Results

Preventing the Down-regulation of κ_2AR Inhibits LPS-stimulated NOS II Expression

α_2AR protein and mRNA levels were markedly decreased in RAW264 cells following LPS stimulation (Fig. 1A). To investigate the role of α_2AR down-regulation in response to LPS, a stable α_2AR transfectant (RAWar) and a vector control (RAWvec) were established. Although the levels of both α_2AR protein and mRNA expression were notably decreased in RAWvec cells following LPS stimulation, the down-regulation of α_2AR expression was prevented in the RAWar cells (Fig. 1B). Because the transfected α_2AR protein did not have a tag sequence capable of modifying α_2AR function, the protein levels of only transfected α_2AR could not be analyzed. The mRNA levels of transfected α_2AR were low in unstimulated RAWar cells but markedly increased in the cells following LPS stimulation (Fig. 1C). In our previous study, we showed that the levels of both protein and mRNA of transfected cDNA cloned into the pcDNA4 vector were low in unstimulated RAW264 cells but markedly increased in the cells following LPS stimulation [17]. Therefore, it appears that the levels of total α_2AR expression in unstimulated RAWar cells were not so higher than those in RAWvec cells and that decrease in the level of intrinsic α_2AR expression in the LPS-stimulated RAWar cells was masked by the increased expression of transfected α_2AR due to the LPS stimulation. Although, the intracellular cAMP concentration in RAWar cells stimulated with salbutamol was similar to those in RAWvec cells, LPS stimulation decreased an accumulation of intracellular cAMP in RAWvec cells but increased it in RAWar cells (Fig. 1D), suggesting that the transfected α_2AR is functionally active. The similar histograms of the distribution of FSC were observed in RAWvec and RAWar cells, suggesting that the α_2AR transfection did not alter the cell size (Fig. 1E). Also, cell viabilities were more than 98 % in both cells.

The effects of forced α_2AR expression on NO production were examined. The nitrite concentration in the culture supernatants of the LPS-stimulated RAWar cells was considerably lower than in the culture supernatants of the RAWvec cells (Fig. 2A). After stimulation with LPS for 6 h, a distinct 130-Kd NOS II protein band was observed in the RAWvec cells but not in the RAWar cells (Fig. 2B). Although a protein band corresponding to NOS II was observed in the RAWar cells after stimulation with LPS for 24 h, the expression level was apparently lower than in the RAWvec cells. Similar results were obtained on RT-PCR analysis of NOS II mRNA expression (Fig. 2B).

Preventing the Down-regulation of κ_2AR Inhibits LPS-stimulated NF-κB Activation.

Next, the effects of forced α_2AR expression on NF-αB activation in response to LPS were analyzed. As illustrated in Fig. 3A, marked NF-αB activation was observed in the RAWvec cells stimulated with LPS for 3 and 6 h but not in the RAWar cells. The level of cytoplasmic IαBα was definitely

decreased in the RAWvec cells after LPS stimulation for 6 h; however, this level was not decreased in the RAWar cells (Fig. 3B). To further confirm the role of α_2AR in LPS-stimulated NF-αB activation, the effects of forced α_2AR expression on NF-αB-dependent gene transcription were analyzed. NF-αB-mediated-luciferase reporter activity (Fig. 3C) and NOS II promoter activity (Fig. 3D) after stimulation with LPS were inhibited in cells co-transfected with the pcDNA4-$\alpha_2$ar construct (AR) as well as in cells co-transfected with pCMV-IαBαM (DN-αB). These findings suggested that α_2AR functions as a negative regulator of NF-αB activation by inhibiting IαBα degradation in LPS-stimulated macrophages. Previously, it has been shown that PDTC blocks NF-αB activation by inhibiting IαBα degradation and subsequently the translocation of NF-αB subunits to the nucleus [22]. Thus, to elucidate the effects of NF-αB activation on the expression of the responsive gene, *Nos2*, PDTC was added to the RAW264 cell cultures at several time points after the addition of LPS, and NO accumulation in the supernatants was analyzed after LPS stimulation for 24 h. As illustrated in Fig. 3E, when PDTC was added to cultures at 0~9 h after the addition of LPS, the NO concentrations in these cultures were markedly lower than those in cultures stimulated with LPS for 24 h without PDTC (right column), indicating that continuous NF-αB activation is essential for adequate NOS II induction.

