10,000 cells was recorded. The error bars denote the SD. Dead cells were excluded from the analysis by counterstaining with propidium iodide. The significance of the effects of Con A and propentofylline were tested by unpaired *t* test. **a:** The stimulation of peritoneal macrophages with Con A (100 μg/ml) leads to a significant ($p < 0.001$) increase in the production of reactive oxygen intermediates after 25 min. Propentofylline (HWA 285; 50 μ*M*) reduces the respiratory burst of Con A-treated cells significantly ($p < 0.001$) down to control levels or below. The control curve shows the spontaneous production of reactive oxygen intermediates at 37°C. **b:** Cultured microglia already have a high spontaneous respiratory burst activity that is considerably higher than in peritoneal macrophages. The microglial production of reactive oxygen intermediates can be significantly ($p < 0.01$) reduced by propentofylline (50 μ*M*) at any time between 20 and 60 min. The high production of toxic free radicals leads to a high rate of cellular death. However, as dead cells were excluded from the analysis by counterstaining with propidium iodide, the marked inhibitory effect of propentofylline remains always clearly visible. **c:** Propentofylline (50 μ*M*) significantly ($p < 0.01$) reduces the Con A-induced (100 μg/ml) cell death. The protective effect is confined to stimulated cells [Con A + HWA 285 (50 μ*M*)]. No influence on unstimulated cells is detected [Control + HWA 285 (50 μ*M*)]. **d:** Unlike unstimulated peritoneal macrophages, the cultured microglial cells with their high spontaneous respiratory burst rate already have a high share of cells with reduced viability, which further increases with time. Despite the high spontaneous loss of cells, a slight but significant ($p < 0.05$) reduction of cell death by propentofylline (50 μ*M*) can still be observed.

ing revealed that in accordance with the high initial respiratory burst activity, the total population of cultured microglia already contains a relatively high number of dead cells that further increases with time. This increase of cell death over time is slightly but significantly (*t* test; $p < 0.05$) returned by propentofylline (Fig. 1d). The drug-independent decrease in respiratory burst activity, which is a characteristic of the highly active cultured microglia, does not interfere with the analysis of the reported effect of propentofylline. At any time point from 20 min onward, the populations of living cells treated with propentofylline have a lower respiratory burst activity than those without treatment. The incubation of microglial cells with 50 μ*M* propentofylline lead to a significant reduction of the respiratory burst activity (*t* test: $p < 0.01$) (Fig. 1b). After 35-min incubation with propentofylline, the inhibition

of respiratory burst activity reached its maximum, reducing the production of reactive oxygen intermediates by 27% of the normal baseline production (*t* test: $p < 0.001$).

When both peritoneal macrophages and microglial cells were stimulated with PMA, no reduction of their respiratory burst activity by propentofylline could be detected. Addition of adenosine deaminase (200 U/mg, 5 μg/ml) to the incubation media had no measurable effects.

In peritoneal macrophages, stimulation with Con A led to a decrease of cellular viability as measured by the counterstaining of dead cells with propidium iodide. After 45-min incubation with Con A (100 μg/ml), the number of dead cells increased to ~35% of the total cell population, whereas Con A stimulation in the presence of 50 μ*M* propentofylline kept the percentage of dead cells down to ~22%. The decrease in Con A-induced cell death became significant from 25 min onward (*t* test: $p > 0.01$). Unstimulated control populations or cell populations incubated only with 50 μ*M* propentofylline persistently contained ~5% dead cells (Fig. 1c).

DISCUSSION

Propentofylline as modulator of respiratory burst activity in mononuclear phagocytes

Propentofylline (HWA 285) is a new xanthine derivate that, unlike other methylxanthines, has neuroprotective properties (Evans et al., 1987; Rudolphi et al., 1987; DeLeo et al., 1988*a*). Pre- and early posttreatment with propentofylline reduces neuronal calcium loading and delayed nerve cell death in the hippocampus and other vulnerable areas of the brain (DeLeo et al., 1987, 1988*a;* Dux et al., 1990) as well as the postischemic increase of glial fibrillary acidic protein in astrocytes (DeLeo et al., 1987). The present study shows that propentofylline also influences the functional properties of microglial cells, which are activated early in ischemia (Gehrmann et al., 1992).

