Free Radical Biology & Medicine, Vol. 32, No. 11, pp. 1071–1075, 2002

0891-5849/02/$–see front matter

PII S0891-5849(02)00803-1

Serial Review: Causes and Consequences of Oxidative Stress in Alzheimer's Disease
Guest Editors: Mark A. Smith and George Perry

APOLIPOPROTEIN E ISOFORM MEDIATED REGULATION OF NITRIC OXIDE RELEASE

CANDICE M. BROWN,*† ELIZABETH WRIGHT,† CAROL A. COLTON,†‡ PATRICK M. SULLIVAN,†‡ DANIEL T. LASKOWITZ,†‡ and MICHAEL P. VITEK†‡§

*University Program in Genetics, †Department of Medicine (Neurology), and ‡Joseph and Kathleen Bryan Alzheimer's Disease Research Center, Duke University Medical Center, Durham, NC, USA; and §Cognosci, Inc., Research Triangle Park, NC, USA

(*Received* 6 *February* 2002; *Accepted* 21 *February* 2002)

Abstract—Progressive dysfunction and death of neurons in Alzheimer's dementia is enhanced in patients carrying one or more APOE4 alleles who also display increased presence of oxidative stress markers. Modulation of oxidative stress is a nontraditional and physiologically relevant immunomodulatory function of apolipoprotein E (apoE). Stimulated peritoneal macrophages from APOE-transgenic replacement (APOE-TR) mice expressing only human apoE3 or human apoE4 protein isoforms were utilized as mouse models to investigate the role of apoE protein isoforms and gender in the regulation of oxidative stress. Macrophages from male APOE4/4-TR mice produced significantly higher levels of nitric oxide than from male APOE3/3-TR mice, while macrophages from female APOE3/3-TR and female APOE4/4-TR mice produced the similar levels of nitric oxide. Primary cultures of microglial cells of APOE4 transgenic mice also produced significantly more nitric oxide than microglia from APOE3 transgenic mice. These data suggest a potentially novel mechanism for gender-dependent and apoE isoform-dependent immune responses that parallel the genetic susceptibility of APOE4 carriers for the development of Alzheimer's disease. © 2002 Elsevier Science Inc.

Keywords—Apolipoprotein E, Nitric oxide, Macrophage, Microglia, Free radicals

INTRODUCTION

Apolipoprotein E (APOE, gene; apoE, protein) is classically regarded as a lipid transport protein that exhibits three common alleles in human populations, $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ [1]. The APOE4 allele is a susceptibility factor for about 50% of the cases of sporadic Alzheimer's disease (AD) [2], and contributes to a poor clinical outcome in patients and in animal models of stroke and head trauma [3]. Some studies suggest that females exhibit a higher incidence of AD [4], but these results are controversial as other studies show no effect of gender and suggest that the higher incidence of AD is related to a longer female life expectancy [5,6]. Alternatively, women receiving perimenopausal estrogen replacement therapy are delayed in their onset of Alzheimer's disease for reasons that are just beginning to be explored [7–9]. In spite of the general acceptance that APOE4 genotype is associated with increased susceptibility for AD, the specific mechanisms and interactions governing apoE isoform specific effects in disease are incompletely understood.

One clue as to the function of APOE4 is the increased presence of oxidative stress markers associated with the amyloid plaques and neurofibrillary tangles that define the presence of Alzheimer's in the patient's brain. Smith et al. report that 3-nitrotyrosine immunostaining of neurofibrillary tangle pathology increases in patients with AD [10]. Since increased susceptibility to AD is associated with the presence of APOE4 genotype and nitric oxide is required to generate the 3-nitrotyrosine modification of proteins, then we hypothesized that nitric oxide production may be higher in APOE4 carriers. Since apoE appears to display nontraditional immunoregulatory properties as a modulator of free radical production

This article is part of a series of reviews on "Causes and Consequences of Oxidative Stress in Alzheimer's Disease." The full list of papers may be found on the homepage of the journal.

Address correspondence to: Dr. Michael P. Vitek, Division of Neurology, Box 2900, Duke University Medical Center, Durham, NC 27710, USA; Tel: (919) 684-0055; Fax: (919) 684-6514; E-Mail: mikevitek@mindspring.com.

throughout the central nervous system (CNS) and the periphery [11], then oxidative stress in diabetes, arthritis, AD, and atherosclerosis [12] may be influenced by APOE genotype. Our previous work supports this view by showing that nitric oxide production in human monocyte-derived macrophages (MDMs) and in RAW cells, a murine macrophage cell line, is modulated by the exogenous application of apoE [13,14].

