described above was 1.2 pg per 100 μg AGE in 1 mL (determined by SRL, Inc., Okayama, Japan). AGE-specific fluorescence was measured at 450 nm after excitation at 390 nm with a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Dibutyryl cAMP (dbcAMP) and forskolin were purchased from Wako Co., Ltd. (Tokyo, Japan). H89 was purchased from Sigma Chemical.

## Results

*The effects of histamine on AGE-2- and AGE-3-induced expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and lymphocyte proliferation during MLR*

In a previous study, to evaluate the binding of AGE subtypes to RAGE, we established an *in vitro* assay using immobilized

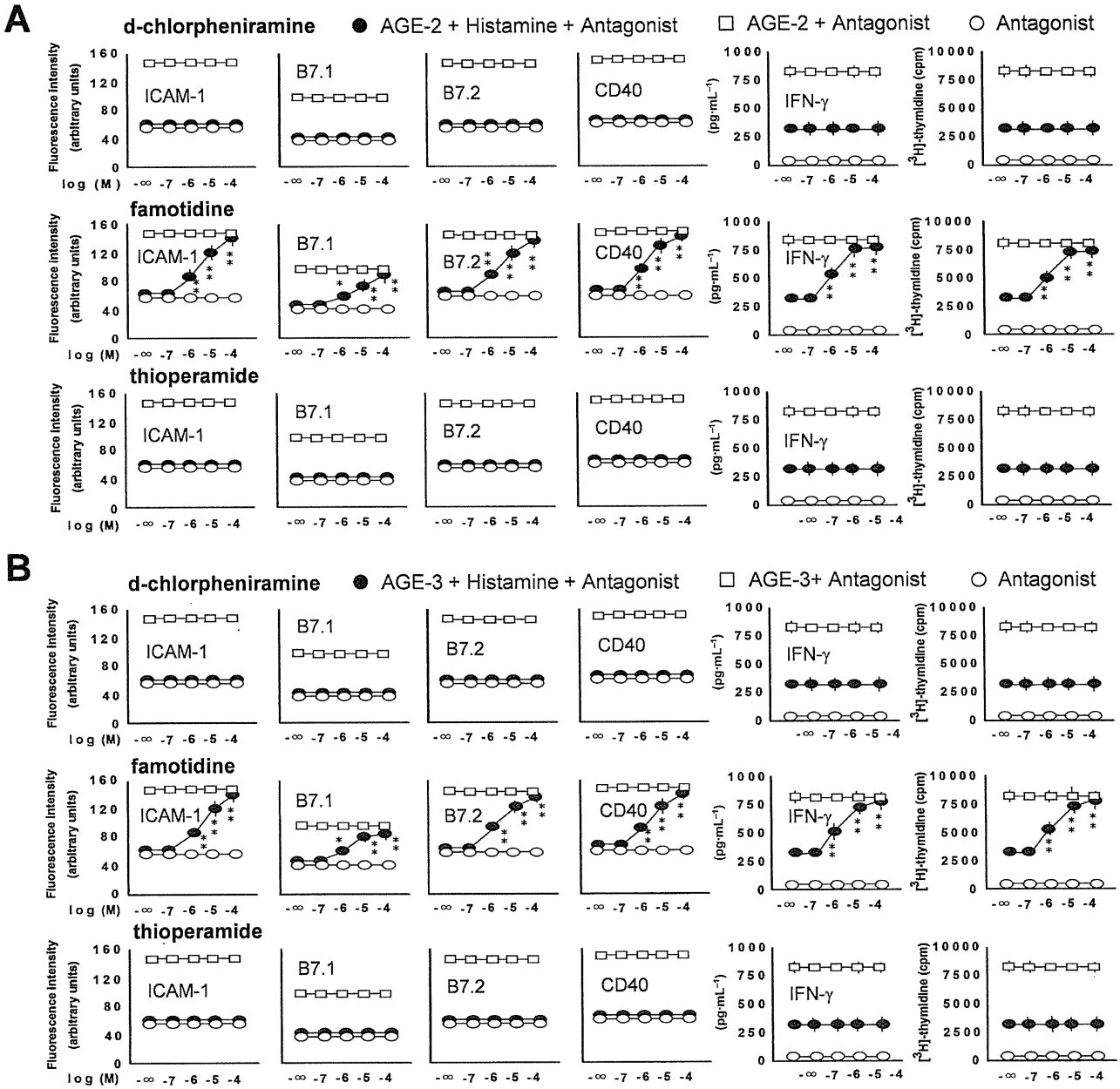


**Figure 2** The effects of histamine receptor antagonists on the actions of histamine. Mixed cells at $2 \times 10^6$ cells·mL$^{-1}$ were incubated with different classes of histamine receptor antagonists, including *d*-chlorpheniramine ($H_1$ antagonist), famotidine ($H_2$ antagonist) and thioperamide ($H_3/H_4$ antagonist), at increasing concentrations from 0.1 to 100 μM. The antagonists were used alone (antagonist), with advanced glycation end product (AGE)-2 or AGE-3 (100 μg·mL$^{-1}$; AGE + antagonist) or with AGEs and histamine (100 μM; AGE + Hist + Ant). The expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. Interferon (IFN)-γ concentration in conditioned media was determined by ELISA. Lymphocyte proliferation was determined by [$^3$H]-thymidine uptake The results are expressed as the means ± SEM of five donors with triplicate determinations. $^*P < 0.05$; $^{**}P < 0.01$ compared with the value for histamine. When an error bar is within a symbol, the bar is omitted.