Propentofylline inhibits the Con A-stimulated respiratory burst of peritoneal macrophages and microglia, indicating an effect on the calcium-dependent free radical formation. As a maximal stimulus for respiratory burst and thus the release of the potentially toxic reactive oxygen intermediates, lectin treatment of macrophages leads to increased cell death. Under this maximal stimulation, the burst-inhibiting, protective effect of propentofylline is maximal. Con A acts via a binding site on phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and 1,2-diacylglycerol, both second messengers. Inositol 1,4,5-triphosphate increases intracellular Ca^{2+}, which in conjunction with 1,2-diacylglycerol induces an activation of protein kinase C (Riches et al., 1988). A sequence of largely unknown metabolic events then leads to the NADPH-dependent formation of reactive oxygen intermediates. In our experiments propentofylline did not affect the respiratory burst induced by direct activation of protein kinase C with PMA. It therefore appears that propentofylline acts in a calcium-dependent manner on those mechanisms that regulate the activation of protein kinase C. An indirect effect of propentofylline via adenosine (DeLeo et al., 1987, 1988*b;* Hagberg et al., 1987; Dux et al., 1990) seems unlikely as the experiments were performed in the presence of adenosine deaminase, which metabolizes adenosine to the ineffective inosine.

Microglial cells as potential target for propentofylline: pathophysiological relevance

Microglial cells are of mesodermal lineage and comprise 5–20% of the total glial population in the brain (Perry and Gordon, 1988; Lawson et al., 1990).

A number of recent studies have identified activated microglial cells as a source of reactive oxygen intermediates (Giulian and Baker, 1986; Colton and Gilbert, 1987; Sonderer et al., 1987; Woodroofe et al., 1989; Banati et al., 1991). Particularly in brain edema and ischemia-induced nerve cell death, reactive oxygen intermediates have been attributed a causative role (Chan and Fishman, 1980; Kogure et al., 1982; Raichle, 1983). Indirect evidence that microglial cells do, in fact, mediate postischemic injury comes from the improved recovery under treatment with chloroquine and colchicine, which both inhibit phagocytic and secretory functions in mononuclear phagocytes (Giulian and Robertson, 1990).

Summary

We provide evidence that propentofylline depresses the intracellular generation of free radicals in peripheral macrophages as well as in isolated microglial cells. As microglial cells are a major source of reactive oxygen intermediates in brain tissue under pathological conditions, the neuroprotective effect of propentofylline might in part be mediated by a reduction of the harmful oxygen intermediates released by microglial cells.

The flow cytometric method employed further allowed us to measure metabolic activity and cellular viability in the same individual cells at the same time. Simultaneously measured viability thus avoids misinterpretations of the tested drug actions because, in principle, a decrease in respiratory

burst activity could be due merely to a decrease in the number of living, i.e., producing, cells. Such a control seems particularly important when potentially toxic metabolites like reactive oxygen intermediates are measured.

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REFERENCES

Banati RB, Rothe G, Valet G, Kreutzberg GW (1991) Respiratory burst in brain macrophages: a flow cytometric study on cultured rat microglia. *Neuropathol Appl Neurobiol* 17:223–230

Banati RB, Gehrmann J, Czech C, Mönning, Jones LL, König G, Beyreuther K, Kreutzberg GW (1993) Early and rapid de novo synthesis of Alzheimer βA4-amyloid precursor protein (APP) in activated microglia. *Glia* 9:199–210

Bellavite P (1988) The superoxide-forming enzymatic system of phagocytes. *Free Rad Biol Med* 4:225–261

Chan PH, Fishman RA (1980) Transient formation of superoxide radicals in polyunsaturated fatty acid-induced brain swelling. *J Neurochem* 35:1242–1245

Colton AC, Gilbert DL (1987) Production of superoxide by a CNS macrophage, the microglia. *FEBS Lett* 223:284–288

DeLeo J, Toth L, Schubert P, Rudolphi K, Kreutzberg GW (1987) Ischemia-induced neuronal cell death, calcium accumulation, and glial response in the hippocampus of the Mongolian gerbil and protection by propentophylline (HWA 285). *J Cereb Blood Flow Metab* 7:745–751

DeLeo J, Schubert P, Kreutzberg GW (1988*a*) Propentofylline (HWA 285) protects hippocampal neurons of Mongolian gerbils against ischemic damage in the presence of an adenosine antagonist. *Neurosci Lett* 84:307–311

DeLeo J, Schubert P, Kreutzberg GW (1988*b*) Protection against ischemic brain damage using propentofylline in gerbils. *Stroke* 19:1535–1539

Dellacourte A, Defossez A, Ceballos I, Nicole A, Sinet PM (1988) Preferential localization of copper-zinc superoxide dismutase in vulnerable cortical neurons in Alzheimer's disease. *Neurosci Lett* 92:247–253

Dux E, Fastbom J, Ungerstedt U, Rudolphi K, Fredholm BB (1990) Protective effect of adenosine and a novel bilateral carotid occlusion in the gerbil hippocampus. *Brain Res* 516: 248–256

Evans MC, Swan JH, Meldrum BS (1987) An adenosine analogue, 2-chloroadenosine, protects against long term development of ischaemic cell loss in the rat hippocampus. *Neurosci Lett* 83:287–292