κ_2AR Regulates NF-κB Activation through κ-arrestins.

As α-arrestin 2 has been reported to interact with IαBα [15, 16], we examined whether α-arrestin 2 participates in the α_2AR-mediated regulation of IαBα degradation and NF-αB activation in response to LPS. α-Arrestin 2 expression was also down-regulated in the LPS-stimulated RAW264 cells (Fig. 4, left panels). Forced α_2AR expression abolished the down-regulation of α-arrestin 2 expression (middle panels), suggesting that α-arrestin 2 expression'was regulated by α_2AR. Deletion of α_2AR by siRNA decreased α-arrestin 2 expression (data not shown), supporting that α-arrestin 2 expression is regulated by α_2AR. To investigate the role of α-arrestin 2 down-regulation in response to LPS, a stable α-arrestin 2 transfectant (RAWarr2) was established (right panels). Since transfection with the vector did not influence NO production (Fig. 1C), cells transfected with α-arrestin 2 were compared with RAW264 cells. As shown in the RAWar cells (Fig. 2), NO production (Fig. 5A) and NOS II protein and mRNA expressions (Fig. 5B) were definitely decreased in the RAWarr2 cells.

Anti-α-arrestin 2 Abs co-immunoprecipitated IαBα in RAW264 cells before but not after LPS stimulation for 6 h (Fig. 6). On the other hand, the amount of IαBα co-precipitated by anti-α-arrestin 2 Abs was not reduced but rather increased in the RAWar and RAWarr2 cells after LPS stimulation, indicating that the LPS-stimulated down-regulation of α_2AR and α-arrestin 2 is essential for IαBα degradation.

Discussion

In this study, we investigated the role played by α_2AR in the anti-microbial responses of macrophages was investigated. First, we demonstrated that α_2AR expression is decreased by LPS stimulation. To directly investigate the role of α_2AR down-regulation in response to LPS, we established a macrophage cell line, RAWar. Prevention of the down-regulation of α_2AR expression in RAWar cells resulted in reduced NO production, suggesting that the LPS-associated down-regulation of α_2AR expression plays an important role in NO production in macrophages.

Decreases in NOS II mRNA expression were observed in the RAWar cells, indicating that NOS II expression was transcriptionally down-regulated by forced α_2AR expression. Prevention of the down-regulation of α_2AR expression in the RAWar cells resulted in a marked decrease in NF-αB activation and inhibited cytosolic IαBα degradation, indicating that the forced α_2AR expression inhibited LPS-induced NF-αB activation by IαBα stabilization.

On the other hand, α-arrestins, which are universally expressed members of the arrestin family, are the major regulators of GPCR signaling that bind to activated GPCRs causing receptor desensitization and internalization [14]. Recently, α-arrestins have been shown to play functional roles in the regulation of a variety of signaling pathways and in the mediation of cross-talk between signaling pathways. Moreover, there is accumulating evidence that α-arrestin 2, which is expressed abundantly in the spleen, is functionally involved in some important immune responses [23-26]. We have demonstrated that α-arrestin 2 is down-regulated in LPS-stimulated RAW264 cells. α-Arrestin 2 down-regulation was abolished in RAWar cells, suggesting that α-arrestin 2 expression is regulated by α_2AR. These findings suggest that α_2AR participates in signal transduction pathways from TLR4 by regulating the level of α-arrestin 2 expression. Meanwhile, the amount of IαBα co-immunoprecipitated by anti-α-arrestin 2 Abs was decreased in the RAW 264 cells after their stimulation with LPS but not in the RAWar or RAWarr2 cells, suggesting that α_2AR inhibited LPS-induced NF-αB activation by stabilizing IαBα through α-arrestin 2. The release of NF-αB following the degradation of IαBα proteins is an essential step in the generation of transcriptionally competent NF-αB. In addition, NF-αB activity following stimulation is dependent on the level of cytoplasmic NF-αB/IαBα complexes free from stabilizing factors. Therefore, the following appear likely: (1) LPS-stimulated signals suppress α_2AR expression, (2) the reduction of α_2AR results in the down-regulation of α-arrestin 2 expression, (3) α-arrestin 2 stabilizes cytoplasmic IαBα and inhibits NF-αB activation; thus, reduction in the level of α-arrestin 2 accelerates IαBα degradation and NF-αB activation in LPS-stimulated cells, and (4) nuclear translocation of NF-αB enhances NOS II expression.

