ELSEVIER

Cardiovascular Research 63 (2004) 31–40

Cardiovascular Research

www.elsevier.com/locate/cardiores

NO suppresses while peroxynitrite sustains NF-κB: a paradigm to rationalize cytoprotective and cytotoxic actions attributed to NO

Yoshiyuki Hattori[a,*], Kikuo Kasai[a], Steven S. Gross[b]

[a] *Department of Endocrinology and Metabolism, Dokkyo University School of Medicine, Mibu, Tochigi, 321-0293, Japan*
[b] *Department of Pharmacology and the Program in Biochemistry and Structural Biology, Weill Medical College of Cornell University, New York, NY, USA*

Received 11 December 2003; received in revised form 8 March 2004; accepted 9 March 2004
Available online 17 April 2004
Time for primary review 26 days

Abstract

Objective: NO has both cytoprotective and cytotoxic effects. A key cytoprotective action of NO is attributed to inhibition of nuclear factor-κB (NF-κB)-mediated gene expression; this potentially endows NO with ubiquitous anti-inflammatory activity. Since immunostimulant-induced iNOS gene expression is itself dependent on NF-κB, NO is expected to limit its own synthesis. On the other hand, many cytotoxic actions of NO have been attributed to the chemical reactivity of peroxynitrite ($ONOO^-$) formed from NO by near diffusion-limited reaction with O_2^-. To assess whether $ONOO^-$ shares the ability of NO to inhibit NF-κB activation and consequent iNOS gene expression, we compared effects of NO donors (NOR3 and SNAP), an $ONOO^-$ donor (SIN-1), and pure $ONOO^-$ on LPS-induced responses in vascular smooth muscle cells (VSMC). **Methods and results:** NO donors, but not $ONOO^-$, suppressed LPS-induced NF-κB activation and expression of a murine iNOS promoter/reporter construct. An NO donor also suppressed NF-κB activation when induced by IL-1β or TNFα. Northern blot and RT-PCR analyses showed that NO, but not $ONOO^-$ or 8-bromo-cGMP, decreases LPS-induced expression of iNOS mRNA. Electrophoretic mobility shift assays (EMSA) and immunocytochemical analyses confirmed that NO but not $ONOO^-$ inhibits nuclear translocation of NF-κB. Although $ONOO^-$ generation from SIN-1 did not inhibit NF-κB activation, conversion of SIN-1 to a pure NO donor (by addition of excess superoxide dismutase) resulted in potent inhibition of NF-κB activation. Dose–response analyses suggest that the inhibitory effect of NO on iNOS gene transcription results specifically from inhibition of NF-κB activation, and is mediated by a G-cyclase-independent mechanism that is unavailable to $ONOO^-$. LPS stimulates IκB-α phosphorylation by inducing IκB kinase (IKK) activity, and NO, but not $ONOO^-$, inhibits LPS-induced IκB-α phosphorylation and IKK activity. **Conclusion:** We demonstrate that only NO inhibits the activation of NF-κB and suppresses iNOS gene expression. This distinction provides a novel paradigm to rationalize cytoprotective and cytotoxic actions attributed to NO.

Keywords: Nitric oxide; Peroxynitrite; Nuclear factor κB; IκB kinase; Vascular smooth muscle cells

1. Introduction

Endothelial-derived nitric oxide (NO) plays a physiological role in regulating vascular tone and blood pressure, maintaining a nonadhesive vasoluminal surface, mediating angiogenesis and preventing inappropriate proliferation of underlying vascular smooth muscle cells (VSMC). In contrast to these beneficial actions of constitutively produced NO, immunostimulants trigger expression of the inducible NO synthase (iNOS) gene product, leading to NO overproduction and vasopathophysiology [1]. iNOS-derived NO overproduction appears to be a ubiquitous mediator of vascular inflammatory conditions, including atherogenesis [2] and various forms of circulatory shock [3,4]. Importantly, the systemic inflammatory response is also defined by the production of oxygen-derived free radicals [5]. There is now substantial evidence that immune stimulus-evoked cytotoxicity involves the concerted action of reactive oxygen- and nitrogen-derived species. Indeed, a major component of injury is associated with the simultaneous production of NO and O_2^-, leading to near diffusion-limited production of the reactive species, peroxynitrite ($ONOO^-$) [6]. Synthesis of $ONOO^-$ has been considered as a channeling

* Corresponding author. Tel./fax: +81-282-87-2150.
E-mail address: yhattori@dokkyomed.ac.jp (Y. Hattori).

0008-6363/$ - see front matter © 2004 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2004.03.014

mechanism that diverts NO from physiological to pathophysiological targets. The ability of immunostimulants to upregulate iNOS gene expression in rodents [7] and man [8] is dependent on activation of the transcription factor, nuclear factor κB (NF-κB). Activated NF-κB serves as the master coordinator of the inflammatory response, binding to consensus elements in the promoters of diverse immunostimulant-inducible genes and upregulating transcription (for review, see Refs. [9,10]). Dormant NF-κB resides in the cytosol of quiescent cells bound to inhibitory proteins, isoforms of IκB. Inflammatory stimuli and oxidative stresses trigger rapid phosphorylation of IκB, resulting in targeted degradation by the proteasome. This results in untethered cytosolic NF-κB that undergoes nuclear translocation, followed by binding to promoter sequences in cognate target genes that elicit transcriptional upregulation. Recently, a cytokine-responsive IκB-α kinase (IKK) that activates NF-κB by phosphorylation of Ser32 and Ser36 residue in IκB-α has been identified [11–13]. In the murine iNOS gene, a 5′-flanking NF-κB binding element was shown to be both necessary and sufficient to initiate transcription in macrophages treated with bacterial lipopolysaccharide (LPS) [7]. In rat VSMC, LPS and interferon-γ (IFN) act synergistically to induce iNOS expression [14] by a process that is inhibited by the NF-κB antagonist, pyrolidine dithiocarbamate [15]. Promoter analyses in rat VSMC confirm that at least one functional NF-κB binding element in the rat iNOS gene mediates immunostimulant-induced transcription [16].

NF-κB-regulated genes additionally include those which encode cytokines, cytokine-receptors, cell adhesion molecules, major histocompatibility complex proteins and enzymes involved in the synthesis and metabolism of inflammatory mediators (e.g., O_2^-). Transcriptional upregulation of this battery of genes constitutes a phenotypic switch that might be propagated to other cells in a feed-forward manner, where it not for the initiation of anti-inflammatory mechanisms that terminate transcription. One important anti-inflammatory mediator may be NO itself. Indeed, it has been reported that NO inhibits NF-κB activation in vascular cells, including human endothelial cells [17] and rodent VSMC [18]. Suppression of NF-κB activation by NO has been attributed to induction and/or stabilization of IκB-α; this may result from NO-mediated inhibition of IκB-α phosphorylation [15]. Paradoxically, NO has also been reported to trigger NF-κB activation in various situations [19,20]. Explanations for these apparently conflicting actions of NO—anti-inflammatory and pro-inflammatory—include differences in rate, source or duration of cell exposure to NO, differences in the time before measuring NF-κB activity, and cell-specific differences in response to NO. An alternative explanation for the apparent opposing actions of NO is offered by an appreciation that NO can be diverted to reaction products with bioactivities that differ from NO itself. As noted above, co-synthesis of O_2^- in the inflammatory environment effectively channels NO to production of $ONOO^-$. In fact, $ONOO^-$ has been reported to increase iNOS through NF-κB in vascular endothelial cells [21]. Accordingly, we hypothesized that reported NF-κB suppressing and potentiating activities of NO may be reconciled by fundamental differences in actions of NO and $ONOO^-$. The present study compares the effects of NO with $ONOO^-$ on NF-κB activation and iNOS gene expression in VSMC.

2. Methods

2.1. Materials

Recombinant rat interferon-γ, TNFα, and IL-1β were obtained from Genzyme (Cambridge, MA, USA). NOR3 ((±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide), SNAP (S-nitroso-*N*-acetyl-DL-penicillamine), SIN-1 (3-(4-morpholinyl)-sydnonimine hydrochloride), and $ONOO^-$ solutions were purchased from Dojin (Kumamoto, Japan). Bacterial lipopolysaccharide (LPS: *Escherichia coli*, serotype No. 0127:B8), pyrrolidine dithiocarbamate (PDTC), *N*-acetylcysteine (NAC), and 8-bromoguanosine 3′:5′-cyclic monophosphate (8-bromo-cGMP) were obtained from Sigma (St. Louis, MO, USA). Cu/Zn-superoxide dismutase (SOD) was purchased from Roche diagnostic (Tokyo, Japan). Bacteria expressing GST-IκBα (1–55) was kindly provided by Dr. Hideaki Kamata (Himeji Institute of Technology, Japan).

2.2. Cell culture and nitrite assay

VSMC were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats, as previously described [22]. Nitrite accumulation, an indicator of NO synthesis, was measured in the cell culture medium of confluent VSMC, as previously described [14].

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1985).

2.3. NF-κB activation

To study NF-κB activation, VSMC were stably transfected with a cis-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (pNFκB-Luc: Stratagene, La Jolla, CA, USA) as previously described [23]. The endothelial cells (YPEN-1 cell line from prostata endothelium, ATCC) were also stably transfected with the same plasmid. Several clones were selected for analysis of NF-κB activation. Luciferase activity was measured using a luciferase assay kit (Stratagene).

For immunohistochemical localization of NF-κB, rat VSMC were grown in wells on glass chamber slides (Nalge Nunc Int., Roskilde, Denmark). Cells were treated with LPS in the absence or presence of NOR3 for 2 h, fixed with 4% formaldehyde/[phosphate-buffered saline (PBS)] for 20 min

at room temperature, and then treated with triton X-100 (0.2% in PBS) for 5 min. Chamber slides were incubated with 1% bovine serum albumin in PBS for 10 min, followed by 30-min exposure to a 1:40 dilution of a rabbit polyclonal antibody that specifically recognizes the p50 subunit of NF-kB (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This p50 antibody did not cross-react with NF-κB subunits p105, p52, or p100 under the conditions employed. Primary antibody was visualized by fluorescence microscopy (Olympus AX 80) after a 30-min incubation with a 1:100 dilution of green-fluorescent Alexa 488 conjugate (goat anti-rabbit IgG, Molecular Probes, Eugene, OR, USA) and washing in PBS.

Electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts, prepared from VSMC that were either untreated or treated with LPS in the absence or presence of NOR3 for 2 h prior to harvesting nuclei. Nuclear extracts were prepared according to the method of Schreiber et al. [24]. EMSA utilized a ^{32}P-labeled double-strand oligonucleotide, containing the NF-κB/c-Rel consensus binding sequence AGTTGAGGGGACTTTCCCAGGC (Promega Biotech, WI, USA). Nuclear proteins were incubated with the labeled oligonucleotide for 30 min and then subjected to polyacrylamide gel electrophoresis and autoradiography.

2.4. iNOS promoter analysis

iNOS promoter activity was studied as previously described [23], using rat VSMC stably transfected with a construct containing a 1.7-kb fragment of the mouse iNOS promoter cloned in front of a reporter gene that encodes the secreted form of human placental alkaline phosphatase (SEAP). SEAP activity in the cell culture medium was measured by a sensitive chemiluminescence assay (Phospha-Light, TROPIX, Bedford, MA, USA).