The interaction of apoE and nitric oxide is probably related to their immune functions in various disease states. Since humans are the only species possessing apoE isoforms, we tested our hypotheses regarding the immunoregulatory functions of apoE isoforms in freshly elicited macrophages and primary microglial cultures from APOE3/3 and APOE4/4 transgenic replacement mice that singularly express human apoE3 or human apoE4 proteins [15,16]. This model system provides an efficient method to examine apoE-mediated inflammatory disease mechanisms that vary with APOE genotype and/or gender, genetic traits that may influence treatment paradigms in patients with inflammatory diseases such as AD.

METHODS

Mice strains

Breeding pairs of homozygous APOE3/3-TR and APOE4/4-TR targeted replacement mice [15,16] backbred three generations onto a C57Bl/6 background were provided by Dr. Patrick Sullivan at Duke University Medical Center. They were generated by replacing exons 2–4 of the endogenous mouse APOE gene with their human APOE3 or APOE4 gene counterparts. All male and female mice used in these studies were aged 15–20 weeks. Mice were housed in Duke University animal facilities under a standard 12 h light:12 h dark cycle; all animals received food and water ad libitum. All experiments were conducted in accordance with protocols approved by the Duke University IACUC Committee.

Elicitation and collection of murine peritoneal macrophages

Mouse macrophages were primed by two separate 1 ml injections of 5 mM sodium periodate (Sigma, St. Louis, MO, USA) administered intraperitoneally on Day 0 and Day 3 of the experiment as previously described [17,18]. Macrophages were collected on Day 4 using peritoneal lavage in 6–9 ml 1× PBS/0.5× heparin. Red blood cells were removed from the macrophage preparation by washing cells for 20–25 s in sterile, distilled water followed by immediate addition of 2× PBS to restore isotonicity. Cells were centrifuged for 10 min at 1000 rpm and resuspended in 15–20 ml macrophage media (MM: 1% HEPES, 1% penicillin-streptomycin, 1% L-glutamine, 10% fetal bovine serum in high glucose DMEM; all purchased from Life Technologies, Rockville, MD, USA). Cells were counted and viability was assessed using trypan blue exclusion; viability was always $\geq$ 97%. Resuspended cells were plated in 96 well dishes at 1×10^5 cells/well.

Extraction and culturing of mouse microglia

Microglial cultures were prepared from the cerebral cortices of postnatal (PN) day 1 pups using standard methods [19,20]. Essentially, cortices are removed and placed into sterile phosphate-buffered saline (PBS) containing 100 μg/ml penicillin-streptomycin. Under a dissecting microscope, the meninges are removed and the cerebral hemispheres (3 brains) are placed into 2 ml of growth media [DMEM containing 5% fetal calf serum (FCS) and 5% horse serum, 2 mM glutamine and 50 μg/ml gentamycin]. The hemispheres are carefully dissociated by mechanical means and the dissociated cells are plated directly into 96 well plates. Microglial enriched ($>$ 90% microglia) cultures are obtained by replacing the media with DMEM supplemented with 10% horse serum after 24 h in culture. After 5 to 7 d, the media is replaced with serum-free media and the cells are used at about 14 d in vitro. Microglia are identified using binding of the lectin, *Griffonia simplifolia* (GS-1) [21].

Treatment of tissue macrophages

Prior to treatment, media was aspirated and cells were washed with warm 1× PBS. Then the following reagents were added to each column of cells in 200 μl serum-free MM as determined by the specific experiment: murine recombinant γ-interferon (γIFN), polyinosinic-polycytidylic (PIC or poly[I]:poly[C]) acid, K+ salt, and lipopolysaccharide (LPS) *E. coli* serotype 055:B5 (all purchased from Sigma). Cells were treated for approximately 72 h at 37°C in 95% O_2/5% CO_2 followed by measurement of nitrite.