The cross-talk between α_2AR and TLR signaling pathways is schematically summarized in Fig. 7. Catecholamines increase cAMP *via* α_2AR activation, and PKA activation inhibits NF-αB-induced

transcription by phosphorylating cAMP responsive element binding protein (CREB), which competes with p65 for the limited amounts of CREB-binding protein (CBP) (Fig. 7A(a)) [27]. However, α_2AR agonists did not suppress NO production (unpublished observation). In the present study, we demonstrated that LPS stimulation suppressed the cAMP accumulation in RAWvec cells stimulated with α_2AR agonist. In addition, we showed that prevention of the down-regulation of α_2AR inhibits the degradation of IκBα through α-arrestin 2, which stabilizes IκBα in the steady state (Fig. 7A (b)). Therefore, the down-regulation of α_2AR and α-arrestin 2 expressions by the TLR4-dependent pathway might provide a mechanism for "escaping" anti-proinflammatory signals, such as the α_2AR-cAMP-PKA pathway [27] or the α_2AR-α-arrestin 2-IκBα pathway. As the levels of α_2AR ligands vary under different conditions, understanding the cross-talk between TLRs and α_2AR pathways may have both physiological and pathophysiological importance. Taken together, the observations of the present study regarding the regulation of TLR4 signaling through α_2AR appear to provide another therapeutic target for the regulation of inflammatory disease conditions.

Acknowledgments

We thank Dr. T. Seya (Hokkaido University, Sapporo. Japan) for providing helpful comments.

Grants: This study was supported in part by Grants-in-Aid for Scientific Research (including the Academic Frontier Project) from the Japanese Ministry of Education, Culture, Sports, Science and Technology and by a Grant-in-Aid for Promotion and Mutual Aid Corporation for Private Schools of Japan (to T.K. and H.O.).

References

1. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003; 21:335-76.

2. Janeway CA,Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002; 20:197-216.

3. Hierholzer C, Harbrecht B, Menezes JM, et al. Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. J Exp Med 1998; 187:917-28.

4. Bogdan C. Nitric oxide and the immune response. Nat Immunol 2001; 2:907-16.

5. Lain de Lera T, Folgueira L, Martin AG, et al. Expression of IkappaBalpha in the nucleus of human peripheral blood T lymphocytes. Oncogene 1999; 18:1581-8.

6. Tergaonkar V, Correa RG, Ikawa M, Verma IM. Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. Nat Cell Biol 2005; 7:921-3.

7. Rodriguez MS, Thompson J, Hay RT, Dargemont C. Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation. J Biol Chem 1999; 274:9108-15.

8. Itoh CE, Kizaki T, Hitomi Y, et al. Down-regulation of beta2-adrenergic receptor expression by exercise training increases IL-12 production by macrophages following LPS stimulation. Biochem Biophys Res Commun 2004; 322:979-84.

9. Downing JE, Miyan JA. Neural immunoregulation: Emerging roles for nerves in immune homeostasis and disease. Immunol Today 2000; 21:281-9.

10. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve--an integrative interface between two supersystems: The brain and the immune system. Pharmacol Rev 2000; 52:595-638.