AGE subspecies and His-tagged soluble RAGE (sRAGE) protein (Takahashi *et al.*, 2009). AGE-2 and AGE-3 showed relatively high affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed moderate affinity for sRAGE. To determine the appropriate incubation time, we examined the kinetics at 0, 4, 16, 24, 48 and 72 h. AGE-2 and AGE-3 (100 $\mu g \cdot mL^{-1}$) induced the expression of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and the proliferation during MLR at 16 h and thereafter up to 24 and 48 h, whereas AGE-4, AGE-5 and BSA at the same concentration had no effect (Ohashi *et al.*, 2010). Moreover, to determine the appropriate concentration of AGEs, the effects of AGE-2 and AGE-3 at concentrations ranging from 100 $ng \cdot mL^{-1}$ to 100 $\mu g \cdot mL^{-1}$ for 48 h were examined. AGE-2 and AGE-3 at 1, 10 and 100 $\mu g \cdot mL^{-1}$ significantly induced the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and proliferation during MLR (Ohashi *et al.*, 2010).

As shown in Figure 1, we observed the effects of histamine (0.1–100 μM) on AGE-enhanced expression of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and lymphocyte proliferation, using 100 $\mu g \cdot mL^{-1}$ AGE-2 or AGE-3 for 48 h in PBMC and MLR. The expression levels of ICAM-1, B7.1, B7.2 and CD40, production of IFN-γ and lymphocyte proliferation were higher during the MLR than in PBMCs cultured alone. Histamine concentration dependently inhibited the effect of the AGEs on adhesion molecule expression, cytokine production and lymphocyte proliferation in both cell systems. $IC_{50}$ values for the inhibitory effect of histamine in the presence of AGE-2 during MLR were 2, 2, 2, 1.5, 1.5 and 1.5 μM, and those in the presence of AGE-3 were 2, 2, 2, 1.5, 1.5 and 1.5 μM respectively. Incubation with histamine alone had no effect on the variables we measured in PBMC or in the MLR (data not shown).

*The involvement of $H_2$ receptors in the actions of histamine*

To determine the histamine receptor subtypes involved in the effects of histamine on these cellular responses to AGE-2 and AGE-3, the effects of an $H_1$-receptor antagonist, we used a range of histamine receptor antagonists, *d*-chlorpheniramine, an $H_2$-receptor antagonist, famotidine, and an $H_3/H_4$-receptor antagonist, thioperamide, at concentrations ranging from 0.1–100 μM. In these experiments, we used a fixed concentration of histamine (100 μM). As shown in Figure 2, among these histamine receptor antagonists, only famotidine inhibited the effects of histamine. Another $H_2$-receptor antagonist, ranitidine, exerted a substantially similar effect to famotidine (data not shown). As shown in Figure 3, the effects of the $H_2/H_4$-receptor agonists, dimaprit and 4-methylhistamine (Parsons *et al.*, 1977), at concentrations ranging from 0.1 to

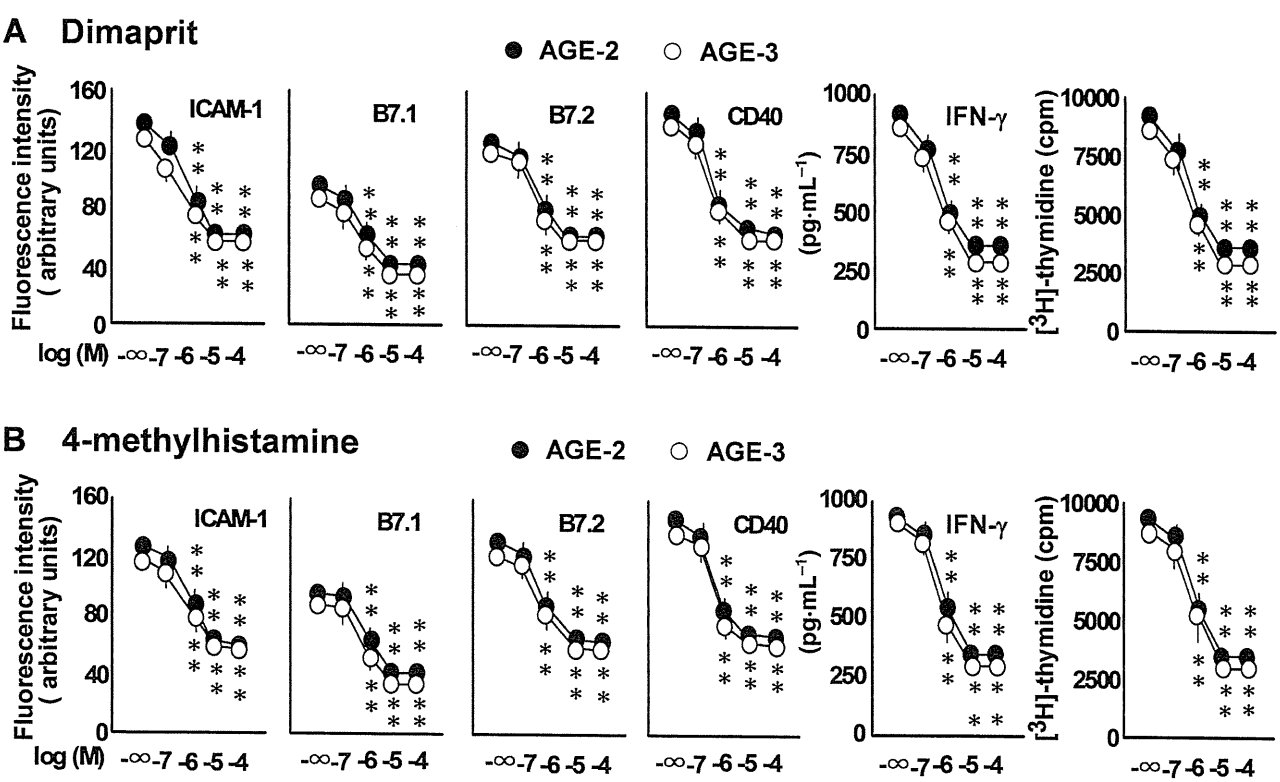


**Figure 3** The effects of histamine receptor agonists on the advanced glycation end product (AGE)-2- and AGE-3-induced expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40, the production of interferon (IFN)-γ and lymphocyte proliferation. Mixed cells at $2 \times 10^6$ $cells \cdot mL^{-1}$ were incubated with histamine $H_2/H_4$-receptor agonists, dimaprit (A) and 4-methylhistamine (B) at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 (100 $\mu g \cdot mL^{-1}$; 48 h). The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry, and IFN-γ concentration in the conditioned media was determined by ELISA. Lymphocyte proliferation was determined by [$^3$H]-thymidine uptake The results are expressed as the means ± SEM of five donors with triplicate determinations. $^{**}P < 0.01$ compared with the value for AGE-2 or AGE-3 alone. When an error bar is within a symbol, the bar is omitted.