2.5. Analysis of iNOS, MCP-1, and IκB-α mRNA expression

Standard Northern blotting was used to investigate the mRNA expression for iNOS, MCP-1, and IκB-α, as previously described [23]. After probing for iNOS, MCP-1, or IκB-α expression, filters were stripped and reprobed for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Expression of iNOS mRNA was also analyzed by reverse transcription-polymerase chain reaction (RT-PCR) as previously described [25]. IκB-α cDNA was synthesized by amplifying cDNA from VSMC using pri-

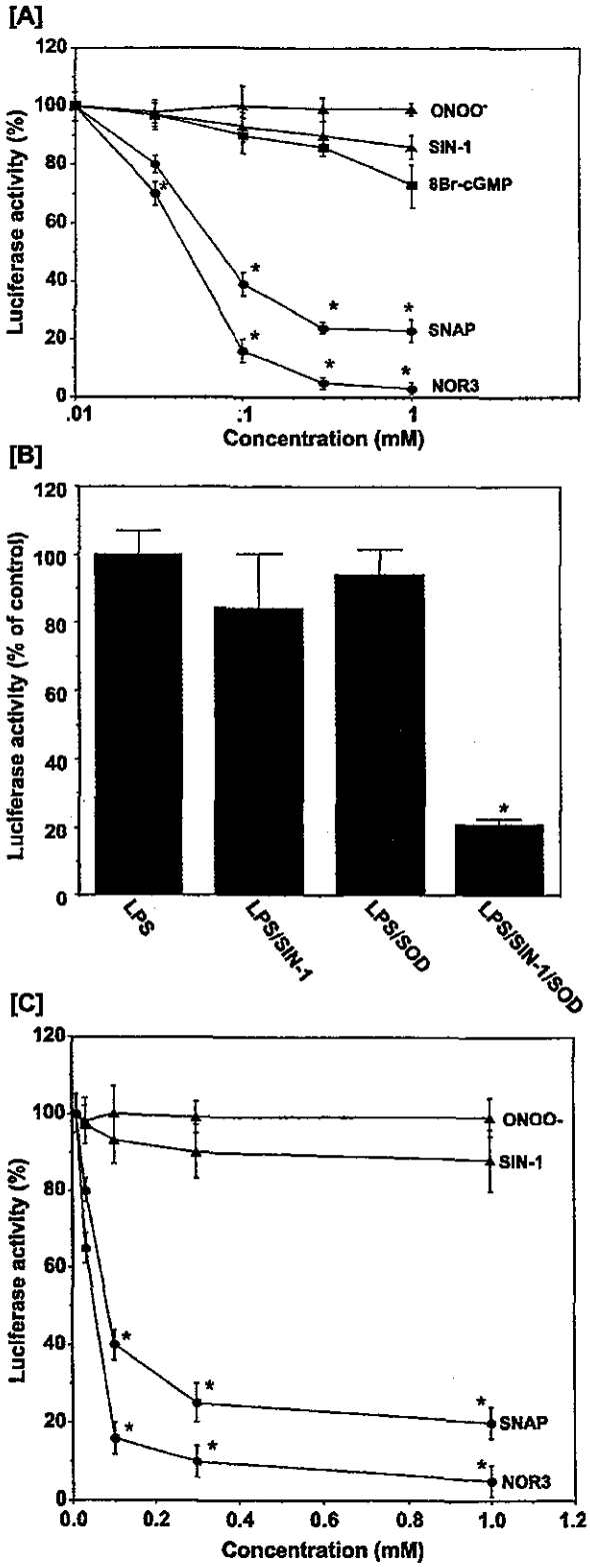


Fig. 1. Effect of NOR3, SNAP, SIN-1, $ONOO^-$, and 8-bromo-cAMP on NF-κB-dependent transcriptional activity/luciferase reporter expression in stably transfected rat vascular smooth muscle cells. (A) Cells were treated with LPS (30 μg/ml) in the presence of indicated concentrations of NOR3, SNAP, SIN-1, $ONOO^-$, or 8-bromo-cAMP for 3 h. (B) Cells were treated with LPS (30 μg/ml) in the presence of SIN-1 (1 mM) or SOD (5000 U/ml) or both for 3 h. After treatment, luciferase activity in the cells was measured. (C) Using endothelial cells (YPEN-1 cell line from prostata endothelium, ATCC), the effect on NF-κB activation was evaluated. Endothelial cells were treated with LPS (1 μg/ml) in the presence of indicated concentrations of NOR3, SNAP, SIN-1, $ONOO^-$, or 8-bromo-cAMP for 3 h. Values are means ± S.E.M. ($n=3$). *$P<0.05$ versus LPS in the absence of each agent.

mers derived from the published cDNA sequence of murine IκB-α [26]. Sequencing of the PCR product (125 bp) showed 96% identical to the corresponding murine IκB-α cDNA sequence.

2.6. Western blot analysis

VSMC treated with LPS in the presence of NOR3 or $ONOO^-$ for various intervals were lysed using cell lysis buffer (Cell Signaling, Beverly, MA, USA) with 1 mM PMSF. The protein concentration of each sample was measured using a Bio-Rad detergent-compatible protein assay. Subsequently, β-mercaptoethanol was added at a final concentration of 1%, and each sample was denatured by boiling for 3 min. Samples containing 15 μg of protein were resolved by electrophoresis on 12% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) incubated with Phospho-IκB-α antibody and IκB-α antibody (1:1000, Cell Signaling). The binding of each of these antibodies was detected using sheep anti-rabbit IgG horseradish peroxidase (1:20,000) and the ECL Plus system (Amersham, Buckinghamshire, UK).

2.7. IKK kinase assay

The kinase activity of IκB kinase (IKK) was analyzed by immune complex kinase assay using a substrate, GST-IκBα (1–55), as described previously [27]. Briefly, the cells were solubilized in ice-cold buffer, and then centrifuged at 15,000 × *g* for 20 min. IKKα and IKKβ were recovered from the lysates by immunoprecipitation, and then the immune complexes were incubated with 20 μl reaction buffer containing 20 mM HEPES/NaOH, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 100 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM DTT, 100 μM ATP, 0.1 μCi [γ-^{32}P]ATP, 10 μg GST-IκBα (1–55) at 30 °C for 20 min. After SDS-polyacrylamide gel electrophoresis (PAGE), the phosphorylation of GST- IκBα was estimated by Imaging plate (Fuji Film).

2.8. Statistical analysis

Comparisons between group means were performed by two-way ANOVA and Tukey post hoc analysis test with InStat software (GraphPad Software). Student's unpaired *t*-test was used for comparisons between two treatment groups. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. NF-κB-dependent transcriptional activation

LPS elicits NF-κB activation in VSMC, thereby stimulating transcription of NF-κB-induced genes. Studies were performed to assess the effect on LPS-induced NF-κB activation of NO-releasing agents (NOR3 and SNAP), an $ONOO^-$ donor (SIN-1), and pure $ONOO^-$. As shown in Fig. 1A, NOR3 and SNAP each caused a dose-dependent and near-complete suppression of NF-κB activation; half-maximal inhibitory concentrations were 50 and 80 μM, respectively. TNFα- and IL-1β also markedly stimulated

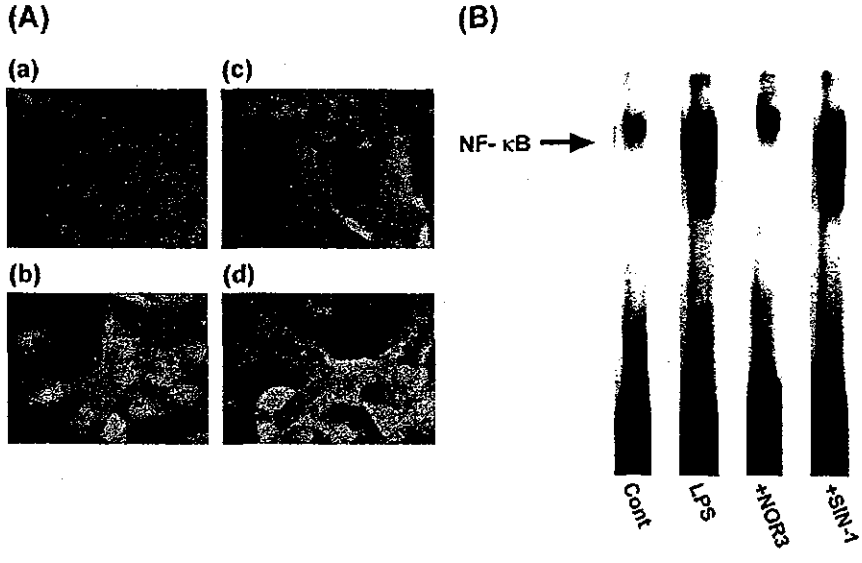


Fig. 2. Effect of NOR3 and SIN-1 on NF-κB nuclear translocation. VSMC were left untreated or treated with LPS (30 μg/ml), alone, or in the presence of NOR3 (500 μM) or SIN-1 (500 μM) for 2 h. (A) Cells were fixed and subjected to immunohistochemical staining for nuclear translocation of NF-κB. (a) Control; (b) LPS; (c) LPS + NOR3; (d) LPS + SIN-1. (B) Cells were treated as in (A), and extracted nuclear protein (10 μg) was subjected to EMSA using a double-stranded oligonucleotide probe for NF-κB binding.

NF-κB activation in VSMC and the NO-donor NOR3 (1 mM) abolished these actions (data not shown). In contrast, no significant inhibition of LPS-induced NF-κB activation was observed when $ONOO^-$ was administered directly or indirectly, as a product of the reaction of SIN-1-derived NO and O_2^-. Notably, when SIN-1 (1 mM) was converted from an $ONOO^-$ donor to an NO donor, by addition of 5000 U/ml SOD, LPS-induced NF-κB activity was potently suppressed (Fig. 1B). Neither SOD on its own (Fig. 1B, 5000 U/ml), nor a cell-permeant analog of cGMP, 8-bromo-cGMP (Fig. 1A), inhibited LPS-induced NF-κB activation. Together, these findings indicate that NO acts by a cGMP-independent mechanism to suppress immunostimulant-induced NF-κB activation and this action is not a property shared with $ONOO^-$.

Using another cell type (endothelial cells), the effect on LPS-induced NF-κB activation of NOR3, SNAP, SIN-1, and pure $ONOO^-$ was examined. As shown in Fig. 1C, NOR3 and SNAP caused a dose-dependent suppression of NF-κB activation in endothelial cells, while SIN-1 and pure $ONOO^-$ had little effect.

3.2. NF-κB nuclear translocation

To examine whether NO prevents nuclear translocation of active NF-κB in LPS treated VSMC, immunohistochemical staining with an anti-p50 antibody was performed. Untreated VSMC displayed a diffuse cytosolic distribution of immunoreactive p50. Following 2-h exposure of rat VSMC to LPS (30 μg/ml), a dense nuclear accumulation of immunoreactive p50 was conspicuous. Notably, the ability of LPS to induce nuclear translocation of NF-κB p50 was abolished by pretreatment of VSMC with NOR3 (500 μM), but unaffected by SIN-1 (500 μM) treatment (Fig. 2A).

EMSA confirmed significant activation of NF-κB by LPS in VSMC. As shown in Fig. 2B, nuclear extracts from LPS-treated cells caused a distinct shift in electrophoretic mobility of a radiolabeled double-stranded oligonucleotide binding probe that recognizes NF-κB; this shifted band was absent from nuclei of untreated cells. This LPS-induced nuclear translocation of active NF-κB was abolished in VSMC that had been pretreated with NOR3 (500 μM), as evidenced by loss of the shifted-band (Fig. 2B). In contrast

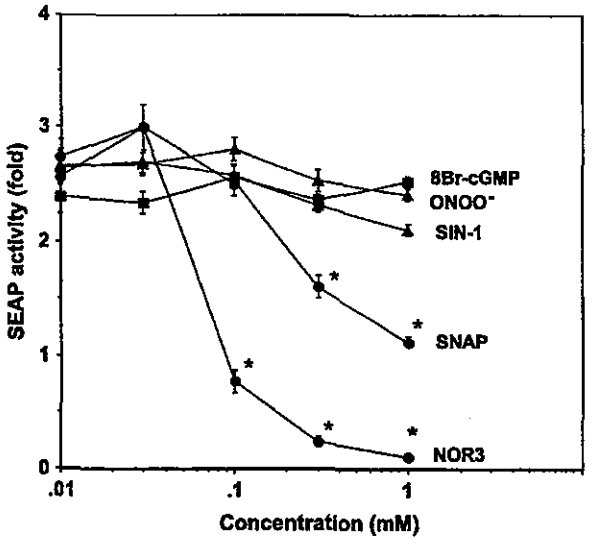


Fig. 3. Effect of NOR3, SNAP, SIN-1, $ONOO^-$, and 8-bromo-cAMP on iNOS promoter/SEAP reporter expression in stably transfected rat VSMC. Cells were treated with LPS (30 μg/ml) alone, or in the presence of indicated concentrations of NOR3, SNAP, SIN-1, $ONOO^-$, or 8-bromo-cAMP for 24 h. After this time, SEAP activity was quantified in the cell culture medium. Values are means ± S.E.M. ($n=3$). *$P<0.05$ versus LPS alone.