Measurement of nitrite and total protein

Nitrite levels were determined by combining 100 μl media with 100 μl Griess reagent and incubated at room temperature for 20 min. Griess reagent was prepared by mixing equal volumes of reagent #1 (1% sulfanilic acid/5% phosphoric acid) and reagent #2 (0.1% napthylethylenediamine) (all purchased from Sigma). Total protein (μg)/well was measured using the BCA method (Pierce, Rockford, IL, USA) according to manufacturer's

instructions with BSA (μg/ml) as standard. Both Griess and BCA values were measured using a Molecular Devices Theromax Microplate Reader (Menlo Park, CA, USA) at OD_{562}. Nitric oxide levels were expressed as μM NO_2-/μg protein. Additional nitrite levels were also measured by injecting 50 μl media sample into a Seivers 280 NOA analyzer (Boulder, CO, USA). Every experiment was replicated at least three times under identical conditions. Cell viability was measured using a standard MTT assay. Briefly, cells were incubated with 25 μl of 5 mg/ml MTT (Sigma) prepared in 1× PBS at 37°C, 95%O_2/5% CO_2 for 4 h, followed by addition of 100 μl lysis buffer (10% SDS in 0.01 M HCl) overnight to solubilize the stable mitochondrial purple formazan product, and read at OD_{562}. Cell death was also measured using the CytoTox Non-Radioactive Cytotoxicity Assay, which uses lactate dehyrogenase activity as a measure of cell lysis, according to manufacturer's instructions (Promega, Madison, WI, USA).

Data analysis

Nitrite as (μM)/μg protein is expressed as mean ± SEM. Each data point represents the mean of at least six wells. Data were analyzed using two-tailed paired or unpaired Student's *t*-test and one-way or repeated measures ANOVA and Tukey's post test as appropriate with Graphpad Prism and Instat software packages (San Diego, CA, USA). Values of $p < .05$ (*) were considered significant and $p < .01$ (**) very significant.

RESULTS

Nitrite production is significantly greater in APOE4/4-TR males than APOE3/3-TR males and similar in APOE3/3-TR and APOE4/4-TR females

Peritoneal macrophages from APOE3/3-TR and APOE4/4-TR mice were treated with γIFN (10 u/ml) in combination with PIC (100 μg/ml), a viral mimetic, or LPS (100 ng/ml), a common bacterial endotoxin, to identify a putative relationship between apoE genotype, oxidative stress, and gender in this mouse model system. Both APOE3/3-TR and APOE4/4-TR mouse strains express identical levels of apoE protein, which eliminates the potentially confounding effect of differing apoE protein levels so that apoE isoform specific effects are observable [15,16].

Peritoneal macrophages from APOE4/4-TR male mice display significantly higher nitrite output (Fig. 1A) than APOE3/3-TR male mice at all treatment conditions. γIFN + PIC treated macrophages displayed lower nitrite production than γIFN + LPS treated macrophages ($p = .001$). Like baseline levels in untreated cells, NO release diminished to basal levels when cells were treated with 1400W (100 μM), a competitive inhibitor of iNOS [22].

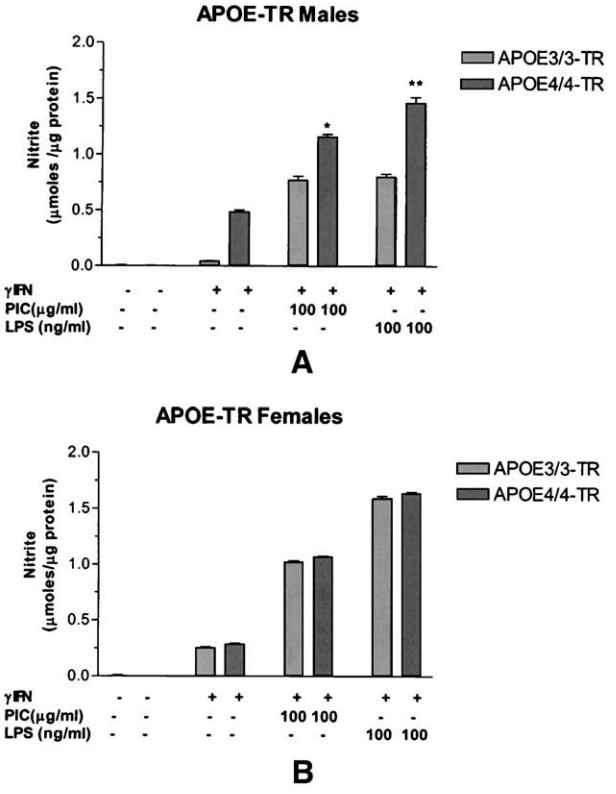

Fig. 1. Nitric oxide release is significantly greater in peritoneal macrophages from APOE4/4-TR mice than APOE3/3-TR mice in males, but is about the same in APOE3/3-TR and APOE4/4-TR females. (A) Average nitrite production (μM)/mg protein was obtained for APOE3/3-TR and APOE4/4-TR male peritoneal macrophages treated with murine recombinant γIFN (γIFN; 10 u/ml), γIFN (10u/ml) + PIC (100 μg/ml), and γIFN (10u/ml) + LPS (100 ng/ml) in serum-free media for 72 h. (B) Peritoneal macrophages from APOE3/3-TR and APOE4/4-TR female mice were treated under identical conditions. Data points represent average supernatant level of nitrite/μg protein (± SEM) from $n = 6$ wells assayed per experimental condition and replicated ≥ 3 times.