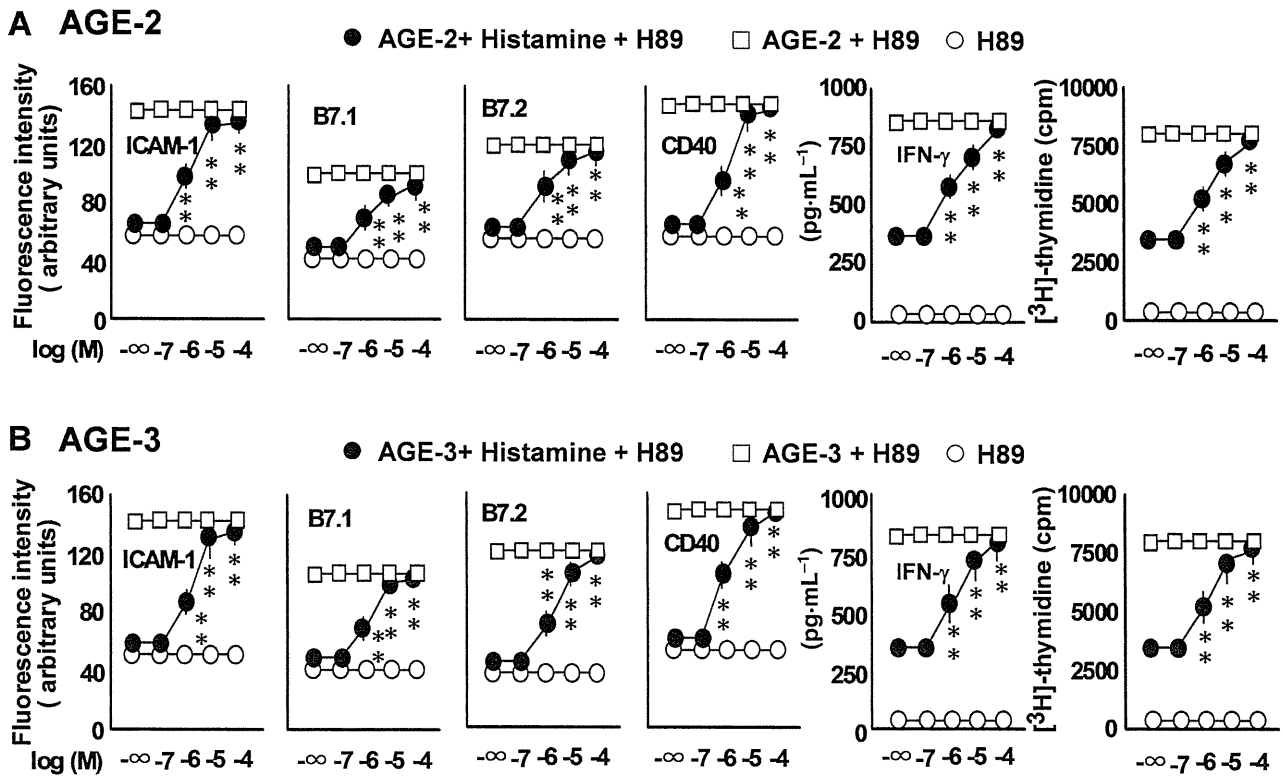


**Figure 4** The effects of the protein kinase A (PKA) inhibitor, H89, on the histamine-induced down-regulation of expression of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40, the production of interferon (IFN)-γ and lymphocyte proliferation. Mixed cells at $2 \times 10^6$ cells·mL$^{-1}$ were incubated with a PKA inhibitor, H89, (0.1–100 μM) in the presence of advanced glycation end product (AGE)-2 (A) and AGE-3 (B) (100 μg·mL$^{-1}$) and histamine (100 μM) for 48 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry, and IFN-γ concentration in the conditioned media was determined by ELISA. Lymphocyte proliferation was determined by [$^3$H]-thymidine uptake. The results are expressed as the means ± SEM of triplicate findings from five donors. $^{**}P < 0.01$ compared with the value in the presence of histamine and AGEs. When an error bar is within a symbol, the bar is omitted.

100 μM were determined in the presence of AGE-2 or AGE-3 (100 μg·mL$^{-1}$). Both agonists inhibited the expression of adhesion molecules, the production of IFN-γ and lymphocyte proliferation in a concentration-dependent manner. The potency and efficacy of the two agonists were quite similar to those of histamine in each response. Moreover, we found that an $H_1$ agonist, 2-(2-pyridyl)ethylamine dihydrochloride (Durant *et al.*, 1975) and an $H_3$ agonist (*R*)-α-methylhistamine dihydrochloride (Arrang *et al.*, 1987) had no effect on the responses to AGE-2 and AGE-3 in our system (data not shown).

*The involvement of cAMP in the action of histamine*

To investigate the involvement of the cAMP/PKA pathway in the action of histamine, the effects of a PKA inhibitor, H89 (0.1–100 μM), on the action of histamine (100 μM) were determined (Figure 4). In the absence of histamine, the PKA inhibitor had no effect on adhesion molecule expression, cytokine production and lymphocyte proliferation, but it did reverse the inhibitory effect of histamine on these responses to AGE-2 or AGE-3. We also assessed the effects of a membrane-permeable cAMP analogue, dbcAMP, and an adenylate cyclase activator, forskolin (0.1–100 μM), on these responses. As shown in Figure 5, both dbcAMP and forskolin inhibited AGE-2- and AGE-3-induced adhesion molecule expression, cytokine production and lymphocyte proliferation in a concentration-dependent manner.