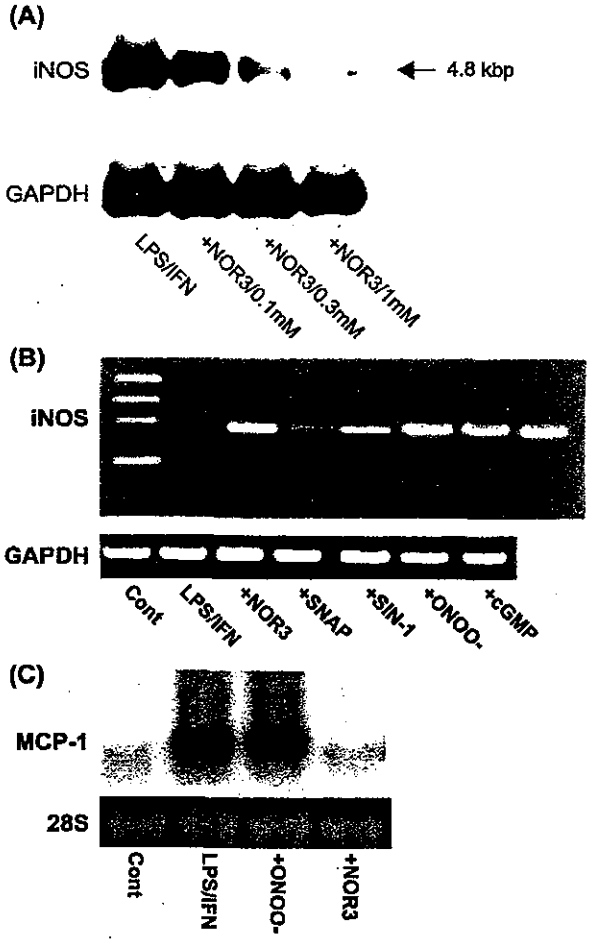


Fig. 4. Effect of NOR3, SNAP, SIN-1, $ONOO^-$, or 8-bromo-cAMP on LPS/IFN-induced increase in iNOS mRNA abundance. Rat VSMC were treated with LPS (30 μg/ml) plus IFN (100 U/ml) in the presence of indicated concentrations of NOR3 (panel A), or 1 mM concentrations of NOR3, SNAP, SIN-1, $OONO^-$, or 8-bromo-cAMP (panel B) for 8 h. For MCP-1 gene expression, VSMC were treated with LPS/IFN in the presence of NOR3 (1 mM) or $ONOO^-$ (1 mM) for 6 h (panel C). Total RNA was isolated and analyzed by northern blot hybridization with an iNOS-specific probe (A) and with MCP-1-specific probe (C), or RT-PCR using iNOS-specific primers (B).

to the complete suppression of NF-κB activation observed with an NO-donor, an $ONOO^-$ donor SIN-1 (500 mM) was without effects (Fig. 2B).

3.3. iNOS promoter activation

The effects of NOR3, SNAP, SIN-1, $ONOO^-$, and 8-bromo-cGMP on LPS-induced iNOS promoter activation in VSMC were evaluated. LPS potently activated the iNOS promoter, as indicated by a >5-fold increase in SEAP reporter gene activity (Fig. 3). This LPS-induced iNOS promoter activity was inhibited in a dose-dependent manner by NOR3 (IC_{50} = 70 μM) and SNAP (IC_{50} = 175 μM). In contrast, SIN-1, peroxynitrite, and 8-bromo-cGMP had no significant inhibitory effect on iNOS promoter activation by LPS (Fig. 3).

3.4. mRAN expression of iNOS and MCP-1

While iNOS mRNA levels approached the detection-limit by northern blot analysis in unstimulated VSMC, the combination of LPS/IFN provided a strong stimulus for iNOS mRNA expression (Fig. 4A). The LPS/IFN-induced increase in iNOS mRNA level was substantially decreased in cells that had been co-treated with NOR3 (0.1 to 1 mM). Fig. 4B shows the effects of NOR3, SNAP, SIN-1, peroxynitrite, and 8-bromo-cGMP on LPS-induced iNOS mRNA levels, as evaluated by RT-PCR. iNOS mRNA was barely detectable by RT-PCR in unstimulated VSMC, but substantially upregulated by 8 h after treatment with LPS/IFN. The LPS/IFN-induced increase in expression of iNOS mRNA was blocked by NOR3 and diminished by SNAP. In contrast, no substantial effect of SIN-1, $ONOO^-$, or 8-bromo-cGMP could be detected on LPS-induced iNOS mRNA expression.

To study the effects of NO and peroxynitrite on other gene expression, MCP-1 mRNA levels were evaluated in LPS/IFN-stimulated VSMC (Fig. 4C). The LPS/IFN-induced increase in MCP-1 mRNA levels was substantially decreased by NOR3, while the levels of MCP-1 mRN levels were not changed in response to $ONOO^-$.

3.5. Antioxidants inhibit NF-κB and NO synthesis

Pyrrolidine dithiocarbamate (PDTC) and *N*-acetylcysteine (NAC) are antioxidants that have been shown to prevent NF-κB activation in many experimental settings. As shown in Fig. 5A, NAC (1 mmol/l) and PDTC (50 μmol/l) markedly attenuated LPS/IFN-induced NF-κB activity in VSMC (62% and 85%, respectively). NAC and PDTC similarly attenuated LPS/IFN-induced NO synthesis (72% and 90%, respectively; Fig. 5B).

3.6. LPS stimulates IκB phosphorylation by inducing IKK activity, and NOR3 inhibits LPS-induced IκB phosphorylation and IKK activity

We first determined whether LPS-induced NF-κB activation is occurred through phosphorylation/degradation of IκB. To determine whether LPS causes IκB-α phosphorylation in rat VSMCs, Western blot analysis using anti-phospho-Ser32 of IκB-α antibody was performed. LPS induces IκB phosphorylation in 15 min, and the levels of phospho-IκB-α disappeared in 60 min. (Fig. 6A, upper panel). The blot was reprobed with anti-IκB antibody, and the data indicated that the maximum LPS-induced degra-

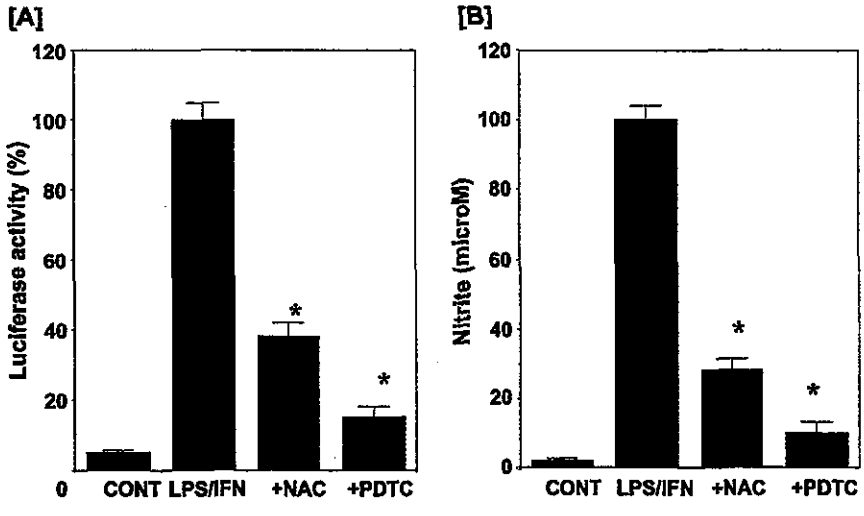


Fig. 5. Effect of NAC or PDTC on nitrite production and NF-κB-dependent transcriptional activity/luciferase reporter expression. Rat VSMC cells were treated with LPS (30 μg/ml) plus IFN (100 U/ml) in the presence of NAC (1 mM) or PDTC (50 μM). (A) Luciferase activity in the cells was measured after a 3 h incubation, and (B) nitrite accumulation in the culture medium was measured after a 24 h. Data are means ± S.E.M. (n = 3). *P < 0.05 versus LPS/IFN in the absence of NAC or PDTC.

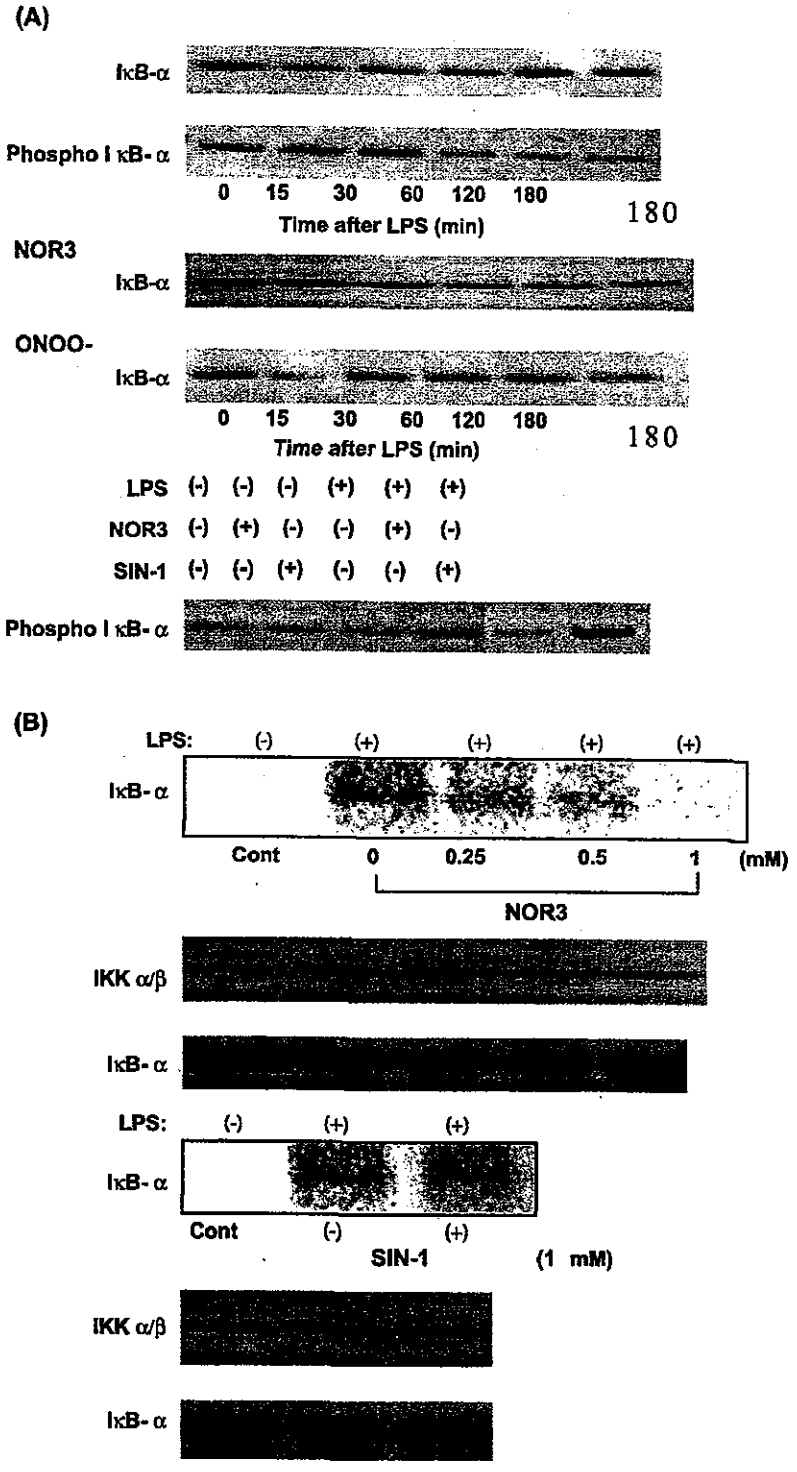


Fig. 6. (A) Effect of NOR3 and SIN-1 on degradation and phosphorylation of I B-α in rat VSMC. Cells were incubated for 45 min with various concentrations of NOR3 (1 mM) or SIN-1 (1 mM), followed by LPS (30 μg/ml) for 0–180 min. Cell were lysed and subjected to Western blot analysis using anti-I B-α antibody and *anti-phospho*-I B-α. (B) Effect of NOR3 and SIN-1 on IKK activity in rat VSMC. Cells were incubated for 45 min with various concentrations of NOR3 (0.25–1 mM) or SIN-1 (1 mM), followed by LPS (30 μg/ml) for 15 min. Cells were lysed and immunoprecipitated with anti-IKKα/β antibody and used for kinase assay using recombinant I B-α as substrate. Note that equal intensities of IKKα/β- and I B-α-specific bands are obtained.

dation was observed within 30 min. After that, IκB synthesis was reactivated possibly by NF-κB in 120 min (upper panel). Next, the effect of NOR3 or ONOO- on LPS-induced IκB-α degradation was determined (middle panel). NOR3 completely blocked this IκB-α degradation. SIN-1 had no effect on LPS-induced IκB-α degradation (middle panel). Lower panel shows the effect of NOR3 and SIN-1 on the phosphorylation of IκB-α. Addition of LPS resulted in a rapid (within 15 min) appearance of phosphorylated IκB-α. NOR3 (10^{-3} mol/l) prevented the LPS-stimulated increase in phosphorylated IκB-α. While SIN-1 had no effect on the LPS-stimulated phosphorylation of IκB-α (lower panel).