In contrast to male macrophages, macrophages from female APOE-TR mice did not display different levels of nitrite production between the APOE genotypes (Fig. 1B, $p = .911$). General trends, however, were observed for gender-specific patterns of nitrite production within the APOE3/3-TR or APOE4/4-TR genotypes. Overall, nitrite production in macrophages from APOE3/3-TR females was significantly higher than nitrite production in macrophages from APOE3/3-TR males ($p = .005$), while nitrite production was not significantly different between macrophages from APOE4/4-TR males and females. All treatments did not significantly change cell number or cell viability (BCA, MTT, Cytox assays) (data not shown).

Table 1. ApoE-isoform Specific Production of Nitric Oxide in Microglia

	APOE3 Nitrite (nM/μg protein) (*n*)	APOE4 Nitrite (nM/μg protein) (*n*)	% increase (APOE4/APOE3) × 100%
γIFN only (100 U/ml)	206 ± 39 (19)	453 ± 83** (16)	220
γIFN + PIC (100 U/ml + 50 μg/ml)	348 ± 89 (12)	794 ± 153** (18)	228

Microglia were cultured in a standard fashion and enriched to greater than 95% microglia by using serum deprivation. To activate iNOS and induce NO production, the microglia were treated for 24 h in serum-free media containing 100 U/ml recombinant murine γIFN alone or in combination with 50 μg/ml polyinosinic: polycytidylic acid (PIC). Supernatant nitrite levels were determined using the Seivers Nitric Oxide Analyzer as standardized against known sodium nitrite concentrations. Protein content of each well was measured using albumin as the standard and data were normalized to nM/μg protein. $^{**}p < .01$ as determined by the unpaired Student's *t*-test; (*n*) = number of wells.

Nitrite production is significantly greater in APOE4/4-TR microglia than in APOE3/3-TR microglia

Neonatal brain microglia from APOE3/3-TR and APOE4/4-TR mice were treated with γIFN (100 u/ml) in combination with PIC (50 μg/ml), a viral mimetic, or LPS (100 ng/ml), a common bacterial endotoxin, to identify a putative relationship between apoE genotype and oxidative stress in this mouse model system. As shown in Table 1, microglia from APOE4/4-TR mice treated with γIFN (100 u/ml) in combination with PIC (50 μg/ml) produced significantly more nitric oxide than from identically treated APOE3/3-TR microglia. All treatments did not significantly change cell number or cell viability (BCA, MTT, Cytox assays) (data not shown).

DISCUSSION

Multiple factors are associated with the development of AD including age, APOE4 genotype, gender (a "non-Y chromosome" genotype) and increased oxidative stress. The present study characterizes a potentially novel mechanism linking APOE genotype to the regulation of oxidative stress in inflammatory disorders such as Alzheimer's disease. Our data clearly show that microglia from APOE4/4 transgenic replacement mice produced significantly higher amounts of nitric oxide than microglia from APOE3/3 transgenic replacement mice. Similarly, macrophages from male APOE4/4-TR mice exhibited significantly higher nitric oxide production than macrophages from male APOE3/3-TR mice. This increase in nitrite reflects an increase in the innate immune inflammatory response of the APOE4/4-TR macrophage that correlates with data obtained from human AD patients, suggesting increased levels of oxidative stress markers in patients with APOE4 alleles [23–25]. Importantly, this effect is observed in both macrophages and microglia, consistent with the close physiological and biochemical relationships between these two types of bone marrow-derived cells, and supports a general characteristic of macrophages from different tissues in that they uniformly respond to a specific apoE-protein isoform.