Frei K, Siepl C, Groscurth P, Bodmer S, Schwerdel C, Fontana A (1987) Antigen presentation and tumor cytotoxicity by interferon-gamma-treated microglial cells. *Eur J Immunol* 17:1271–1278

Gehrmann J, Bonnekoh P, Miyazawa T, Hossmann K-A, Kreutzberg GW (1992) Immunocytochemical study of an early microglial activation in ischemia. *J Cereb Blood Flow Metab* 12:257–269

Gendelman HE, Orenstein JM, Baca LM, Weiser B, Burger H, Kalter DC, Meltzer MS (1989) Editorial review. The macrophage in the persistence and pathogenesis of HIV infection. *AIDS* 3:475–495

Giulian D (1987) Ameboid microglia as effectors of inflammation in the central nervous system. *J Neurosci Res* 18:155–171

Giulian D, Baker TJ (1986) Characterization of ameboid microglia isolated from developing mammalian brain. *J Neurosci* 6:2163–2178

Giulian D, Robertson C (1990) Inhibition of mononuclear phagocytes reduces ischemic injury in the spinal cord. *Ann Neurol* 27:33–42

Graeber MB, Tetzlaff W, Streit WJ, Kreutzberg GW (1988) Microglial cells but not astrocytes undergo mitosis following rat facial nerve axotomy. *Neurosci Lett* 85:317–321

Haga S, Akai K, Ishii I (1989) Demonstration of microglial cells in and around senile (neuritic) plaques in the Alzheimer brain. *Acta Neuropathol* 77:569–575

Hagberg H, Andersson P, Lacarewicz J, Jacobson I, Butcher S, Sandberg M (1987) Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. *J Neurochem* 49:227–231

Halliwell B, Gutteridge JMC (1985) Oxygen radicals and the nervous system. *Trends Neurosci* 8:22–26

Halliwell B, Hoult JR, Blake DR (1988) Oxidants, inflammation and anti-inflammatory drugs. *FASEB J* 2:2867–2873

Kogure K, Watson BD, Busto R, Abe K (1982) Potentiation of lipid peroxides by ischemia in rat brain. *Neurochem Res* 7:437–454

Konat GW, Wiggins RC (1985) Effects of reactive oxygen species on myelin membrane proteins. *J Neurochem* 45:1113–1118

Konno H, Yamamato T, Iwasaki Y, Saitoh T, Suzuki H, Terunuma H (1989) Ia-expressing microglial cells in experimental allergic encephalomyelitis rats. *Acta Neuropathol* 77: 472–479

Lawson LJ, Perry VH, Dri P, Gordon S (1990) Heterogeneity in the distribution and morphology of microglia in the normal, adult mouse brain. *Neuroscience* 39:151–170

McGeer PL, Itagaki S, McGeer EG (1988*a*) Expression of the histocompatibility glycoprotein HLA-DR in neurological disease. *Acta Neuropathol* 76:550–557

McGeer PL, Itagaki S, Boyes BE, McGeer EG (1988*b*) Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* 38: 1285–1291

Morioka T, Kalehua AN, Streit WJ (1991) The microglial reaction in the rat dorsal hippocampus following transient forebrain ischemia. *J Cereb Blood Flow Metab* 10:850–859

Perry VH, Gordon S (1988) Macrophages and microglia in the nervous system. *Trends Neurosci* 11:273–277

Raichle ME (1983) The pathophysiology of brain ischemia. *Ann Neurol* 13:2–10

Riches DWH, Channon JY, Leslie CC, Henson PM (1988) Receptor-mediated signal transduction in mononuclear phagocytes. *Prog Allergy* 42:65–122

Rothe G, Valet G (1994) Flow cytometric assays of oxidative burst activity in phagocytes. *Methods in Enzymol* 233:539–548

Rothe G, Oser A, Valet G (1988) Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. *Naturwissenschaften* 75:354–355

Rudolphi KA, Keil M, Hinze HJ (1987) Effect of theophylline on ischemically induced hippocampal damage in Mongolian gerbils: a behavioral and histopathological study. *J Cereb Blood Flow Metab* 7:74–81

Sonderer B, Wild P, Fontana A, Peterhans E, Schwyzer M (1987) Murine glia cells in culture can be stimulated to generate reactive oxygen. *J Leukocyte Biol* 42:463–473

Woodroofe MN, Hayes GM, Cuzner ML (1989) Fc receptor density, MHC antigen expression and superoxide production are increased in interferon-gamma-treated microglia isolated from adult rat brain. *Immunology* 68:421–426

Zemlan FP, Thienhaus OJ, Bosmann HB (1989) Superoxide dismutase activity in Alzheimer's disease: possible mechanism for paired helical filament formation. *Brain Res* 476:160–162