11. Kohm AP, Sanders VM. Norepinephrine and beta 2-adrenergic receptor stimulation regulate CD4+ T and B lymphocyte function in vitro and in vivo. Pharmacol Rev 2001; 53:487-525.

12. Ferguson SS, Downey WE,3rd, Colapietro AM, Barak LS, Menard L, Caron MG. Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 1996; 271:363-6.

13. Ogasawara J, Sanpei M, Rahman N, Sakurai T, Kizaki T, Hitomi Y, Ohno H, Izawa T.

Beta-adrenergic receptor trafficking by exercise in rat adipocytes: Roles of G-protein-coupled receptor kinase-2, α-arrestin-2, and the ubiquitin-proteasome pathway. FASEB J 2006; 20:350-2.

14. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 2002; 115:455-65.

15. Witherow DS, Garrison TR, Miller WE, Lefkowitz RJ. Beta-arrestin inhibits NF-kappaB activity by means of its interaction with the NF-αB inhibitor IαBα. Proc Natl Acad Sci U S A 2004; 101:8603-7.

16. Gao H, Sun Y, Wu Y, Luan B, Wang Y, Qu B, Pei G. Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-αB pathways. Mol Cell 2004; 14:303-17.

17. Kizaki T, Suzuki K, Hitomi Y, et al. Uncoupling protein 2 plays an important role in nitric oxide production of lipopolysaccharide-stimulated macrophages. Proc Natl Acad Sci U S A 2002; 99:9392-7.

18. Kizaki T, Ookawara T, Iwabuchi K, et al. Age-associated increase of basal corticosterone levels decreases ED2^{high}, NF-kappaB^{high} activated macrophages. J Leukoc Biol 2000; 68:21-30.

19. Raje CI, Kumar S, Harle A, Nanda JS, Raje M. The macrophage cell surface glyceraldehyde-3-phosphate dehydrogenase is a novel transferrin receptor. J Biol Chem 2007; 282:3252-61.

20. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. comparison of activating cytokines and evidence for independent production. J Immunol 1988; 141:2407-12.

21. Kizaki T, Suzuki K, Hitomi Y, et al. Negative regulation of LPS-stimulated expression of inducible nitric oxide synthase by AP-1 in macrophage cell line J774A.1. Biochem Biophys Res Commun 2001; 289:1031-8.

22. Staal FJ, Roederer M, Herzenberg LA, Herzenberg LA. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc Natl Acad Sci U S A 1990; 87:9943-7.

23. Sun Y, Cheng Z, Ma L, Pei G. α-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. J Biol Chem 2002; 277:49212-9.

24. Barlic J, Andrews JD, Kelvin AA, et al. Regulation of tyrosine kinase activation and granule release through α-arrestin by CXCRI. Nat Immunol 2000; 1:227-33.

25. Fong AM, Premont RT, Richardson RM, Yu YR, Lefkowitz RJ, Patel DD. Defective lymphocyte chemotaxis in α-arrestin2- and GRK6-deficient mice. Proc Natl Acad Sci U S A 2002; 99:7478-83.

26. Walker JK, Fong AM, Lawson BL, Savov JD, Patel DD, Schwartz DA, Lefkowitz RJ. α-arrestin-2 regulates the development of allergic asthma. J Clin Invest 2003; 112:566-74.

27. Parry GC, Mackman N. Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-αB-mediated transcription. J Immunol 1997; 159:5450-6.