The radiolabeled, phosphorylated IκB-α-specific band is detected in LPS-treated cells while it was undetectable in the untreated cells, demonstrating that LPS induces the IKK activity (Fig. 6B) This IKK activity was dose-dependently decreased by treatment of the cells with NOR3 (upper panel). However, SIN-1 had no effect on LPS-induced IKK activity (lower panel). The remaining half of the immunoprecipitated samples were analyzed by Western blot analysis using anti-IKKα/β antibody, showing the identical level of expression of IKK, suggesting that IKK is expressed in these cells. The identical amount of IκB was detected when the equal volume of kinase reaction mixture was loaded into SDS-PAGE and analyzed by Western blot using anti-IκB antibody (Fig. 6B).

3.7. IκB-α mRNA expression

IκB-α mRNA expression was expressed at a relatively low level in unstimulated VSMC, as measured by northern blot analysis. Treatment of cells with LPS for 2 h substantially increased levels of IκB-α mRNA. NOR3 (1 to 1000 μM) alone neither increased the abundance of IκB-α mRNA, nor did it (100 μM) influence the extent of upregulation by LPS (data not shown).

4. Discussion

A wealth of literature supports the notion that ONOO is a key mediator of cytotoxicity arising from NO synthesis. Herein we reveal a fundamental and potentially critical distinction between NO and $ONOO^-$: while NO acts to inhibit NF-κB activation, $ONOO^-$ allows for sustained activation of NF-κB. This conclusion in VSMC is uniformly supported by results from several lines of investigation: (a) NF-κB-driven reporter gene expression, (b) EMSA studies of NF-κB nuclear translocation, and (c) immunocytochemical analysis of NF-κB p50 subcellular localization. Given that iNOS gene transcription itself requires NF-κB for upregulation by immunostimulants [7,8,15,16], it is also revealing that NO, but not $ONOO^-$, suppressed immunostimulant-evoked iNOS promoter activity and mRNA expression. The ability of NO to suppress NF-κB activation extends from iNOS to the numerous NF-κB-dependent gene products that contribute to the inflammatory response. That endogenously produced NO can indeed function as an anti-inflammatory molecule in biological systems is supported by reports that NOS inhibitors potentiate cytokine-induced expression of NF-κB-regulated genes in endothelial cells [28], macrophages [29] and in tissues from animals treated with immunostimulants [30] or exposed to ischemia–reperfusion injury [31].

Is the inhibition of NF-κB that we observe with NO-donors actually mediated by NO? Amperometric detection indicates that all NO donors tested—NOR3, SNAP and SIN-1 (in the presence of SOD)—release NO in physiological buffers at 37 °C with a $T1/2 < 2$ h [32,33]. Given the diverse structure of these compounds, it is unlikely that their shared ability to prevent NF-κB activation resides in either the parent or product molecules. We cannot however exclude the possibility that a NO-derived species, other than $ONOO^-$, (e.g., a nitrosothiol) is responsible or contributes to NF-κB inhibition.

High reactivity would predict that all added $ONOO^-$ is consumed within seconds of addition to cells. Accordingly, the failure of pure $ONOO^-$ to elicit effects on NF-κB activation or iNOS gene expression could conceivably be due to a more limited exposure of cells to $ONOO^-$, compared with NO from donor compounds. Studies with SIN-1 argue against this view. SIN-1 has the unique property of generating $OONO^-$ by releasing equimolar quantities of O_2^- and NO, essentially in a simultaneous manner. Although SIN-1 delivers a continuous flux of $ONOO^-$ to VSMC, no effect on LPS-induced NF-κB activation and iNOS gene expression was observed. The concentration of SIN-1 used in our study (1 mM) was reported to produce $ONOO^-$ under the conditions employed, but no detectable NO [34]. However, release of NO by SIN-1 can be observed to occur as an increasing function of added SOD, with ~5000 units achieving complete scavenging of O_2^- and maximal stimulation of NO production [35]. Our data show that only when SIN-1 was transformed into a pure NO donor (upon addition of 5000 units of SOD) did it substantially inhibit LPS-induced NF-κB activation. Thus, SIN-1 generated a sufficient flux of $ONOO^-$, under the experimental conditions used, that diversion to NO resulted in potent suppression of NF-κB activation. Accordingly, findings with SIN-1 provide strong support for the view that $ONOO^-$ supports, rather than suppresses, NF-κB activation.

A cGMP-independent effect of NO is suggested by our observation that a cell-permeable cGMP analogue failed to elicit a detectable effect on either NF-κB activation or iNOS gene expression. We confirmed that NO inhibits iNOS gene expression by directly blocking phosphorylation and subsequent degradation of IκB-α [18]. We further showed that LPS stimulates IκB-α phosphorylation by inducing IKK activity, and NO inhibits LPS-induced IκB-α phosphorylation and IKK activity. These data suggested that NO suppressed the

Clinical Endocrinology (2004) 61, 619–625 doi: 10.1111/j.1365-2265.2004.02145.x

Apolipoprotein E phenotype affects the malondialdehyde-modified LDL concentration and forearm endothelial function in postmenopausal women

Mikio Tsuda*, Mitsuhiro Sanada*, Yukihito Higashi†, Yoshiko Hara‡, Ichiro Kodama*, Kazuaki Chayama‡, Masao Yoshizumi† and Koso Ohama*

**Department of Obstetrics and Gynaecology, †Department of Physiology and ‡First Department of Internal Medicine, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan*

(Received 8 May 2004; returned for revision 18 July 2004; finally revised 12 August 2004; accepted 17 September 2004)

Summary

OBJECTIVE We investigated whether the apolipoprotein E (apo E) phenotype affects the serum concentration of malondialdehyde-modified low-density lipoprotein (MDA-LDL) or forearm endothelial function in postmenopausal women.

PATIENTS AND MEASUREMENTS Individuals were divided into three groups according to their apo E phenotype: E2 (E2/2 and E2/3, $n = 12$); E3 (E3/3, $n = 71$); and E4 (E3/4 and E4/4, $n = 27$). The serum concentrations of lipids and MDA-LDL were measured. Forearm blood flow during reactive hyperaemia and after sublingual nitroglycerin administration was measured by strain-gauge plethysmography.

RESULTS The serum concentrations of total and LDL cholesterol were significantly higher in the E4 group than in the E2 group ($P < 0.05$) or in the E3 group ($P < 0.05$). The serum apo B concentration was significantly higher in the E4 group than in the E2 group ($P < 0.05$). The serum concentrations of high density lipoprotein (HDL) cholesterol and nitrite/nitrate were significantly lower in the E4 group than in the E2 group ($P < 0.05$). Other lipid concentrations did not differ in the three groups. The serum MDA-LDL concentration was highest in the E4 group, and was lowest in the E2 group (E2: 91·1 ± 6·9 IU/l, E3: 112·3 ± 5·9 IU/l, E4: 128·8 ± 9·9 IU/l; $P < 0.05$). The forearm blood flow response to reactive hyperaemia was lowest in the E4 group, and highest in the E2 group (E2: 52·2 ± 5·8 ml/min per 100 ml tissue, E3: 40·7 ± 1·7 ml/min per 100 ml tissue, E4: 33·4 ± 2·4 ml/min per 100 ml tissue; $P < 0.05$). The forearm blood flow changes in response to nitroglycerine were similar between all three groups.

CONCLUSIONS The apo E phenotype affects the serum MDA-LDL concentration and forearm endothelial function in postmenopausal women.

Postmenopausal women are at an increased risk for coronary heart disease (CHD), consistent with a typically adverse plasma lipid profile (Coldits *et al.*, 1987; Matthews *et al.*, 1989) and the presence of endothelial dysfunction (Taddei *et al.*, 1996). However, the risk of CHD does not increase in all of these women, because individual variations exist for genetic factors that affect the lipid profile or endothelial function. Furthermore, it is not well known how genetic factors affect the risk of CHD in postmenopausal women.

Apolipoprotein (apo E), a protein constituent of chylomicrons, very low density lipoprotein (VLDL) and high density lipoprotein (HDL), is important in the clearance of triglyceride-rich lipoproteins, acting as a ligand for the LDL receptor and the lipoprotein remnant receptor (Mahley & Innerarity, 1983). The three common alleles (ε2, ε3, ε4) of the gene for apo E code for the isoforms E2, E3 and E4, resulting in the presence of six phenotypically distinct forms of apo E: E2/2, 2/3, 3/3, 3/4, 4/4 and 2/4 (Utermann *et al.*, 1977; Zannis *et al.*, 1981). These phenotypes have substantially different effects on lipid and lipoprotein metabolism (Davignon *et al.*, 1988; Dallongeville *et al.*, 1992; Kamboh *et al.*, 1993), and influence the response to therapy with hypolipidaemic agents (Ordovas *et al.*, 1995; Tsuda *et al.*, 2001).

Malondialdehyde-modified LDL (MDA-LDL) is one of the oxidative products of LDL metabolism, and this aldehyde may react with lysine residues in the LDL apo B-100 moiety, resulting in a decreased affinity of apo B-100 for the LDL receptor and an increased affinity for scavenger receptors (Holvoet *et al.*, 1995). MDA-LDL has been demonstrated in atherosclerotic lesions, and an elevated serum concentration of MDA-LDL is associated with CHD (Holvoet *et al.*, 1998). It is well known that

Correspondence: Mikio Tsuda, Department of Obstetrics and Gynaecology, Graduate School of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Tel.: 81 82 257 5262; Fax: 81 82 257 5264; E-mail: mtsuda@hiroshima-u.ac.jp

endothelium-dependent vasorelaxation is attenuated by hyperlipidaemia and in atherosclerotic vessels. Furthermore, oxidized LDL might play a causative role in the development of endothelial dysfunction (Cox & Cohen, 1996). Endothelial dysfunction has been implicated in the atherosclerotic process from early atherogenesis to ischaemic cardiomyopathy (Healy, 1990), and the degree of endothelial dysfunction is related to the clinical severity of heart failure (Carville *et al.*, 1998). The development of endothelial dysfunction is associated with the progression of myocardial diastolic dysfunction in patients with CHD (Ma *et al.*, 2000).

In the present study, we evaluated the relationship between apo E phenotype and MDA-LDL concentration and endothelium-dependent vasodilatation in postmenopausal women.

Methods

From January 2000 to December 2003, 110 consecutive postmenopausal Japanese women, 49–58 years of age, participating in the annual gynecological health check-up at our clinic, were enrolled in this study. Each individual had experienced natural menopause for at least 1 year but for no more than 5 years. They were of normal weight, with a body mass index < 25 kg/m^2. Menopausal status was confirmed by the presence of a serum FSH concentration > 40 mIU/ml, and a serum oestradiol concentration < 20 pg/ml. None of the women smoked, used caffeine or alcohol regularly, or had a history of thyroid disease, liver disease or diabetes mellitus. None had ever received hormone replacement therapy, other steroid hormones or any medications known to affect lipoprotein metabolism. The ethics committee of the Department of Obstetrics and Gynaecology of Hiroshima University approved the study protocol. Each individual gave her informed consent for participation.

The vasodilator responses to reactive hyperaemia, an index of endothelium-dependent vasodilation, and to the sublingual administration of nitroglycerine (NTG), an index of endothelium-independent vasodilation, were evaluated in each participant. This evaluation began at 0830 h. Each participant had fasted the previous night for at least 14 h. After the overnight fast, the participant rested supine in a quiet, air-conditioned room (constant temperature, 22–25°C). After the participant had rested for 30 min in that position, the basal forearm blood flow (FBF) was measured as described below. Next, the effects of reactive hyperaemia and of the sublingual administration of NTG on FBF were evaluated by inflating a cuff over the left upper arm to 280 mmHg for 5 min. After the cuff occlusion was released, FBF was measured for 3 min. An NTG tablet (0·3 mg) (Nihonkayaku Co., Tokyo, Japan) was then administered sublingually, and the FBF was again measured for 3 min. In a preliminary study, we confirmed the reproducibility of the FBF response to reactive hyperaemia and sublingual NTG on two separate occasions in seven postmenopausal women (mean age 65 ± 7 years). The coefficients of variation were 6·5% and 5·8%, respectively.