Consistent with human and rodent studies showing a greater inflammatory response in females than in males [26–28], peritoneal macrophages from APOE3/3 female mice produced significantly more nitric oxide than their male APOE3/3 counterparts. Interestingly, macrophages from female APOE3/3 mice produced the same levels of nitric oxide as macrophages from female APOE4/4 mice. The similar levels of nitric oxide production in APOE3/3 females and APOE4/4 females also match the high levels of nitric oxide production in APOE4/4 males. An increased inflammatory response is beneficial in acute situations such as shock, viral infection, and bacterial infection when a rapid, robust response is required, but can be deleterious when prolonged in chronic states such as AD, multiple sclerosis, diabetes, and arthritis [12,29].

Based on our data, we interpret this female pattern of nitric oxide production in premenopausal mice as being independent of APOE genotype. This interpretation suggests that apoE, in an isoform-specific manner, may regulate nitric oxide production differently in the presence or absence of sex steroids. In a recent report, nitric oxide activity was increased following castration and decreased following dihydrotestosterone treatment, suggesting that androgens do modulate nitric oxide production [30]. Such a finding sets the stage for testing of the mechanism by which apoE-isoform-specific production of nitric oxide can occur. Thus, we are currently measuring nitric oxide production in gonadoectomized female and male APOE-TR mice to determine whether the apoE-isoform-specific-dependent production of nitric oxide is dependent or independent of the presence of sex steroids.

Acknowledgements — We wish to thank Michele V. Eyster and Michael H. Herbstreith for excellent technical assistance. This work was supported by NIH grants AG 15307, AG15383, P50 AG05128 and Alzheimer's Association Zenith award (98-012) to M.P.V., NIH grant (P50 AG36718) to C.A.C., and NSF Graduate Fellowship to C.M.B.

REFERENCES

[1] Weisgraber, K. H.; Mahley, R. W. Human apolipoprotein E: the Alzheimer's disease connection. *FASEB J.* **10:**1485–1494; 1996.

[2] Saunders, A. M.; Strittmatter, W. J.; Schmechel, D.; St. George-Hyslop, P. H.; Pericak-Vance, M. A.; Joo, S. H.; Rosi, B. L.; Gusella, J. F.; Crapper-MacLachlan, D. R.; Alberts, M. J.; Hulette, C.; Crain, B.; Goldgaber, D.; Roses, A. D. Association of apolipoprotein E allele E4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* **43:**1467–1472; 1993.

[3] Laskowitz, D. T.; Roses, A. D. Apolipoprotein E: an expanding role in the neurobiology of disease. *Alzheimers Rep.* **1:**5–12; 1998.

[4] Bretsky, P. M.; Buckwalter, J. G.; Seeman, T. E.; Miller, C. A.; Poirer, J.; Schellenberg, G. D.; Finch, C. E.; Henderson, V. W. Evidence for an interaction between apolipoprotein E genotype, gender, and Alzheimer disease. *Alzheimer Dis. Assoc. Disord.* **13:**216–221; 1999.

[5] Hebert, L. E.; Scherr, P. A.; McCann, J. J.; Beckett, L. A.; Evans, D. A. Is the risk of developing Alzheimer's disease greater for women than for men? *Am. J. Epidemiol.* **153:**132–136; 2001.

[6] Turner, R. S. Alzheimer's disease in man and transgenic mice. *Am. J. Pathol.* **158:**797–801; 2001.

[7] Tang, M.-X.; Jacobs, D.; Stern, Y.; Marder, K.; Scholfield, P.; Gurland, B.; Andrews, H.; Mayeux, R. Effect of oestrogen during menopause on risk and age at onset of Alzheimer's diesease. *Lancet* **348:**429–432; 1996.

[8] Mortensen, E. L.; Hogh, P. A gender difference in the association between APOE genotype and age-related cognitive decline. *Neurology* **57:**89–95; 2001.

[9] Ghebremedhin, E.; Schultz, C.; Thal, D. R.; Rub, U.; Ohm, T. G.; Braak, E.; Braak, H. Gender and age modify the association between APOE and AD-related neuropathology. *Neurology* **56:** 1696–1701; 2001.

[10] Smith, M. A.; Richey Harris, P. L.; Sayre, L. M.; Beckamn, J. S.; Perry, G. Widespread peroxynitrite damage in Alzheimer's disease. *J. Neurosci.* **17:**2653–2657; 1997.

[11] Mahley, R. W.; Rall, S. C. Apolipoprotein E: far more than a lipid transport protein. *Annu. Rev. Genomics Hum. Genet.* **1:**507–537; 2000.

[12] Zamora, R.; Vodovotz, Y.; Billiar, T. R. Inducible nitric oxide synthase and inflammatory diseases. *Mol. Med.* **6:**347–373; 2000.