Legends for figures

Fig. 1. LPS stimulation down-regulates α_2AR expression. (A) RAW264 cells were stimulated with LPS. The protein levels of α_2AR and GAPDH (loading control) in the plasma membrane were analyzed by Western blotting (left panel). The α_2AR mRNA and 18S rRNA (loading control) were analyzed by RT-PCR (right upper panel). Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean ± SEM) (right lower panel). *P < 0.01 vs. 0 h. (B) RAW264 cells were transfected with the *κ₂ar* construct or vector alone. The protein levels of α_2AR and GAPDH (left panel) and mRNA expressions of α_2AR and 18S rRNA (right upper panel) were analyzed as in A. Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean ± SEM) (right lower panel). *P < 0.01 vs. 0 h. (C) mRNA expressions of α_2AR and 18S rRNA (upper panel) were analyzed as in A. Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean ± SEM) (lower panel). *P < 0.01 vs. 0 h. (D) Cells were cultured with or without LPS for 6 h and were stimulated with Salbutamol (1×10^{-6} M) for final 30 min. Then, intracellular cAMP concentrations were analyzed. *P < 0.05 vs. without LPS. (E) Cell size was measured by flow cytometric analysis of forward light scatter characteristics (FSC).

Fig. 2. Forced α_2AR expression suppresses NO production and NOS II expression. (A) Cells were stimulated with LPS for 24 h, and nitrite accumulation in the supernatants was measured using the Griess reagent. The results are expressed as means ± SEM from three-well cultures. *P < 0.001 vs. LPS-stimulated RAW264 or RAWvec cells. (B) The protein levels of NOS II and GAPDH (left panel) and mRNA expressions of NOS II and 18S rRNA were analyzed as in A (right upper panel). Bar graphs show the relative intensity of the PCR bands from four separate experiments (mean ± SEM) (right lower panel). *P < 0.01 vs. corresponding RAWvec cells. Data shown are representative of three–four separate experiments.

Fig. 3. Forced α_2AR expression suppresses NF-αB activation.
(A) The vector control cells and α_2AR transfectant were stimulated with LPS, and NF-αB activation was analyzed by EMSA. (B) The vector control cells and α_2AR transfectant were stimulated with LPS, and cytoplasmic IαBα and GAPDH (loading control) was analyzed by Western blotting. (C, D) RAW264 cells were co-transfected with the pNF-αB-Luc vector (C) or NOS II promoter-luciferase construct (D) and vector (Vec), pcDNA4-α_2AR (AR) or pCMV-IαBαM (DN-IαB). The cells were cultured with LPS for 24 h, and luciferase activities were determined. The results are expressed as means ± SEM from six-well cultures. *P < 0.001 vs. cells co-transfected with Vec. (E) PDTC was

added to the cultures at the indicated time points after addition of LPS. Nitrite accumulation in the supernatants at 24 h of culture was measured using the Griess reagent. The results are expressed as means ± SEM from three-well cultures. The error bars are too small to be distinguishable in the figure (numeric data from the left bar: 3.75 ± 0.18, 5.07 ± 0.22, 4.22 ± 0.07, 5.69 ± 0.12, 10.38 ± 0.06, 15.00 ± 0.05, and 25.20 ± 0.28). $^*P < 0.001$ vs. LPS-stimulated cells without PDTC. Data shown are representative of two–three separate experiments.

Fig. 4. LPS stimulation down-regulates α-arrestin 2 expression. RAW264, RAWar, and RAWarr2 cells were stimulated with LPS, and the protein levels of α-arrestin 2 and GAPDH (upper panel) and mRNA expressions of α-arrestin 2 and 18S rRNA (middle panel) were analyzed as in Fig. 1A. Bar graphs show the relative intensity of the band from three separate experiments (mean ± SEM) (lower panel). $^*P < 0.01$ vs. 0 h.

Fig. 5. Forced α-arrestin 2 expression suppresses NO production and NOS II expression.
(A) Cells were stimulated with LPS for 24 h, and nitrite accumulation in the supernatants was measured using the Griess reagent. The results are expressed as means ± SEM from three-well cultures. $^*P < 0.001$ vs. LPS-stimulated RAW264 cells. (B) The protein levels of NOS II and GAPDH (left panel) and mRNA expressions of NOS II and 18S rRNA (light upper panel) were analyzed as in Fig. 1A. Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean ± SEM) (right lower panel). $^*P < 0.01$ vs. corresponding RAW264 cells. Data shown are representative of three–four separate experiments.