The fasting serum concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol, triglyceride, lipoprotein (a), creatinine, glucose, electrolytes, nitrite/nitrate and MDA-LDL, and the plasma renin activity (PRA) and angiotensin-converting enzyme (ACE) activity were obtained after a 30-min rest period. Body weight, blood pressure and heart rate were also measured.

apo E phenotyping

The apo E phenotype was determined by isoelectric focusing electrophoresis (Phenotyping Apo E IEF System, Jyoukou Co. Ltd, Tokyo, Japan). In brief, the plasma sample was pretreated with Tween 20 and dithiothreitol for 15 min. The sample was then subjected to isoelectric focusing electrophoresis. Fifteen minutes of prefocusing followed by 30 min of initial focusing were carried out, and subsequent focusing (final focusing) was conducted for an additional 90 min. Immunoblotting was performed according to the method described by Kamboh *et al.* (1988), in which the primary antibody for human apo E was raised in goat and labelled with alkaline phosphatase. Data from a blinded set of samples that were analysed by the present technique (Kataoka *et al.*, 1994) showed a close relationship (421 of 431 samples: 98%) to data obtained in the genetic phenotyping conducted by the polymerase chain reaction (Hixon & Vernier, 1990).

Measurement of forearm blood flow

FBF was measured with a mercury-filled Silastic strain-gauge plethysmograph (EC-5R, D.E. Hokanson, Inc., Issaquah, WA, USA) as described previously (Panza *et al.*, 1990; Higashi *et al.*, 1999). In brief, the strain gauge was attached to the left upper arm supported above the level of the right atrium and connected to a plethysmography device. A wrist cuff was inflated to a pressure of 50 mmHg above the systolic blood pressure to exclude the hand circulation from the measurements 1 min before each measurement and throughout the measurement of the FBF. The upper arm cuff was inflated to 40 mmHg for 7 s in each 15-s cycle to occlude venous outflow from the forearm, using a rapid cuff inflator (EC-20, D. E. Hokanson, Inc.). The FBF output signal was transmitted to a recorder (U-228, Advance Co., Nagoya, Japan). FBF is expressed as ml/min/100 ml of forearm tissue volume. The FBF was calculated by two independent observers who had no knowledge of the patient's clinical data or the results from the linear portions of the plethysmographic recordings. The intraobserver coefficient of variation was 3·0 ± 1·6%. Four plethysmographic measurements were averaged to obtain the FBF at baseline, during reactive hyperaemia, and after the administration of sublingual NTG.

Analytical methods

Samples of venous blood were placed in polystyrene tubes that contained sodium ethylenediaminetetraacetic acid (EDTA) (1 mg/ml). The EDTA-containing tubes were chilled immediately in an ice bath. Next, the plasma and the serum were separated by centrifugation at 4°C and room temperature, respectively, for 10 min. Samples were stored at −80°C until the time of assay. The serum concentrations of total cholesterol, triglyceride and HDL cholesterol were measured by enzymatic assays (McNamara & Shaefer, 1987) (BM-2250, Nippon Denshi, Tokyo, Japan). The serum concentration of LDL cholesterol was calculated using the Friedewald formula (Friedewald *et al.*, 1972). Serum concentrations of oestradiol and FSH were measured by using a conventional radioimmunoassay kit (oestradiol; ACS-180, Bayer Medical, NY, FSH; ES-600, Roche Diagnostics, Basel, Switzerland). Nitrite/nitrate concentrations were measured with an autoanalyser (flow injection analyser, TCI-NOX1000, Tokyo Kasei Kogyo, Japan), which uses a protocol based on the Griess reaction (Green *et al.*, 1982). The ACE activity (IU/l at 37°C) was measured with ACE Colour (Fujirebio Co., Ltd, Tokyo, Japan). Plasma renin activity (PRA) was determined using a radioimmunoassay kit. The serum lipoprotein (a) concentration was determined by an enzyme-linked immunosorbent assay (ELISA). A monoclonal antibody 1H11-based competition ELISA was used for the quantification of MDA-LDL in the serum (Kotani *et al.*, 1994). The intra-assay coefficients of variation for total cholesterol, triglyceride, HDL cholesterol, oestradiol, FSH, lipoprotein (a), serum ACE activity, nitrite/nitrate, PRA and MDA-LDL were all under 5%.

Statistical analysis

All data are expressed as the mean ± SE. Differences between the three groups with differing apo E phenotypes were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni correction. Log transformations of the data using data for triglycerides were used when the values were not normally distributed. The comparison of the time-course curves of FBF during reactive hyperaemia between the three apo E phenotypes was performed using two-way ANOVA for repeated measures of one factor, followed by the Bonferroni correction for multiple, paired comparisons. Pearsons' correlation coefficient was used to determine correlations between variables. The following factors were considered as independent variables: age; systolic or diastolic blood pressure; total cholesterol; triglycerides; HDL cholesterol; LDL cholesterol; lipoprotein (a); serum ACE activity; nitrite/nitrate, PRA, MDA-LDL; maximal FBF response to reactive hyperaemia. Data were processed using the Software package Statview V (SAS Institute Inc., NC) or Super ANOVA (Abacus Concepts, Berkeley, CA). A value of $P < 0{\cdot}05$ was accepted as statistically significant.

Results

The frequencies of the different apo E phenotypes in our population were as follows: 0·9% ($n = 1$) for E2/2, 10% ($n = 11$) for E2/3, 64·5% ($n = 71$) for E3/3, 0% ($n = 0$) for E2/4, 22·7% ($n = 25$) for E3/4 and 1·8% ($n = 2$) for E4/4. These frequencies are similar to those reported previously for normal Japanese (Eto *et al.*, 1986; Shiraki *et al.*, 1997) and Western (Sing & Davignon, 1985) populations.

The individuals were divided into three apo E groups: one group containing all subjects with the E2/2 ($n = 1$) or E2/3 ($n = 11$) phenotypes (E2 group), a second group containing all subjects with the E3/3 ($n = 71$) phenotype (E3 group) and a third group containing all subjects with the E3/4 ($n = 25$) or E4/4 ($n = 2$) phenotype (E4 group).

Within the whole population, MDA-LDL was positively correlated with total cholesterol ($r = 0{\cdot}56$, $P < 0{\cdot}001$), LDL cholesterol ($r = 0{\cdot}54$, $P < 0{\cdot}001$) and triglycerides ($r = 0{\cdot}45$, $P < 0{\cdot}001$), and negatively correlated with HDL cholesterol ($r = -0{\cdot}45$, $P < 0{\cdot}001$). Maximal FBF responses to reactive hyperaemia were negatively correlated with LDL cholesterol ($r = -0{\cdot}26$, $P < 0{\cdot}01$) and triglycerides ($r = -0{\cdot}21$, $P < 0{\cdot}05$).

Effect of apo E phenotype on clinical variables

The clinical characteristics of the three groups are summarized in Table 1. There were no statistically significant differences between the three groups with regard to mean age, body mass index, age at menopause, blood pressure or heart rate or between the serum oestradiol concentration, or serum FSH concentration. The serum concentrations of total and LDL cholesterol were significantly higher in the E4 group than in the E2 group ($P < 0{\cdot}05$), or in the E3 group ($P < 0{\cdot}05$). The serum apo B concentration was significantly higher in the E4 group than in the E2 group ($P < 0{\cdot}05$). The serum concentrations of HDL cholesterol and nitrite/nitrate were significantly lower in the E4 group than in the E2 group ($P < 0{\cdot}05$). Other lipid concentrations did not differ in the three groups.

Effect of apo E phenotype on MDA-LDL

The serum MDA-LDL concentration was highest in the E4 group, and was lowest in the E2 group (E2: 91·1 ± 6·9 IU/l, E3: 112·3 ± 5·9 IU/l, E4: 128·8 ± 9·9 IU/l; $P < 0{\cdot}05$; Fig. 1). The ratio of MDA-LDL and LDL cholesterol did not differ in the three groups.

Effect of apo E phenotype on endothelial function

Basal FBF was similar between all three groups. The FBF response to reactive hyperaemia, an index of endothelium-dependent

Table 1 Clinical characteristics in each phenotype group

Variable	E2 group ($n = 12$)	E3 group ($n = 71$)	E4 group ($n = 27$)
Age (years)	55·5 ± 2·1	54·1 ± 0·6	55·4 ± 1·1
Age at menopause (years)	48·2 ± 1·1	49·6 ± 0·4	49·0 ± 0·8
Body mass index (kg/m^2)	21·1 ± 0·7	21·8 ± 0·3	21·8 ± 0·6
Systolic blood pressure (mmHg)	123 ± 5	124 ± 2	133 ± 5
Diastolic blood pressure (mmHg)	78 ± 4	76 ± 1	79 ± 2
Heart rate (beats/min)	66 ± 3	65 ± 1	66 ± 2
Total cholesterol (mmol/l)	5·38 ± 0·10	5·67 ± 0·05	6·15 ± 0·08ab
LDL cholesterol (mmol/l)	2·85 ± 0·22	3·37 ± 0·11	3·82 ± 0·18ab
HDL cholesterol (mmol/l)	2·05 ± 0·13	1·89 ± 0·05	1·74 ± 0·08a
Apolipoprotein A1 (g/l)	1·69 ± 0·04	1·62 ± 0·03	1·56 ± 0·04
Apolipoprotein A2 (g/l)	0·32 ± 0·02	0·33 ± 0·01	0·32 ± 0·01
Apolipoprotein B (g/l)	0·88 ± 0·05	1·01 ± 0·03	1·10 ± 0·05a
Triglyceride (mmol/l)	1·09 ± 0·33	1·24 ± 0·09	1·50 ± 0·23
Lipoprotein (a) (mg/dl)	20·5 ± 6·4	26·7 ± 5·6	23·6 ± 3·9
PRA (ng/ml/h)	1·3 ± 0·3	1·1 ± 0·1	1·0 ± 0·1
Serum ACE activity (IU/l)	12·5 ± 1·7	11·5 ± 0·3	12·8 ± 0·9
Nitrite/nitrate (μmol/l)	52·2 ± 8·8	43·1 ± 2·2	33·8 ± 5·1a
Serum creatinine (μmol/l)	61·8 ± 2·7	61·0 ± 0·9	64·5 ± 3·5
Serum glucose (mmol/l)	4·9 ± 0·2	4·7 ± 0·1	4·9 ± 0·1
Oestradiol (pmol/l)	40·7 ± 2·9	52·5 ± 6·6	51·4 ± 11·4
FSH (IU/l)	85·0 ± 14·8	73·5 ± 3·0	73·1 ± 9·1
Basal FBF (ml/min per 100 ml tissue)	6·5 ± 0·5	6·1 ± 0·2	6·2 ± 0·6

Results are presented as the mean ± SE. a: $P < 0·05$ vs. E2 group. b: $P < 0·05$ vs. E3 group.

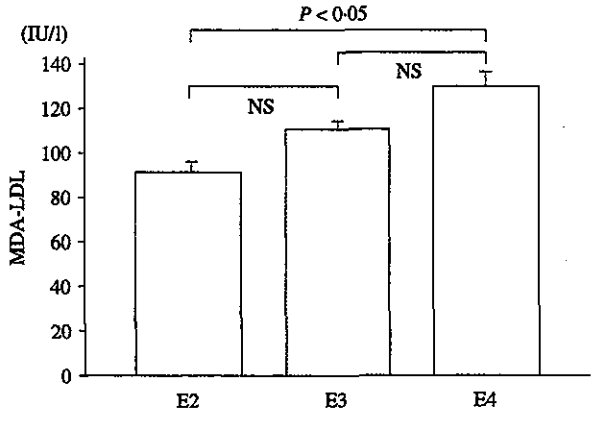


Fig. 1 The serum concentrations of MDA-LDL in the E2 ($n = 12$), E3 ($n = 71$) and E4 ($n = 27$) groups. Results are presented as the mean value ± SE. NS = not significant. The serum concentration of MDA-LDL was significantly higher in the E4 group than in the E2 group ($P < 0·05$).

vasorelaxation, was lowest in the E4 group, and highest in the E2 group (E2: 52·2 ± 5·8 ml/min per 100 ml tissue, E3: 40·7 ± 1·7 ml/min per 100 ml tissue, E4: 33·4 ± 2·4 ml/min per 100 ml tissue; $P < 0·05$; Fig. 2). The changes in FBF after sublingual administration of GTN, an index of endothelium-independent vasodilation, were similar between all three groups.

Discussion

In the present study, we evaluated the relationship between the apo E phenotype and serum MDA-LDL concentration, and the forearm endothelial function in postmenopausal women. We found that the apo E4 phenotype was associated with a higher serum MDA-LDL concentration and lower FBF response to reactive hyperaemia in postmenopausal women.

apo E phenotype and MDA-LDL

The apo E phenotype influences lipid metabolism. Specifically, in normal population, individuals with the E2 allele have lower concentrations of plasma total and LDL cholesterol than individuals with the E3/3 allele (Utermann *et al.*, 1979), and individuals with the E4 allele have higher plasma total and LDL cholesterol concentrations than individuals with the E3/3 allele (Bouthillier *et al.*, 1983). Our findings support these previous results. While it is not known whether the apo E phenotype affects the MDA-LDL concentration, which is considered to be one of the oxidative products of LDL metabolism, our present study shows that the apo E phenotype does affect the serum MDA-LDL concentration in postmenopausal women.