[13] Vitek, M. P.; Snell, J.; Dawson, H.; Colton, C. A. Modulation of nitric oxide prodution in human macrophages by apolipoprotein-E and amyloid-beta protein. *Biochem. Biophys. Res. Commun.* **240:** 391–394; 1997.

[14] Colton, C. A.; Czapiga, M.; Snell-Callanan, J.; Chernyshev, O. N.; Vitek, M. P. Apolipoprotein E acts to increase nitric oxide in macrophages by stimulating arginine transport. *Biochim. Biophys. Acta* **1535:**134–144; 2001.

[15] Knouff, C.; Hinsdale, M. E.; Mezdour, H.; Altenburg, M. K.; Watanable, M.; Quarfordt, S. H.; Sullivan, P. M.; Maeda, N. Apo E structure determines VLDL clearance and atherosclerosis risk in mice. *J. Clin. Invest.* **103:**1579–1586; 1999.

[16] Sullivan, P. M.; Mezdour, H.; Aratani, Y.; Knouff, C.; Nijib, J.; Reddick, R. L.; Quarfordt, S. H.; Maeda, N. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atheroschlerosis. *J. Biol. Chem.* **272:**17972–17980; 1997.

[17] Weinberg, J. B. In vivo modulation of macrophage tumoricidal activity: enhanced tumor cell killing by peritoneal macrophages from mice given injections of sodium periodate. *J. Natl. Cancer Inst.* **66:**529–533; 1981.

[18] Weinberg, J. B. Macrophage polykaryon formation in vitro by peritoneal cells from mice given injections of sodium periodate. *Am. J. Pathol.* **110:**182–192; 1983.

[19] Colton, C.; Yao, J.; Keri, J.; Gilbert, D. Regulation of microglial function by interferons. *J. Neuroimmunol.* **40:**89–98; 1992.

[20] Guilian, D.; Baker, T. Characterization of amoeboid microglia isolated from developing mammalian brain. *J. Neurosci.* **6:**2163–2178; 1986.

[21] Colton, C.; Abel, C.; Patchett, J.; Keri, J.; Yao, J. Lectin staining of cultured CNS microglia. *J. Histochem. Cytochem.* **40:**505–512; 1992.

[22] Garvey, E. P.; Oplinger, J. A.; Furfine, E. S.; Kiff, R. J.; Laszlo, F.; Whittle, B. J. R.; Knowles, R. G. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J. Biol. Chem.* **272:**4959–4963; 1997.

[23] Ramassamy, C.; Averill, D.; Beffert, U.; Bastianetto, S.; Theroux, L.; Lussier-Cacan, S.; Cohn, J.; Christen, Y.; Davignon, J.; Quirion, R.; Poirier, J. Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. *Free Radic. Biol. Med.* **27:**544–553; 1999.

[24] Ramassamy, C.; Averill, D.; Beffert, U.; Theroux, L.; Lussier-Cacan, S.; Cohn, J. S.; Christen, Y.; Schoofs, A.; Davignon, J.; Poirier, J. Oxidative insults are associated with apolipoprotein E genotype in Alzheimer's disease brain. *Neurobiol. Dis.* **7:**23–37; 2000.

[25] Ihara, Y.; Hayabara, T.; Sasaki, K.; Kawada, R.; Nakashima, Y.; Kuroda, S. Relationship between oxidative stress and apoE phenotype in Alzheimer's disease. *Acta Neurol. Scand.* **102:**346–349; 2000.

[26] Miller, L.; Hunt, J. S. Sex steroid hormones and macrophage function. *Life Sci.* **59:**1–14; 1996.

[27] Chao, T.-C.; Van Alten, P. J.; Walter, R. J. Steroid sex hormones and macrophage function: modulation of reactive oxygen intermediates and nitrite release. *Am. J. Reprod. Immunol.* **32:**43–52; 1994.

[28] Savita, U. Sex steroid hormones modulate the activation of murine peritoneal macrophages: receptor mediated modulation. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **119:**199–204; 1998.

[29] Weinberg, J. B. Nitric oxide production and nitric oxide synthase type 2 expression by human mononuclear phagocytes: a review. *Mol. Med.* **4:**557–591; 1998.

[30] Singh, R.; Pervin, S.; Shryne, J.; Gorski, R.; Chaudhuri, G. Castration increases and androgens decrease nitric oxide synthase activity in the brain: physiologic implications. *Proc. Natl. Acad. Sci. USA* **97:**3672–3677; 2000.