## Discussion

The levels of AGE-2 are about 17 μg·mL$^{-1}$ in the serum of diabetic patients (Enomoto *et al.*, 2006; Nakamura *et al.*, 2007). AGEs at concentrations ranging from 50 to 200 μg·mL$^{-1}$ markedly induced human monocyte adhesion to bovine retinal endothelial cells (Mamputu and Renier, 2004). AGEs at 200 μg·mL$^{-1}$ induced the expressions of CD40, CD80 and CD86 and the production of IFN-γ in dendritic cells (Ge *et al.*, 2005). In the previous study, we found that AGE-2 and AGE-3 over a range of concentrations (1–100 μg·mL$^{-1}$) significantly up-regulated the expression of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and lymphocyte proliferation during MLR (Ohashi *et al.*, 2010). Therefore, the concentration (100 μg·mL$^{-1}$) used in the present study may not be far above the pathological concentration of AGEs in the serum of diabetic patients previously reported (Enomoto *et al.*, 2006; Nakamura *et al.*, 2007).

In the present study, we clearly demonstrated for the first time that histamine inhibited AGE-2- and AGE-3-induced

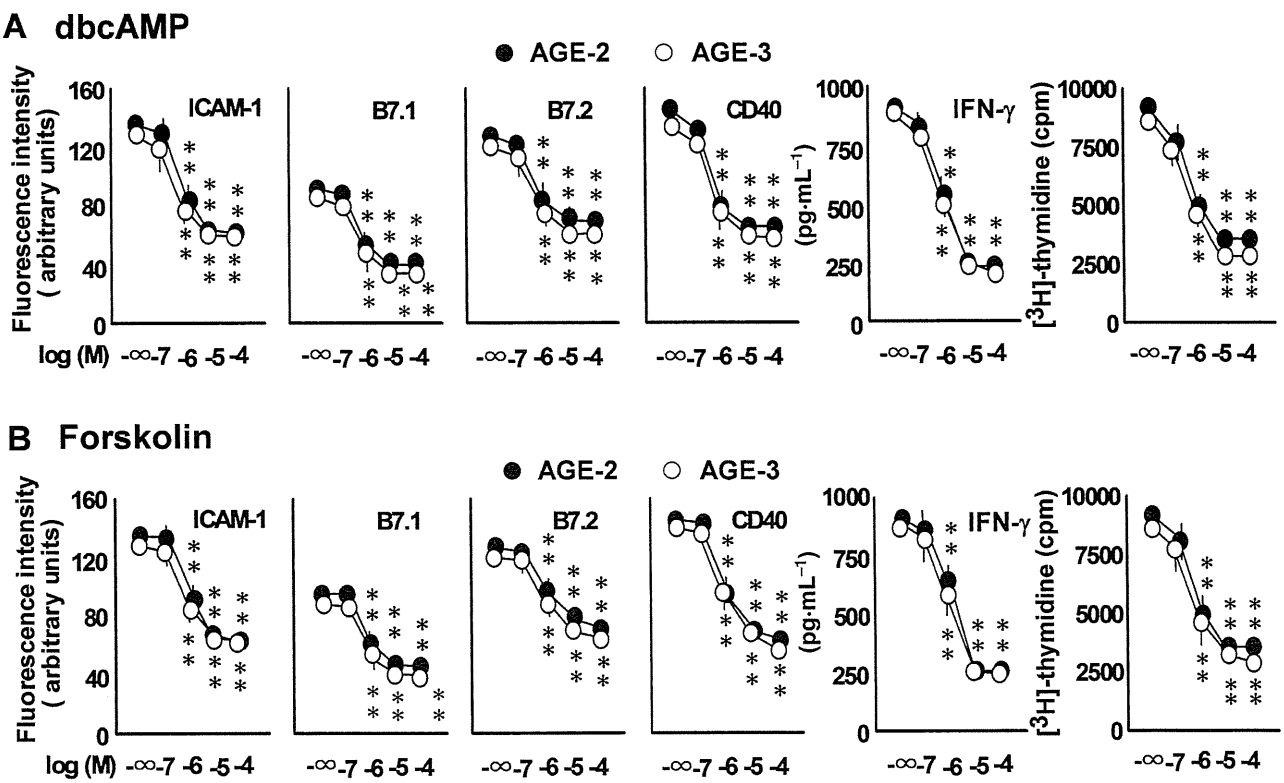


**Figure 5** The effects of forskolin and dibutyryl cAMP (dbcAMP) on advanced glycation end product (AGE)-induced expression of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40, the production of interferon (IFN)-γ and lymphocyte proliferation. Mixed cells at $2 \times 10^6$ cells·mL$^{-1}$ were incubated with a cAMP analogue, dbcAMP (A), or an adenylate cyclase activator, forskolin (B), at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 (100 μg·mL$^{-1}$; 48 h). The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry, and IFN-γ concentration in the conditioned media was determined by ELISA. Lymphocyte proliferation was determined by [$^3$H]-thymidine uptake The results are expressed as the means ± SEM of five donors with triplicate determinations. $^{**}P < 0.01$ compared with the value for AGE-2 or AGE-3 alone. When an error bar is within a symbol, the bar is omitted.

expressions of ICAM-1, B7.1, B7.2 and CD40 on human monocytes, the production of IFN-γ and lymphocyte proliferation during MLR (Figure 1). The action of histamine was inhibited by an $H_2$-receptor antagonist, famotidine, but not an $H_1$-receptor antagonist, *d*-chlorpheniramine, or an $H_3/H_4$ antagonist, thioperamide (Figure 2). The histamine $H_2/H_4$-receptor agonists dimaprit and 4-methylhistamine mimicked the action of histamine (Figure 3). As the $IC_{50}$ values of histamine and the $H_2/H_4$-receptor agonists to prevent the up-regulation of adhesion molecule expression and cytokine production were consistent with the affinity of those agonists to typical histamine $H_2$ receptors (Elenkov *et al.*, 1998; Itoh *et al.*, 2002; Wake *et al.*, 2009), it was concluded that the inhibitory effect of histamine was mediated by the stimulation of $H_2$ receptors rather than the $H_1$, $H_3$ and $H_4$ receptors for histamine.