The mechanisms responsible for the apparent differences in MDA-LDL concentrations between the apo E phenotypes are not known. Several possibilities should be considered. First, it is well known that significant differences in the mean concentrations of

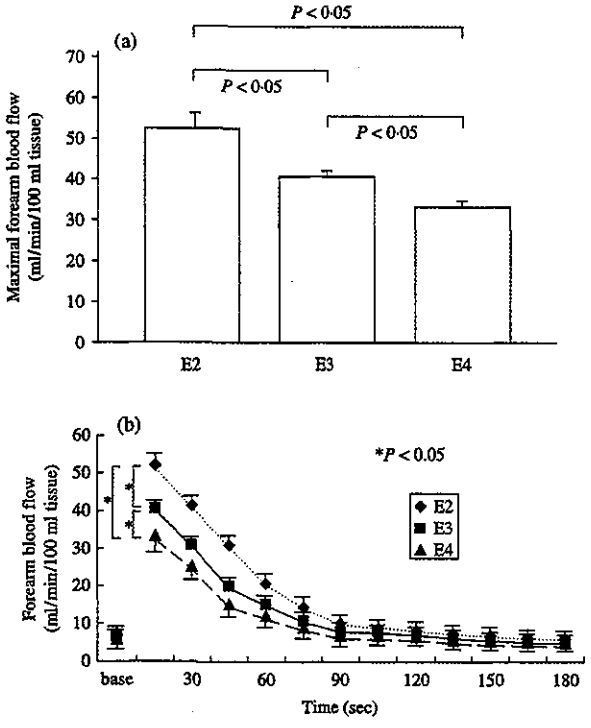


Fig. 2 (a) Maximal forearm blood flow (FBF) during reactive hyperaemia in the E2 ($n = 12$), E3 ($n = 71$) and E4 ($n = 27$) groups. The results are presented as the mean value ± SE. There was a significant difference in reactive hyperaemia between each group ($P < 0·05$). (b) Forearm blood flow at rest and during reactive hyperaemia in the E2 ($n = 12$), E3 ($n = 71$) and E4 ($n = 27$) groups. There was a significant difference in reactive hyperaemia between each group ($P < 0·05$).

LDL cholesterol and apo B exist between groups with differing apo E phenotypes. These concentrations are lowest in subjects with the E2 allele and highest in subjects with the E4 allele. In the present study, the mean of LDL cholesterol and apo B concentrations were highest in the E4 group and lowest in the E2 group. Because the MDA-LDL concentration is significantly correlated with LDL and apo B concentrations, MDA-LDL concentrations would be expected to be highest in the E4 group and lowest in the E2 group.

Second, the apo E phenotype might influence the oxidation of lipoproteins. Significant differences in the activities of enzymatic antioxidants, such as catalase and glutathione peroxidase, exist between groups with differing apo E phenotypes. These activities are highest in subjects with the E2 allele and lowest in subjects with the E4 allele (Miyata & Smith, 1996; Ramassamy *et al.*, 1999). E4, with less antioxidant activity than E2 or E3, might be the least effective in protecting lipids from oxidative damage. Therefore, MDA-LDL concentrations would be expected to be highest in the E4 group and lowest in the E2 group.

apo E phenotype and endothelial function

Our results also showed that the apo E phenotype is associated with endothelium-dependent vasodilatation in postmenopausal women. Hypercholesterolaemia, especially increased concentrations of oxidized LDL, induces microvascular dysfunction characterized by the loss of endothelium-derived nitric oxide (NO), just as MDA-LDL inhibits endothelium-dependent relaxation by inhibiting NO production and endothelium-derived hyperpolarizing factor (EDHF) secretion (Holvoet, 1999). The present study showed that the concentration of nitrite/nitrate was highest in the E2 group and lowest in the E4 group. In the E4 group, the higher concentrations of MDA-LDL and LDL might inhibit the production/release of NO and might induce less of an FBF response to reactive hyperaemia.

However, recent data suggest that HDL cholesterol has direct beneficial effects on endothelial function. HDL cholesterol activates endothelial nitric oxide synthase (eNOS) (Yuhanna *et al.*, 2001) and reverses oxidized LDL-mediated inhibition of eNOS activity (Uittenbogaard *et al.*, 2000). *In vivo* studies suggest that peripheral endothelial function is abnormal in subjects with low HDL cholesterol concentrations and is preserved in those with high HDL cholesterol concentrations (Kuvin *et al.*, 2003). Plasma triglyceride concentration is also associated with endothelial function. Lewis *et al.* (1999) suggested that endothelium-dependent relaxation by acetylcholine is impaired in hypertriglyceridaemic subjects with normal concentrations of plasma LDL cholesterol. The plasma triglyceride concentration is inversely related to LDL particle size (McNamara *et al.*, 1992). Because small dense LDL particles are prone to oxidative modification (Chait *et al.*, 1993), increases in triglycerides can impair endothelium-dependent vasodilatation. Our results showed that the HDL cholesterol concentration was highest in the E2 group and lowest in the E4 group, and the triglyceride concentration was lowest in the E2 group and highest in the E4 group. The differences in FBF based on apo E phenotypes might be influenced by the HDL cholesterol and triglyceride concentrations.

Haraki *et al.* (2002) reported that there was no difference between apo E phenotypes and flow-mediated dilatation of the brachial artery in healthy men. The discrepancy between their study and our results might be due to the sex-related difference or difference in the methods of vasodilation.

Study limitations

A definitive way to assess endothelial function may be the direct intra-arterial infusion of a vasoactive agent in the forearm. However, Celermajer *et al.* (1992) demonstrated that the noninvasive assessment of reactive hyperaemia is useful in assessing endothelial function. In the present study, this noninvasive method was simple to perform, reproducible and caused no adverse effects.

We recommend use of this noninvasive technique to assess endothelial function in routine clinical examinations and in future studies.

The limitation of the present study is that the number of subjects in each group was relatively small, especially in the E2 group. Our findings will therefore need to be confirmed in larger studies.

Conclusions

The present study suggests that the apo E phenotype affects the serum MDA-LDL concentration and FBF response to reactive hyperaemia in postmenopausal women. Therefore, apo E phenotyping may be important in predicting the risk of CHD in postmenopausal women.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (no. 1477085300).

References

Bouthillier, D., Sing, C.F. & Davignon, J. (1983) Apolipoprotein E phenotyping with a single gel method – application to the study of informative matings. *Journal of Lipid Research*, **24**, 1060–1069.

Carville, C., Adnot, S., Sediame, S., Benacerraf, S., Castaigne, A., Calvo, F., de Cremou, P. & Dubois-Rande, J.L. (1998) Relation between impairment in nitric oxide pathway and clinical status in patients with congestive heart failure. *Journal of Cardiovascular Pharmacology*, **32**, 562–570.

Celermajer, D.S., Sorensen, K.E., Gooch, V.M., Spiegelhalter, D.J., Miller, O.I., Sullivan, I.D., Lloyd, J.K. & Deanfield, J.E. (1992) Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet*, **40**, 1111–1115.

Chait, A., Brazg, R.L., Tribble, D.L. & Krauss, R.M. (1993) Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. *American Journal of Medicine*, **94**, 350–356.

Coldits, G.A., Willet, W.C., Stampfer, M.J., Rosner, B., Speizer, F.E. & Hennekens, C.H. (1987) Menopause and the risk of coronary heart disease in women. *New England Journal of Medicine*, **316**, 1105–1110.

Cox, D.A. & Cohen, M.L. (1996) Effects of oxidized low-density lipoprotein on vascular contraction and relaxation: clinical and pharmacological implications in atherosclerosis. *Pharmacological Research*, **48**, 3–19.

Dallongeville, J., Lussier-Cacan, S. & Davignon, J. (1992) Modulation of plasma triglyceride levels by apo E phenotype: a meta-analysis. *Journal of Lipid Research*, **33**, 447–454.

Davignon, J., Gregg, R.E. & Sing, S.F. (1988) Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis*, **8**, 1–21.

Eto, M., Watanabe, K. & Ishii, K. (1986) A racial difference in apolipoprotein E allele frequencies between the Japanese and Caucasian populations. *Clinical Genetics*, **30**, 422–427.

Friedewald, W.T., Levy, R.I. & Fredrickson, D.S. (1972) Estimation of the concentration of low density lipoprotein cholesterol in plasma without use the preparative ultracentrifuge. *Clinical Chemistry*, **18**, 499–502.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. & Tannenbaum, S.R. (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry*, **126**, 131–138.

Haraki, T., Takegoshi, C., Kitoh, C., Wakasugi, T., Saga, T., Hirai, J.I., Aoyama, T., Inazu, A. & Mabuchi, H. (2002) Carotid artery intima-media thickness and brachial artery flow-mediated vasodilation in asymptomatic Japanese male subjects amongst apolipoprotein E phenotypes. *Journal of International Medicine*, **252**, 114–120.##

Healy, B. (1990) Endothelial cell dysfunction: an emerging endocrinopathy linked to coronary disease. *Journal of the American College of Cardiology*, **16**, 357–358.

Higashi, Y., Sasaki, S., Kurisu, S., Yoshimizu, A., Sasaki, N., Matsuura, H., Kajiyama, G. & Oshima, T. (1999) Regular aerobic exercise augments endothelium-dependent vascular relaxation in normotensive as well as hypertensive subjects: role of endothelium-derived nitric oxide. *Circulation*, **100**, 1194–1202.

Hixon, J.E. & Vernier, D.T. (1990) Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with Hha I. *Journal of Lipid Research*, **31**, 545–548.

Holvoet, P. (1999) Endothelial dysfunction, oxidation of low-density lipoprotein, and cardiovascular disease. *Therapeutic Apheresis*, **3**, 287–293.

Holvoet, P., Perez, G., Zhao, Z., Brouwers, E., Bernar, H. & Collen, D. (1995) Malondialdehyde-modified low density lipoproteins in patients with atherosclerotic disease. *Journal of Clinical Investigation*, **95**, 2611–2619.

Holvoet, P., Vanhaecke, J., Janssens, S., Van de Werf, F. & Collen, D. (1998) Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation*, **98**, 1487–1494.

Kamboh, M.I., Ferrell, R.E. & Kottke, B. (1988) Genetic studies of human apolipoprotein. A novel rapid procedure to screen apolipoprotein E polymorphism. *Journal of Lipid Research*, **29**, 1535–1543.

Kamboh, M.I., Aston, C.E., Ferrell, R.E. & Hamman, R.F. (1993) Impact of apolipoprotein E polymorphism in determining inter-individual variation in total cholesterol and low density lipoprotein cholesterol in Hispanics and non-Hispanic whites. *Atherosclerosis*, **98**, 201–211.

Kataoka, S., Paidi, M. & Howard, B.V. (1994) Simplified iso-electric focusing/immunoblotting determination of apoprotein E phenotype. *Clinical Chemistry*, **40**, 11–13.

Kotani, K., Maekawa, M., Kanno, T., Kondo, A., Toda, N. & Manabe, M. (1994) Distribution of immunoreactive malondialdehyde-modified low-density lipoprotein in human serum. *Biochimica et Biophysica Acta*, **1215**, 121–125.

Kuvin, J.T., Patel, A.R., Sidhu, M., Rand, W.M., Sliney, K.A., Pandian, N.G. & Karas, R.H. (2003) Relation between high-density lipoprotein cholesterol and peripheral vasomotor function. *American Journal of Cardiology*, **92**, 275–279.

Lewis, T.V., Dart, A.M. & Chin-Dusting, J.P. (1999) Endothelium-dependent relaxation by acetylcholine is impaired in hypertriglyceridemic humans with normal levels of plasma LDL cholesterol. *Journal of the American College of Cardiology*, **33**, 805–812.

Ma, L.N., Zhao, S.P., Gao, M., Zhou, Q.C. & Fan, P. (2000) Endothelial dysfunction associated with left ventricular diastolic dysfunction in patients with coronary heart disease. *International Journal of Cardiology*, **72**, 275–279.

Mahley, R.W. & Innerarity, B. (1983) Lipoprotein receptors and cholesterol homeostasis. *Biochimica et Biophysica Acta*, **737**, 197–222.

Matthews, K.A., Meilahn, E., Kuller, L.H., Kelsey, S.F., Caggiula, A.W. & Wing, R.R. (1989) Menopause and risk factors for coronary heart disease. *New England Journal of Medicine*, **321**, 641–646.