Fig. 6. α-arrestin 2 interacts with cytosolic IαBα. Before and after stimulation with LPS for 6 h, cells were lyzed and immunoprecipitated with anti-α-arrestin 2 Abs. Western blotting analysis was performed using anti-IαBα Abs (upper panel). The protein levels of GAPDH in equal amounts of lyzates were used for control (lower panel).

Fig. 7. Cross-talk between α_2AR and TLRs signaling pathways.
(A) α_2AR agonists suppress NF-αB activation by increasing cytoplasmic α-arrestin 2, which stabilizes the NF-αB/IαBα complexes in cytoplasm (a) or by activating CREB which competes CBP with NF-αB in the nucleus (b). (B) TLR4-dependent signals lead to the following steps both in the presence or absence of α_2AR agonists: ① TLR4-dependent down-regulation of α_2AR expression, ② down-regulation of α-arrestin 2, ③ release of NF-αB/IαBα complexes in the cytoplasm, ④ degradation of IαBα, and ⑤ translocation of NF-αB to the nucleus and transcription of its target genes.

Figure 1

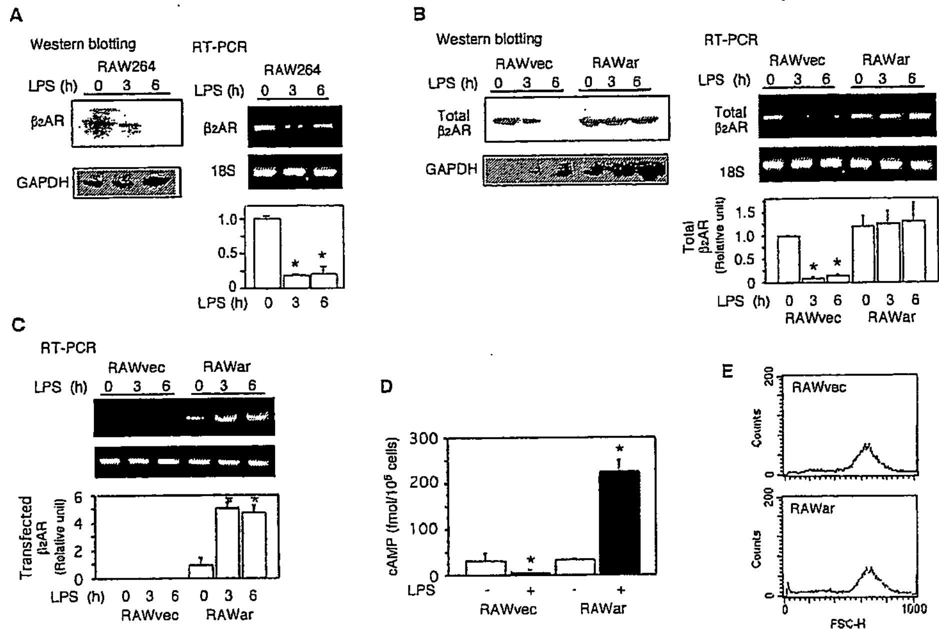


Figure 2

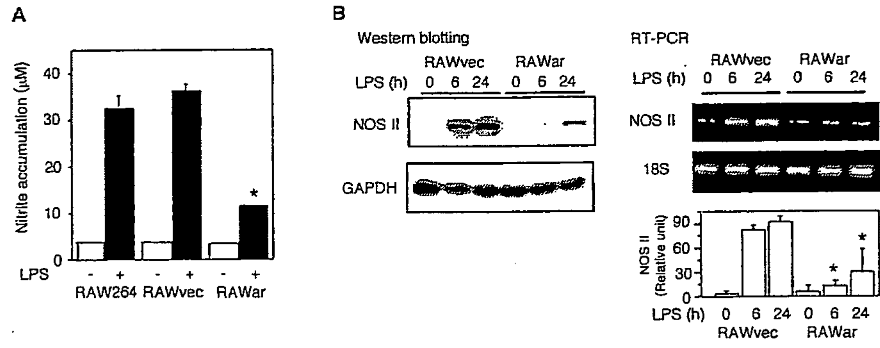


Figure 3

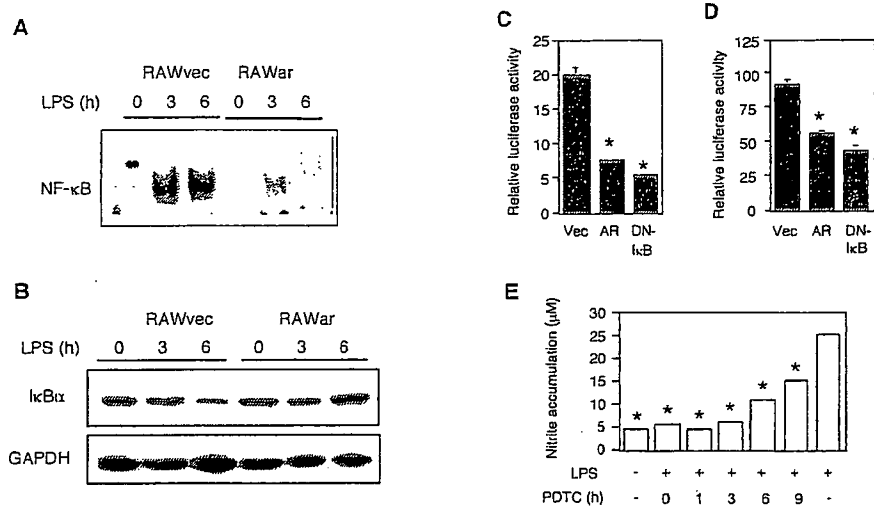


Figure 4

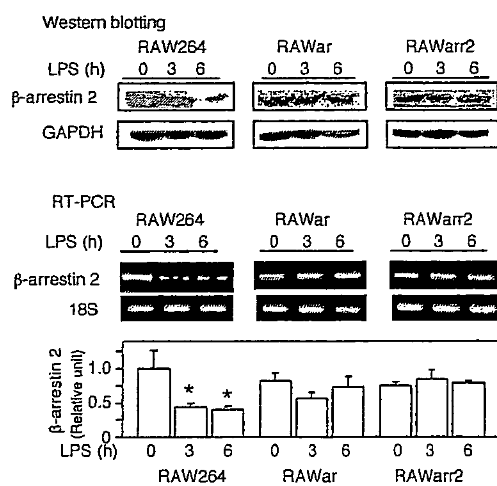


Figure 5

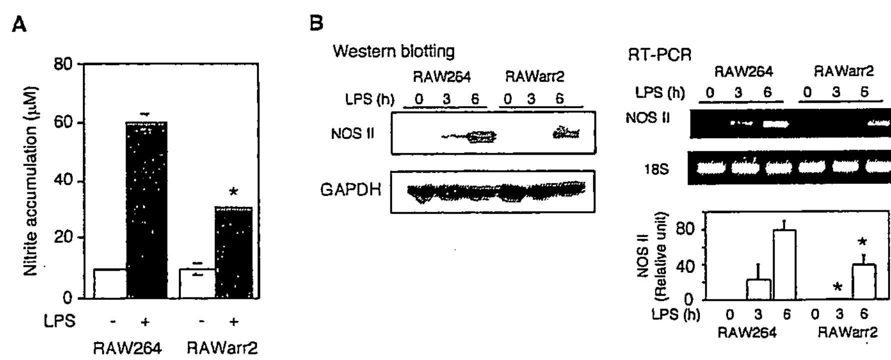


Figure 6

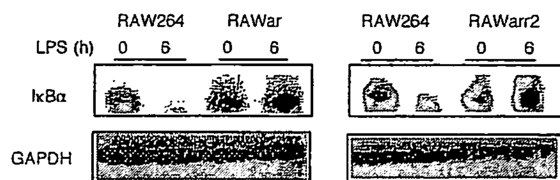
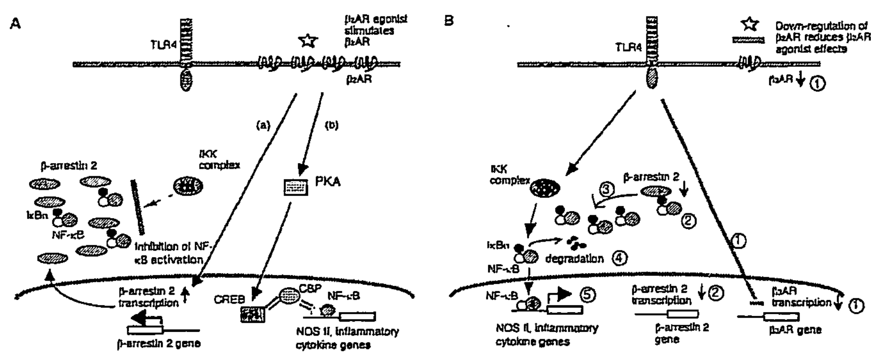


Figure 7



Original article

Title: White matter lesions as a feature of cognitive impairment, low vitality, and other symptoms of the geriatric syndrome in the elderly