In the previous study, we found that histamine induced the production of cAMP in monocytes via $H_2$ receptors (Wake *et al.*, 2009). Here we found that the PKA inhibitor, H89, inhibited the action of histamine (Figure 4) and that the cAMP analogue, dbcAMP, and the adenylate cyclase activator, forskolin, mimicked the effect of histamine (Figure 5), suggesting the involvement of the cAMP/PKA pathway in these actions of histamine. We observed a similar pattern of inhibitory effects of histamine on IL-18-induced activation of monocytes during MLR via $H_2$ receptors (Itoh *et al.*, 2002). Thus, a common pathway triggered by IL-18 and AGEs may be regulated by the $H_2$-receptor cAMP/PKA system. Further work is necessary on this issue.

Although histamine alone had no effect on basal levels of the adhesion molecules, the production of IFN-γ and lymphocyte proliferation during MLR, that is, in the absence of AGE-2 and AGE-3, dbcAMP and forskolin inhibited these basal responses (data not shown). Moreover, none of the histamine receptor antagonists affected this lack of response to histamine alone, and no histamine receptor agonists altered the basal responses (data not shown). These results indicated that histamine used alone inhibited cAMP-initiated regulation during the MLR, in a histamine receptor-independent manner.

It is known that PTDM is a common side effect of cyclosporin A and tacrolimus (Marchetti, 2005). Many patients develop diabetes mellitus early after transplantation when they are exposed to tacrolimus and steroids (Filler *et al.*, 2000; van Hooff *et al.*, 2004). The cause of impaired graft survival in PTDM patients depends on the use of lower dosages of immunosuppressive agents in diabetic patients. The accumulation of AGEs was elevated in recipients with chronic renal dysfunction and cardiovascular disease after renal transplantation (Hartog *et al.*, 2006). Immunological

rejection and ischaemia was reported as mechanisms of the induction of mast cell infiltration in renal allograft (Shoskes *et al.*, 1990). The relation between mast cells and rejection has been described in the heart (Ly *et al.*, 1992), lung (Yousem, 1997), intestine (Walgenbach *et al.*, 1996), graft-versus-host disease (Nagler *et al.*, 1995) and acute cellular rejection of the human kidney (Lajoie *et al.*, 1996). However, little is known about the effect of histamine and $H_2$-receptor stimulation on AGE-induced monocyte activation during MLR.

In conclusion, histamine inhibited the AGE-2- and AGE-3-induced expression of the adhesion molecules ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and lymphocyte proliferation, via histamine $H_2$ receptors and the cAMP/PKA pathway. Through the inhibition of toxic AGE-induced responses in monocytes, the stimulation of histamine $H_2$ receptors may partially contribute to down-regulating the immune response in patients with PTDM.

## Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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DOI:10.1111/j.1476-5381.2010.00880.x
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RESEARCH PAPER

# Ciprofloxacin inhibits advanced glycation end products-induced adhesion molecule expression on human monocytes

S Mori[1], HK Takahashi[2], K Liu[2], H Wake[2], J Zhang[2], R Liu[2], T Yoshino[3] and M Nishibori[2]

[1]*School of Pharmacy, Shujitsu University, Okayama, Japan, Departments of* [2]*Pharmacology, and* [3]*Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*

**Correspondence**
Dr Hideo Kohka Takahashi, Department of Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama, 700-8558, Japan. E-mail: kohka611@md.okayama-u.ac.jp

**Keywords**
advanced glycation end products; ciprofloxacin; cyclooxygenase-2; cyclic adenosine monophosphate; prostaglandins E2; protein kinase A; adhesion molecule; peripheral blood mononuclear cells; human; monocytes

**Received**
6 November 2009
**Revised**
14 April 2010
**Accepted**
19 April 2010

## BACKGROUND AND PURPOSE
Advanced glycation end products (AGEs) subtypes, proteins or lipids that become glycated after exposure to sugars, can induce complications in diabetes. Among the various AGE subtypes, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3) are involved in inflammation in diabetic patients; monocytes are activated by these AGEs. Ciprofloxacin (CIP), a fluorinated 4-quinolone, is often used clinically to treat infections associated with diabetis due to its antibacterial properties. It also modulates immune responses in human peripheral blood mononuclear cells (PBMC) therefore we investigated the involvement of AGEs in these effects.

## EXPERIMENTAL APPROACH
Expression of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 was examined by flow cytometry. The production of tumour necrosis factor (TNF)-α, interferon (IFN)-γ, prostaglandin $E_2$ ($PGE_2$) and cAMP were determined by enzyme-linked immunosorbent assay. Cyclooxygenase (COX)-2 expression was determined by Western blot analysis. Lymphocyte proliferation was determined by [$^3$H]-thymidine uptake.

## KEY RESULTS
CIP induced $PGE_2$ production in monocytes, irrespective of the presence of AGE-2 and AGE-3, by enhancing COX-2 expression; this led to an elevation of intracellular cAMP in monocytes. Non-selective and selective COX-2 inhibitors, indomethacin and NS398, inhibited CIP-induced $PGE_2$ and cAMP production. In addition, CIP inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40 in monocytes, the production of TNF-α and IFN-γ and lymphocyte proliferation in PBMC. Indomethacin, NS398 and a protein kinase A inhibitor, H89, inhibited the actions of CIP.

## CONCLUSIONS AND IMPLICATIONS
CIP exerts immunomodulatory activity via $PGE_2$, implying therapeutic potential of CIP for the treatment of AGE-2- and AGE-3-induced inflammatory responses.