McNamara, J.R. & Shaefer, E.J. (1987) Automated enzymatic standardized lipid analyses for plasma and lipoprotein functions. *Clinica et Chimica Acta*, **166**, 1–8.

McNamara, J.R., Jenner, J.L., Li, Z., Wilson, P.W. & Schaefer, E.J. (1992) Change in LDL particle size is associated with change in plasma triglyceride concentration. *Arteriosclerosis and Thrombosis*, **12**, 1284–1290.

Miyata, M. & Smith, J.D. (1996) Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. *Nature Genetics*, **14**, 55–61.

Ordovas, J.M., Lopez-Miranda, J., Perez-Jimenez, F., Rodriguez, C., Park, J.S., Cole, T. & Schaefer, E.J. (1995) Effect of apolipoprotein E and A-IV phenotypes on the low density lipoprotein response to HMG CoA reductase inhibitor therapy. *Atherosclerosis*, **113**, 157–166.

Panza, J.A., Quyyumi, A.A., Brush, J.E. & Epstein, S.E. (1990) Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *New England Journal of Medicine*, **353**, 22–27.

Ramassamy, C., Averill, D., Beffert, U., Bastianetto, S., Theroux, L., Lussier-Cacan, S., Cohn, J.S., Christen, Y., Davignon, J., Quirion, R. & Poirier, J. (1999) Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. *Free Radical Biology and Medicine*, **27**, 544–553.

Shiraki, M., Shiraki, Y., Aoki, C., Hosoi, T., Inoue, S., Kaneki, M. & Ouchi, Y. (1997) Association of bone mineral density with apolipoprotein E phenotype. *Journal of Bone and Mineral Research*, **12**, 1438–1445.

Sing, C.F. & Davignon, J. (1985) Role of the apolipoprotein E polymorphism in determining normal plasma lipid and lipoprotein variation. *American Journal of Human Genetics*, **37**, 268–285.

Taddei, S., Virdis, A., Ghiadoni, L., Mattei, P., Sudano, I., Bernini, G., Pinto, S. & Salvetti, A. (1996) Menopause is associated with endothelial dysfunction in women. *Journal of Hypertension*, **28**, 576–582.

Tsuda, M., Sanada, M., Nakagawa, H., Kodama, I., Sakashita, T. & Ohama, K. (2001) Phenotype of apolipoprotein E influences the lipid metabolic response of postmenopausal women to hormone replacement therapy. *Maturitas*, **38**, 297–304.

Uittenbogaard, A., Shaul, P.W., Yuhanna, I.S., Blair, A. & Smart, E.J. (2000) High density lipoprotein prevents oxidized low density lipoprotein-induced inhibition of endothelial nitric-oxide synthase localization and activation in caveolae. *Journal of Biological Chemistry*, **275**, 11278–11283.

Utermann, G., Hees, M. & Steinmertz, A. (1977) Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinemia in man. *Nature*, **269**, 604–607.

Utermann, G., Pruin, N. & Steinmetz, A. (1979) Polymorphism of apolipoprotein E. III. Effect of a single polymorphic gene locus an plasma lipid levels in men. *Clinical Genetics*, **15**, 63–72.

Yuhanna, I.S., Zhu, Y., Cox, B.E., Hahner, L.D., Osborne-Lawrence, S., Lu, P., Marcel, Y.L., Anderson, R.G., Mendelsohn, M.E., Hobbs, H.H. & Shaul, P.W. (2001) High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nature Medicine*, **7**, 853–857.

Zannis, V.I., Just, P.W. & Breslow, J.L. (1981) Human apolipoprotein E isoprotein subclasses are genetically determined. *American Journal of Human Genetics*, **256**, 9077–9083.

Autologous Bone-Marrow Mononuclear Cell Implantation Improves Endothelium-Dependent Vasodilation in Patients With Limb Ischemia

Yukihito Higashi, MD; Masashi Kimura, MD; Keiko Hara, MD; Kensuke Noma, MD; Daisuke Jitsuiki, MD; Keigo Nakagawa, MD; Tetsuya Oshima, MD; Kazuaki Chayama, MD; Taijiro Sueda, MD; Chikara Goto, RTP, MS; Hiroaki Matsubara, MD; Toyoaki Murohara, MD; Masao Yoshizumi, MD

Background—Patients with limb ischemia were associated with endothelial dysfunction. The purpose of this study was to determine whether autologous bone-marrow mononuclear cell (BM-MNC) implantation improves endothelial dysfunction in patients with limb ischemia.

Methods and Results—We evaluated the leg blood flow (LBF) response to acetylcholine (ACh), an endothelium-dependent vasodilator, and sodium nitroprusside (SNP), an endothelium-independent vasodilator, before and after BM-MNC implantation in 7 patients with limb ischemia. LBF was measured with a mercury-filled Silastic strain-gauge plethysmograph. The number of BM-MNCs implanted into ischemic limbs was $1.6\times10^9\pm0.3\times10^9$. The number of $CD34^+$ cells included in the implanted BM-MNCs was $3.8\times10^7\pm1.6\times10^7$. BM-MNC implantation improved the ankle-brachial pressure index (0.33±0.21 to 0.39±0.17, $P=0.06$), transcutaneous oxygen pressure (28.4±11.5 to 36.6±5.2 mm Hg, $P=0.03$), and pain-free walking time (0.8±0.6 to 2.9±2.2 minutes, $P=0.02$). After BM-MNC implantation, LBF response to ACh was enhanced (19.3±6.8 versus 29.6±7.1 mL/min per 100 mL; $P=0.002$). The vasodilatory effect of SNP was similar before and after BM-MNC implantation.

Conclusions—These findings suggest that BM-MNC implantation augments endothelium-dependent vasodilation in patients with limb ischemia. (***Circulation.* 2004;109:1215-1218.**)

Key Words: angiogenesis ■ cells ■ endothelium ■ ischemia

Recent studies have shown that bone-marrow mononuclear cell (BM-MNC) implantation increases collateral vessel formation in both ischemic limb models and patients with limb ischemia.[1,2] However, it is not clear whether these collateral arteries have normal vascular function, especially endothelial function. Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis and plays an important role in development and maintenance of atherosclerosis.[3] Limb ischemia is generally associated with endothelial dysfunction.[4,5] Therefore, it is clinically important to evaluate the vascular function of collateral arteries induced by BM-MNC implantation. We hypothesized that BM-MNC implantation would improve impaired endothelial function in patients with limb ischemia.

To determine the effect of BM-MNC implantation on endothelial function in patients with limb ischemia, we evaluated endothelium-dependent vasodilation induced by acetylcholine (ACh) and endothelium-independent vasodilation induced by sodium nitroprusside (SNP) before and after BM-MNC implantation.

Methods

Subjects

Seven patients with peripheral arterial disease (6 men and 1 woman; mean age, 64±9 years) who had rest pain and nonhealing ulcers and who were not candidates for angioplasty or surgical revascularization were enrolled in this study. The diagnosis of limb ischemia was confirmed by angiography. Patients with diabetes mellitus, coronary artery disease, and history of malignant disorders were excluded. Four of the 7 patients had smoking habits, and those 4 patients stopped smoking 2 months before BM-MNC implantation. The drugs used were not changed throughout the study. Lifestyle also was regulated throughout the study. The study protocol was approved by the Ethics Committee of the Hiroshima University Graduate School of Medicine. Written informed consent for participation was obtained from all subjects.

Received March 5, 2003; de novo received December 15, 2003; revision received January 28, 2003; accepted January 28, 2003.

From the Department of Cardiovascular Physiology and Medicine (Y.H., M.Y.), Department of Medicine and Molecular Science (M.K., K.H., K. Noma, D.J., K. Nakagawa, K.C.), Division of Physical Therapy, Institute of Health Sciences (C.G.), and the Department of Clinical Laboratory Medicine (T.O.), Department of Surgery (T.S.), Hiroshima University Graduate School of Biomedical Sciences, Department of Cardiovascular Medicine (H.M.), Kyoto Prefectural University of Medicine, and Department of Cardiology (T.M.), Nagoya University Graduate School of Medicine.

Correspondence to Yukihito Higashi, MD, PhD, FAHA, Department of Cardiovascular Physiology and Medicine, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail yhigashi@hiroshima-u.ac.jp

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000121427.53291.78

Patient	Age, y	Sex	Disorders	Drugs	BM-MNC Count, No. of Cells	CD34 (+) Cells in BM-MNC	ABI	TcO_2, mm Hg	Pain-Free Walking, min	Basal LBF mL · min^{-1} · 100 mL^{-1}
Patient 1										
Before	74	Male	HL	CaB, statin, APA	1.8×10^9	2.5×10^7	0.35	36	0.91	1.3
After 4 wk							0.44	41	1.22	2.4
After 24 wk							0.41	40	1.35	2.3
Patient 2										
Before	62	Male	HL	Statin	1.4×10^9	3.3×10^7	0.60	42	2.14	3.5
After 4 wk							0.62	45	6.89	3.9
After 24 wk							...	...	...	...
Patient 3										
Before	73	Male	HT, HL	CaB, ACEI, APA	1.6×10^9	4.2×10^7	0.23	25	1.21	2.2
After 4 wk							0.21	31	3.35	2.1
After 24 wk							0.22	32	2.98	2.2
Patient 4										
Before	56	Male	None	APA	2.2×10^9	6.2×10^7	ND	9	0	0.2
After 4 wk							0.26	37	2.34	0.6
After 24 wk							0.28	33	2.99	0.6
Patient 5										
Before	73	Female	HT	CaB, APA	1.5×10^9	1.3×10^7	0.39	38	0.56	1.9
After 4 wk							0.40	37	1.63	3.8
After 24 wk							0.38	36	1.35	3.2
Patient 6										
Before	51	Male	HL	Statin, APA	1.4×10^9	3.9×10^7	0.19	20	0.33	0.3
After 4 wk							0.22	31	0.98	0.3
After 24 wk							0.21	29	1.01	0.3
Patient 7										
Before	59	Male	HT	CaB, APA	1.2×10^9	5.1×10^7	0.52	29	0.76	2.6
After 4 wk							0.61	34	4.75	3.3
After 24 wk							0.59	32	5.11	3.1

ABI indicates ankle-brachial pressure index; TcO_2, transcutaneous oxygen; HL, hyperlipidemia; HT, hypertension; CaB, calcium blocker; APA, antiplatelet agent; ACEI, angiotensin-converting enzyme inhibitor; and ND, not detected.

BM-MNC Implantation

BM-MNCs were sorted and implanted in patients with limb ischemia as previously described.[2]

Effect of BM-MNC Implantation on Endothelial Function in Patients With Limb Ischemia

Leg vascular responses to ACh (Daiichi Pharmaceutical Co) and SNP (Maluishi Pharmaceutical Co) were evaluated by use of a mercury-filled Silastic strain-gauge plethysmograph (EC-5R, D.E. Hokanson, Inc) before and at 4 weeks after BM-MNC implantation in all subjects and at 24 weeks after BM-MNC implantation in 6 of the 7 subjects. Subjects fasted for at least 12 hours before cell implantation. They were kept in the supine position in a quiet, dark, air-conditioned room (temperature, 22°C to 25°C) throughout the study. A 23-gauge polyethylene catheter was inserted into the BM-MNC–implanted femoral artery for the infusion of ACh and SNP under local anesthesia. After each patient had spent 30 minutes in the supine position, we measured leg blood flow (LBF) and arterial blood pressure. Then, the effects of the ACh and SNP infusion on leg hemodynamics were measured. ACh (7.5, 15, and 30 μg/min) and SNP (0.75, 1.5, and 3.0 μg/min) were infused intra-arterially for 5 minutes at each dose. The infusions of ACh and SNP were performed in random order. Each study proceeded after the LBF had returned to baseline.

To evaluate the drug-related effect on endothelium-dependent vasodilation, the infusion of ACh and SNP was performed using a protocol identical to that used for the study of limb ischemic patients with implanted MN-MNCs before and after 4 weeks of follow-up in 5 patients with limb ischemia (4 men and 1 woman; mean age, 65±7 years) as a control group. Five patients were taking ACE inhibitors and antiplatelet agents; 3 of those 5 patients were taking calcium antagonists, and 2 were taking statins. The patients were subjected to 4 weeks of follow-up without any drug treatment or lifestyle modification.

Measurement of LBF

The blood flow was measured using a mercury-filled Silastic strain-gauge plethysmograph (EC-5R, D.E. Hokanson, Inc) as previously described.[6,7]

Statistical Analysis

Results are presented as the mean±SD. All reported probability values were 2-tailed. Values of $P<0.05$ were considered significant. Comparisons of parameters before and after BM-MNC implantation were performed with adjusted means by ANCOVA using baseline data as covariates. Comparisons of time-course curves of parameters during the infusions of ACh and SNP were analyzed by 2-way ANOVA for repeated measures on 1 factor followed by the Bonferroni correction for multiple-paired comparisons.