Authors: Kazuki Sonohara, MD; Koichi Kozaki, MD; Masahiro Akishita, MD; Kumiko Nagai, PhD; Hiroshi Hasegawa, MD; Masafumi Kuzuya, MD; Koutaro Yokote, MD; Kenji Toba, MD

Institutions: From the Department of Geriatric Medicine (K.S., K.K., K.N., H.H., K.T.), Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan.

Department of Geriatric Medicine (M.A.), University of Tokyo Graduate School of Medicine, Tokyo, Japan.

Department of Geriatrics (M.K.), Nagoya University Graduate School of Medicine, Nagoya, Japan.

Department of Clinical Cell Biology and Medicine (K.Y.), Chiba University Graduate School of Medicine, Chiba, Japan.

Corresponding author: Koichi Kozaki

Department of Geriatric Medicine, Kyorin University School of Medicine

6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, JAPAN

Phone: +81-422-44-1917 Fax: +81-422-44-1917

E-mail: kozaki-tky@umin.ac.jp

Running title: White matter lesions in the elderly

The authors claim no commercial interest concerning this article.

Abstract

White matter lesions (WMLs) are common findings on MRI in elderly persons. In this study, we analyzed the relation of WMLs with global cognitive function, depression, vitality/volition, and nineteen symptoms of the geriatric syndrome in Japanese elderly patients who attended three university geriatric outpatient clinics. Two hundred and eighty six subjects (103 men and 183 women, mean±SD age 74.5±7.8 years old) were included in this study. MRI scans were performed for the diagnosis of WMLs, and the severity of periventricular and subcortical white matter hyperintensities (PVHs and DWMHs) was rated semi-quantitatively. Concurrently, all subjects underwent tests of cognitive function, depressive state, and vitality, and were examined for nineteen symptoms of the geriatric syndrome. The study subjects showed cognitive decline, depression, and low vitality, all to a mild extent. Univariate linear regression analysis showed a negative correlation between the severity of WMLs and cognitive function or vitality. Multiple logistic analysis revealed that the severity of WMLs was a significant determinant of cognitive impairment and low vitality, after adjustment for confounding factors such as age, sex, and concomitant diseases. PVH and/or DWMH score was significantly greater in

subjects who exhibited 13 out of 19 symptoms of the geriatric syndrome. Logistic regression analysis indicated that WMLs were associated with psychological disorders, gait disturbance, urinary problems, and parkinsonism. In conclusion, WMLs were associated with various symptoms of functional decline in older persons. Evaluating WMLs in relation to functional decline would be important for preventing disability in elderly people.

Key words: white matter lesion, periventricular hyperintensity, deep white matter hyperintensity, geriatric syndrome