## Abbreviations
AGEs, advanced glycation end products; AH6809, 6-isopropoxy-9-oxaxanthene-2-carboxylic acid; AH23848, (4Z)-7-[(rel-1S,2S,5R)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid; BSA, bovine serum albumin; CIP, ciprofloxacin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; H-89, N-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulphonamide dihydrochloride; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; m, monoclonal; PBMC, peripheral blood mononuclear cells; $PGE_2$, prostaglandins $E_2$; PKA, protein kinase A; RAGE, receptor for AGEs; TNF, tumour necrosis factor

## Introduction

It is known that advanced glycation end products (AGEs) are products of the non-enzymatic glycation of proteins/lipids that accumulate during natural aging and are also greatly augmented in disorders such as diabetes, renal failure and Alzheimer's disease (Schmidt *et al.*, 1994; Brownlee, 1995; Takedo *et al.*, 1996). The formation and accumulation of AGEs occur at an accelerated rate in diabetic patients and may participate in the pathogenesis of diabetic microvascular and macrovascular complications (Bierhaus *et al.*, 1998; Fukami *et al.*, 2004). Direct immunochemical evidence for the existence of some AGE structures was provided within AGE-modified proteins and peptides (Takeuchi and Yamagishi, 2004). Among the various subtypes of AGE, it has been shown that toxic AGE structures, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3), are the main AGE structures detectable in the serum of diabetic patients. AGE-2 plays a role in the development of atherosclerosis (Takeuchi *et al.*, 2000). The interaction between AGEs and the receptor for AGEs (RAGE) perturbs a variety of vascular homeostatic functions, and thus may contribute to diabetic vasculopathy (Schmidt *et al.*, 1994; Park *et al.*, 1998). AGEs and RAGE are detected in atherosclerotic plaque of diabetic patients (Cuccurullo *et al.*, 2006). A recent study reported that RAGE expression is associated with the apoptotis of smooth muscle cells and macrophages, suggesting that RAGE may promote plaque destabilization (Burke *et al.*, 2004). AGEs up-regulate RAGE expression in various tissues, facilitating the AGE–RAGE response by forming a positive feedback loop (Yamagishi and Imaizumi, 2005). In a previous study, we found that AGE-2 and AGE-3 induced the expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes, the production of tumour necrosis factor (TNF)-α and interferon (IFN)-γ, and lymphocyte proliferation in human peripheral blood mononuclear cells (PBMC), but AGE-4 and AGE-5 had no effect (Takahashi *et al.*, 2009a). The effect of AGE-2 and AGE-3 on the production of IFN-γ and TNF-α was mediated by RAGE and dependent on cell-to-cell interaction via the engagement between ICAM-1, B7.1, B7.2 and CD40 on monocytes and their ligands on T-cells (Wake *et al.*, 2009; Takahashi *et al.*, 2009a).

A major product of cyclooxygenase (COX)-initiated arachidonic acid metabolism, prostaglandin $E_2$ ($PGE_2$), which is released from antigen-presenting cells, primes naive human T-cells and enhances production of anti-inflammatory cytokines while inhibiting synthesis of pro-inflammatory cytokines (Coleman *et al.*, 1994). Among the four $PGE_2$ receptor subtypes E-prostanoid $(EP)_1$, $EP_2$, $EP_3$ and $EP_4$, activation of the $EP_2$ and $EP_4$ receptors leads to an increase in cAMP levels and protein kinase A (PKA) activity (Bastien *et al.*, 1994). It has been reported that the enhanced expression of adhesion molecules, including ICAM-1, B7.1, B7.2 and CD40, on monocytes results in the enhanced activation of T-cells (Durie *et al.*, 1994; Ranger *et al.*, 1996; Camacho *et al.*, 2001). We also found that cell-to-cell interactions mediated by the engagement between ICAM-1, B7.1, B7.2 and CD40, respectively, on monocytes and their ligands on T-cells were involved in T-cell activation, inducing the production of IFN-γ and TNF-α in PBMC (Takahashi *et al.*, 2003). In a previous study, we found that $PGE_2$ inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation via $EP_2$ and $EP_4$ receptors (Takahashi *et al.*, 2009b).

Fluoroquinolone antibacterial agents are known to exert their bactericidal activity by inhibiting bacterial type II topoisomerases, a major component of mitotic chromosomes. It has been well documented that this group of agents have the immunomodulatory effects (Riesbeck, 2002). The synthesis of interleukin (IL)-1β and TNF-α by lipopolysaccharide-stimulated human monocytes is significantly inhibited by ciprofloxacin (CIP) (Riesbeck and Forsgren, 1990). Recently, we found that CIP induced the production of $PGE_2$ in monocytes in a concentration-dependent manner, regardless of the presence of IL-18, by enhancing the expression of COX-2 protein and the elevation of intracellular cAMP in monocytes (Takahashi *et al.*, 2005).

In the present study, we examined the effects of CIP on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation in human PBMC to better understand the immunomodulatory effects of CIP.

## Methods

### *Isolation of PBMC and monocytes*

Normal human PBMC were obtained from 10 healthy volunteers after acquiring Institutional Review Board (IRB) approval (Okayama Univ. IRB No.106). Samples of 20–50 mL peripheral blood were withdrawn from a forearm vein; after which, PBMC were prepared and monocytes isolated from PBMC were separated by counterflow centrifugal elution as previously described (Takahashi *et al.*, 2003). PBMC and monocytes were then suspended at a final concentration of

$1 \times 10^6$ cells·mL$^{-1}$ in the medium as previously described (Takahashi *et al.*, 2003).

### *Flow cytometric analysis for adhesion molecule expression*

Changes in the expressions of human leukocyte antigens, ICAM-1, B7.1, B7.2, CD40 and CD40L, on monocytes were examined by multicolour flow cytometry using a combination of anti-CD14 Ab with anti-ICAM-1, anti-B7.1, anti-B7.2, anti-CD40 or anti-CD40L Ab. PBMC at $1 \times 10^6$ cells·mL$^{-1}$ were incubated for 24 h. Cultured cells at $5 \times 10^5$ cells·mL$^{-1}$ were prepared for flow cytometric analysis as previously described (Takahashi *et al.*, 2003) and analysed with a FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were processed using the CELL QUEST program.