Results

Clinical Characteristics

The baseline clinical characteristics before and at 4 weeks and 24 weeks after BM-MNC implantation of patients with limb ischemia are summarized in the Table. The number of BM-MNCs implanted into ischemic limbs was $1.6\times10^9\pm0.3\times10^9$. The number of $CD34^+$ cells included in the implanted BM-MNCs was $3.8\times10^7\pm1.6\times10^7$. BM-MNC implantation improved the ankle-brachial pressure index from 0.33 ± 0.21 to 0.39 ± 0.17 after 4 weeks ($P=0.06$) and to 0.35 ± 0.38 after 24 weeks ($P=0.16$), transcutaneous oxygen pressure from 28.4 ± 11.5 to 36.6 ± 5.2 mm Hg after 4 weeks ($P=0.03$) and to 33.7 ± 3.8 mm Hg after 24 weeks ($P=0.06$), pain-free walking time from 0.8 ± 0.6 to 2.9 ± 2.2 minutes after 4 weeks ($P=0.02$) and to 2.5 ± 1.6 minutes after 24 weeks ($P=0.03$), and basal LBF from 1.7 ± 1.2 to 2.4 ± 1.4 mL/min per 100 mL tissue after 4 weeks ($P=0.04$) and to 2.0 ± 1.2 mL/min per 100 mL tissue after 24 weeks ($P=0.05$).

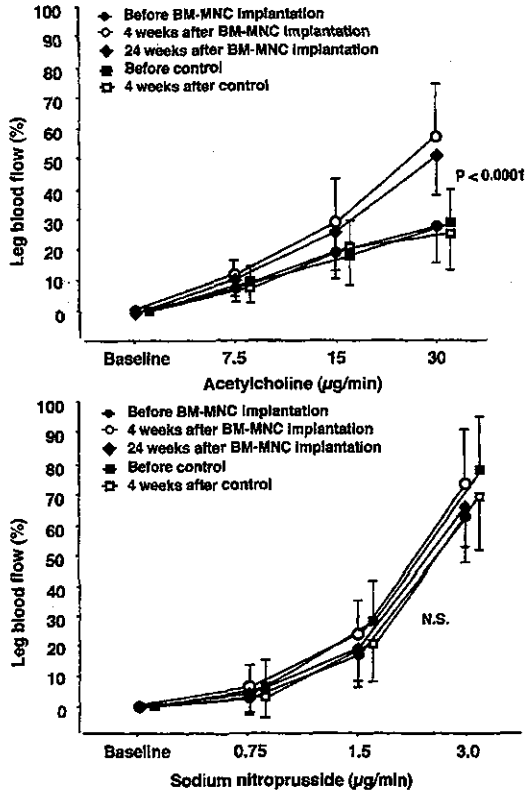
BM-NMC implantation did not alter blood pressures (mean blood pressure, from 86.2 ± 10.3 to 88.1 ± 11.2 mm Hg after 4 weeks and to 87.3 ± 12.1 mm Hg after 24 weeks) or serum concentrations of total cholesterol (from 5.28 ± 1.24 to 5.22 ± 1.06 mmol/L after 4 weeks and to 5.23 ± 1.18 mmol/L after 24 weeks), LDL cholesterol (from 3.88 ± 0.78 to 3.78 ± 0.72 mmol/L after 4 weeks and to 3.72 ± 0.81 mmol/L after 24 weeks), glucose (from 4.6 ± 0.4 to 4.5 ± 0.5 mmol/L after 4 weeks and to 4.6 ± 0.6 mmol/L after 24 weeks), and insulin (from 41.8 ± 9.8 to 42.3 ± 10.1 pmol/L after 4 weeks and to 43.6 ± 11.4 pmol/L after 24 weeks).

Effect of BM-MNC Implantation on Endothelial Function in Patients With Limb Ischemia

The intra-arterial infusion of ACh increased LBF in a dose-dependent manner. After BM-MNC implantation, LBF responses to ACh were enhanced in patients with limb ischemia (Figure, top). There was no significant difference in LBF response to ACh after 4 weeks and 24 weeks of follow-up (Figure, top). The intra-arterial infusion of SNP also increased LBF in a dose-dependent manner. The LBF response to SNP was unaffected by BM-MNC implantation (Figure, bottom). In the control group, there was no significant difference in LBF responses to ACh and SNP before and those after 4 weeks and 24 weeks of follow-up (Figure). No significant change was observed in arterial blood pressure or heart rate in response to intra-arterial infusion of either ACh or SNP before or after BM-MNC implantation and after 4 weeks of follow-up.

Discussion

In the present study, BM-MNC implantation improved not only limb ischemic symptoms and findings of angiography but also endothelium-dependent vasodilation in patients with limb ischemia. This beneficial effect of BM-MNC implantation on vascular function may be selective in endothelium-dependent vasodilation (endothelial cell function) but not in endothelium-independent vasodilation (smooth muscle cell function).



Comparison of LBF (as % change from basal flow) response to ACh administration (top) and SNP administration (bottom) before and after BM-NMC implantation of 4 weeks and 24 weeks of follow-up in patients with limb ischemia.

Our results showed that BM-MNC implantation increased the ankle-brachial pressure index, transcutaneous oxygen pressure, and basal LBF per se. Therefore, one possible mechanism by which BM-MNC implantation augments endothelium-dependent vasodilation is by increasing shear stress results from blood flow. Acute or chronic increases in shear stress stimulate the release of nitric oxide in isolated vessels and cultured cells through the enhanced expression of endothelial nitric oxide synthase gene.[8,9]

BM-MNCs ($CD34^+$ fraction) include endothelial progenitor cells and various angiogenetic growth factors, such as the vascular endothelial growth factor (VEGF) and angiopoietin families. Supplementation of the progenitor endothelial cells results in augmentation of neovascularization of ischemic tissue and repair of mature endothelial cells that release nitric oxide.[10] VEGF induces the formation of collateral vessels and increases collateral blood flow, leading to improvement in endothelium-dependent vasodilation.[11] In addition, VEGF directly upregulates endothelial nitric oxide synthase expression and increases subsequent nitric oxide release.[12] Rajagopalan et al[5] recently reported that gene therapy using an adenoviral vector encoding a 121-amino-acid isoform of VEGF augmented ACh-induced vasodilation in lower-leg circulation in patients with peripheral arterial disease. Although the mechanism by which BM-MNC implantation improves endothelial function in patients with limb ischemia is not clear, the multiplier effect of progenitor endothelial cells and VEGF may contribute to the angiogenesis-induced improvement in endothelium-dependent vasodilation.

We have recently shown that antihypertensive agents, such as ACE inhibitors, restore endothelial function in patients with mild to moderate hypertension but not in patients with severe hypertension.[13] It is clinically important that endothelial dysfunction is reversible by BM-MNC implantation in patients with severe atherosclerosis. BM-MNC implantation is expected to prevent the development of atherosclerosis through improvement in endothelial function.

Although a drastic change in endothelial function was observed after BM-MNC implantation, the number of subjects in this study was small, and the observation period is relatively short. In addition, this phase 1 clinical trial was not placebo-controlled. Controlled studies using a large population of patients and with long observation periods are needed to determine the role of BM-MNC implantation in endothelial function in patients with severe atherosclerosis.

Although the effectiveness of therapeutic angiogenesis with VEGF gene therapy in patients with peripheral arterial diseases has been established, BM-MNC implantation therapy may provide a new aspect of therapeutic angiogenesis in such patients.

References

1. Shintani S, Murohara T, Ikeda H, et al. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. *Circulation.* 2001;103:897–895.
2. Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomized controlled trial. *Lancet.* 2002;360:427–435.
3. Ross R. The pathogenesis of atherosclerosis. *N Engl J Med.* 1999;340: 115–126.
4. Boger RH, Bode-Boger SM, Brandes R, et al. Biochemical evidence for impaired nitric oxide synthase in patients with peripheral arterial occlusive disease. *Circulation.* 1997;95:2068–2074.
5. Rajagopalan S, Shah M, Luciano A, et al. Adenovirus-mediated gene transfer of $VEGF_{121}$ improves lower-extremity endothelial function and flow reserve. *Circulation.* 2001;104:753–755.
6. Panza JA, Quyyumi AA, Brush JE Jr, et al. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med.* 1990;323:22–27.
7. Higashi Y, Sasaki S, Nakagawa K, et al. Endothelial function and oxidative stress in renovascular hypertension. *N Engl J Med.* 2002;346: 1954–1962.
8. Miller VM, Vanhoutte PM. Enhanced release of endothelium-derived factors by chronic increases in blood flow. *Am J Physiol.* 1988;255: H446–H451.
9. Uematsu M, Ohara Y, Navas JP, et al. Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *Am J Physiol.* 1995; 269:C1371–C1378.
10. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997;275:964–967.
11. Bauters C, Asahara T, Zheng LP, et al. Recovery of disturbed endothelium-dependent flow in the collateral-perfused rabbit ischemic hindlimb after administration of vascular endothelial growth factor. *Circulation.* 1995;91:2802–2809.
12. Shen B-Q, Lee DY, Zioncheck TZ. Vascular endothelial growth factor governs endothelial nitric-oxide synthase expression via a KDR/FLK-1 receptor and a protein kinase C signaling pathway. *J Biol Chem.* 1999; 274:33057–33063.
13. Higashi Y, Sasaki S, Nakagawa K, et al. The severity of hypertension affects improved resistance artery endothelial function by angiotensin converting enzyme inhibition. *J Cardiovasc Pharmacol.* 2002;39: 668–676.

Geriatrics and Gerontology International 2004; 4: 151–156

ORIGINAL ARTICLE

Strategy for treating elderly Japanese with hypercholesterolemia*

Hisanori Horiuchi,[1] Yuji Matsuzawa,[2] Hiroshi Mabuchi,[3] Hiroshige Itakura,[4] Jun Sasaki,[5] Mitsuhiro Yokoyama,[6] Yuichi Ishikawa,[7] Shinji Yokoyama,[8] Seijiro Mori,[9] Takashi Ohrui,[10] Masahiro Akishita,[11] Toshio Hayashi,[12] Kiminori Yamane,[13] Genshi Egusa[14] and Toru Kita[15]

[1]Departments of Geriatric Medicine and [15]Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, [2]Department of Internal Medicine, The Sumitomo Hospital, Osaka, [3]The Second Department of Internal Medicine, School of Medicine, Kanazawa University, Kanazawa, [4]Ibaraki Christian University, Ibaraki, [5]International University Graduate School of Health and Welfare, Fukuoka, [6]Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine and [7]Faculty of Health Sciences, Kobe University School of Medicine, Kobe, [8]Department of Biochemistry, Cell Biology and Metabolism, Nagoya City University Graduate School of Medical Sciences, and [12]Department of Geriatrics, Nagoya University Graduate School of Medicine, Nagoya, [9]Department of Internal Medicine, Matsudo Municipal Hospital, Matsudo, [10]Department of Geriatric and Respiratory Medicine, Tohoku University School of Medicine, [11]Department of Geriatric Medicine, Kyorin University School of Medicine, Mitaka, [13]The Second Department of Internal Medicine, Hiroshima University School of Medicine, and [14]The Egusa Genshi Clinic, Hiroshima, Japan

Background: It has been widely accepted that control of serum cholesterol levels is effective for prevention of cardiovascular events. Recent data have suggested that this is also the case in the elderly.

Methods: A research group (chaired by T. Kita) was organized as part of the Comprehensive Research on Aging and Health conducted by the Japanese Ministry for Health, Labour, and Welfare in 1999–2002 to determine the best strategy for control of cholesterol levels in elderly Japanese with hypercholesterolemia. In order to do this a review of the literature was conducted.

Conclusion: The research group concluded: (i) Japanese patients aged 65–74 years with hypercholesterolemia should be treated by following the Guideline for Diagnosis and Treatment of Atherosclerotic Cardiovascular Diseases by the Japan Atherosclerosis Society (2002), as cholesterol-lowering therapy would bring a similar, or even larger, preventive effect to the elderly, whose absolute risk of cardiovascular events is higher than that in the younger population; (ii) target cholesterol levels in elderly Japanese aged ≥ 75 years with

Accepted for publication 17 February 2004.

Correspondence: Dr Toru Kita, Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, 606–8507, Japan. Email: tkita@kuhp.kyoto-u.ac.jp

*Based on the report of the Research Group for 'Long-term prognosis of elderly Japanese with hypercholesterolemia' in the Comprehensive Research on Aging and Health.