### *Enzyme-linked immunosorbent assay*

PBMC at $1 \times 10^6$ cells·mL$^{-1}$ were used to analyse IFN-γ and TNF-α production, and monocytes at $1 \times 10^6$ cells mL$^{-1}$ were used to analyse $PGE_2$ production. After being cultured for 24 h at 37°C in a 5%$CO_2$/air mixture, the cell-free supernatant was assayed for IFN-γ, TNF-α (R&D Systems, Minneapolis, MN, USA) and $PGE_2$ protein (Cayman Chemical, Ann Arbor, MI, USA) by enzyme-linked immunosorbent assay (ELISA) employing the multiple Abs sandwich principle. The detection limits of ELISA for IFN-γ, TNF-α and $PGE_2$ were 10 pg·mL$^{-1}$.

### *Proliferation assay*

PBMC were treated under various conditions. Cultures were incubated for 48 h, during which they were pulsed with [$^3$H]-thymidine (3 Ci per well) for the final 16 h. Cells were then divided into 96-well microplates, 200 μL per well, resulting in 1 μCi [$^3$H]-thymidine per well, and harvested by the Micro-Mate 196 Cell Harvester (Perkin Elmer Life Science Inc., Boston, MA, USA). Thymidine incorporation was measured by a beta-counter (Matrix 9600; Perkin Elmer Life Science Inc.).

### *Western immunoblotting*

Monocytes at $1 \times 10^6$ cells·mL$^{-1}$ were incubated with CIP in the presence or absence of AGE-2 and AGE-3 at 37°C in a 5% $CO_2$–air mixture for 30 min. After the incubation, the cells were washed twice in phosphate-buffered saline before the addition of 60 mL ice-cold lysis buffer (HEPES-buffered Hank's balanced salt solution, pH 7.4, 0.5% Triton X-100, 10 mg·mL$^{-1}$ leupeptin, 10 mg·mL$^{-1}$ aprotinin) and 60 μL sample buffer (0.125 M Trizma base, pH 6.8, 20% glycerol, 4% sodium dodecyl sulphate, 10% 2-mercaptoethanol). The samples were then heated at 95°C for 7 min before being stored at 20°C. Sample proteins (50 μL per lane) were separated on 9% acrylamide gel and transferred onto Trans-Blot membranes at 4°C for 16 h at 300 mA; after which, the membranes were blocked for 1 h at 25°C in Tris-buffered saline (25 mM Tris–HCl, 0.2 M NaCl, 0.15% Tween 20, pH 7.6) containing 5% dried milk (wt. vol$^{-1}$). Next, the membranes were treated with horseradish peroxidase-conjugated rabbit polyclonal Ab against human COX-2 (Cayman Chemical) and β-actin (Sigma Chemical).

### *Measurement of cAMP production in monocytes*

Monocytes at $1 \times 10^6$ cells·mL$^{-1}$ were incubated at 37°C in a 5%$CO_2$/air mixture. After 1 h, cells at $2 \times 10^5$ cells 200 μL$^{-1}$ per well were supplemented with trichloroacetic acid to a final concentration of 5% and 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase at 100 μM, and frozen at –80°C. Frozen samples were subsequently sonicated and assayed for cAMP using a cAMP enzyme immunoassay kit (Cayman Chemical) according to the manufacturer's instructions, for which no acetylation procedures were performed.

### *Statistical analysis*

Statistical significance was evaluated using ANOVA followed by Dunnett's test. A probability value of less than 0.05 was considered to indicate statistical significance. The results are expressed as the means ± SEM of triplicate findings from five donors.

### *Reagents and drugs*

AGE-modified bovine serum albumin (BSA) (Sigma Aldrich, St Louis, MO, USA) was prepared as previously described (Takeuchi *et al.*, 2000; Takahashi *et al.*, 2009a). Briefly, each protein was incubated under sterile conditions with glyceraldehyde 3-phosphate (AGE-2) (Sigma Aldrich) or glycolaldehyde (AGE-3) (Sigma Aldrich) in 0.2 M phosphate buffer (pH 7.4) at 37°C for 7 days. AGE-BSA was dialyzed for 2 days at 4°C. The endotoxin concentration of AGEs at 100 μg/mL described above was measured at SRL (Okayama, Japan) and was found to be 1.2 pg/mL. AGE-specific fluorescence was measured at 450 nm after excitation at 390 nm with a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). CIP (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid hydrochloride hydraye) was

kindly provided by Bayer Yakuhin, Ltd. (Osaka, Japan). N-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-89), an $EP_2$ receptor antagonist (Alexander *et al.*, 2008), 6-isopropoxy-9-oxaxanthene-2-carboxylic acid (AH6809) and an $EP_4$ receptor antagonist, (4Z)-7-[(rel-1S,2S,5R)-5-((1,1'-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid (AH23848) were purchased from Sigma-Aldrich. NS398 and indomethacin were from Cayman Chemical. For flow cytometric analysis, fluorescein isothiocyanate (FITC)-conjugated mouse $IgG_1$ mAb against ICAM-1/CD54 and phycoerythrin-conjugated anti-CD14 mAb were purchased from DAKO (Glostrup, Denmark). FITC-conjugated mouse $IgG_1$ mAb against B7.2 and CD40 was obtained from Pharmingen (San Diego, CA, USA), and FITC-conjugated $IgG_1$, an isotype-matched control, was obtained from Sigma Chemical.

## Results

### *The effect of CIP on the expression of COX-2 protein and the production of $PGE_2$ in monocytes*

In a previous study, we established an *in vitro* binding assay using immobilized AGE subspecies and the His-tagged soluble form of RAGE (sRAGE) protein to evaluate the binding of AGE subtypes to RAGE (Takahashi *et al.*, 2009a). AGE-2 and AGE-3 showed relatively high affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed moderate affinity for sRAGE. The appropriate incubation time and concentration of AGEs were determined according to these previous studies (Wake *et al.*, 2009; Takahashi *et al.*, 2009a).

The effect of CIP 100 μg $mL^{-1}$ on COX-2 protein expression in monocytes in the presence or absence of AGE-2 and AGE-3 at 100 μg $mL^{-1}$ was determined by Western blot analysis 30 min after the addition of CIP (Figure 1A). COX-2 expression in monocytes treated with BSA was marginal, but the addition of CIP markedly increased the expression of COX-2 irrespective of the presence of AGE-2 and AGE-3.

Whereas AGE-2 and AGE-3 had no effect on the production of $PGE_2$, CIP at 100 μg·$mL^{-1}$ increased the production of $PGE_2$ in a time-dependent manner, with a maximum level at 24 h (Figure 1B). CIP concentration-dependently increased the production of $PGE_2$ both in the presence and absence of AGE-2 and AGE-3 at 24 h (Figure 1C). At 100 μg·$mL^{-1}$, CIP induced the production of 20 nM $PGE_2$ irrespective of the presence of AGE-2 and AGE-3.

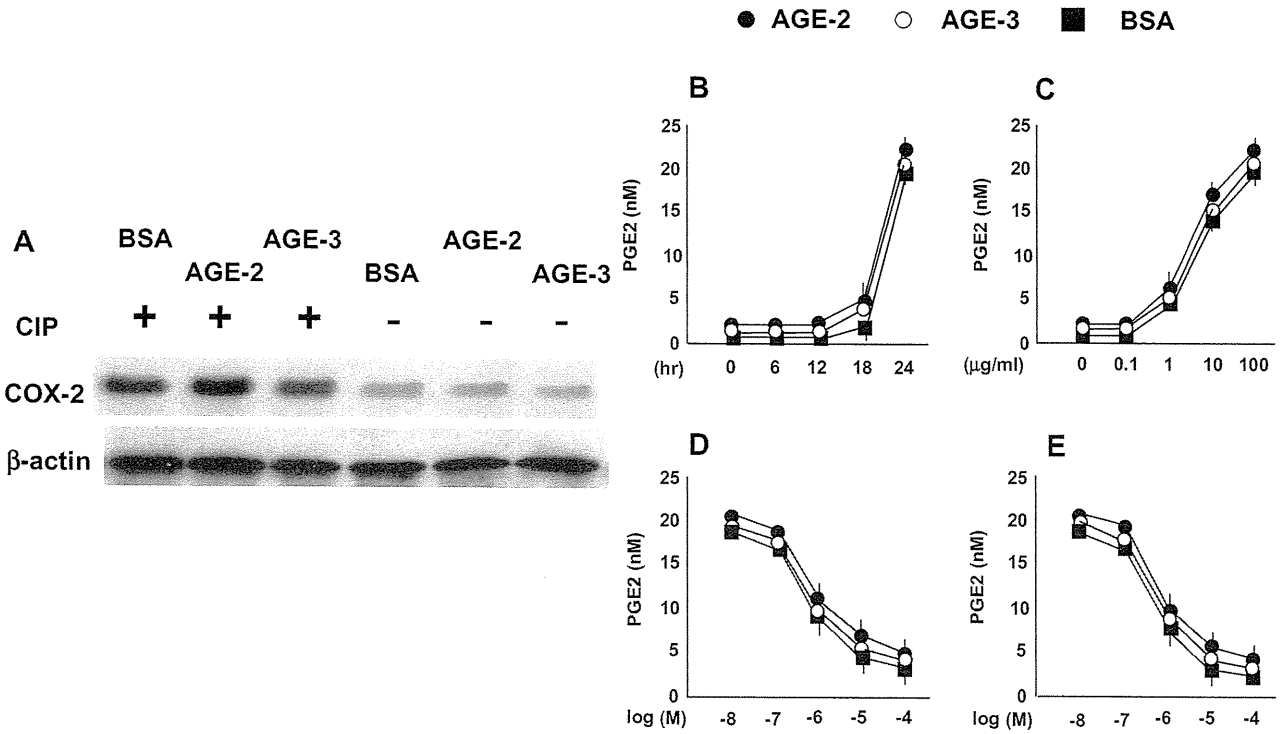
The effects of a non-selective COX-2 inhibitor, indomethacin, and a selective COX-2 inhibitor, NS398, at concentrations ranging from 0.01 to 100 μM on CIP-enhanced production of $PGE_2$ in monocytes were determined after the 24 h incubation (Figure 1D,E). Indomethacin and NS398 inhibited the production of $PGE_2$, irrespective of the presence of AGE-2 and AGE-3, in a concentration-dependent manner.

### *The effect of CIP on cAMP production in monocytes*

The effect of CIP 100 μg·$mL^{-1}$ on intracellular cAMP in monocytes was determined in the presence and absence of AGE-2 and AGE-3 at 100 μg·$mL^{-1}$ (Figure 2). AGE-2 and AGE-3 did not induce the production of cAMP, whereas CIP and $PGE_2$ elicited the production of cAMP irrespective of the presence of AGE-2 and AGE-3. NS398 100 μM blocked the production of cAMP induced by CIP.

### *The effects of CIP on AGE-2- and AGE-3-induced adhesion molecule expression, cytokine production and lymphocyte proliferation*

To determine an appropriate incubation time for investigating the effects of AGE-2 and AGE-2 on these cells, we examined the kinetics at 0, 4, 16, 24 and 48 h as reported previously (Wake *et al.*, 2009; Takahashi *et al.*, 2009a). In the absence of AGEs, the expression of ICAM-1, B7.1, B7.2 and CD40 moderately increased at 16 h and, thereafter, up to 24 and 48 h. AGE-2 and AGE-3 at 100 μg·$mL^{-1}$ significantly increased the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α in PBMC and their proliferation at 16 h and, thereafter, up to 24 and 48 h, whereas AGE-4, AGE-5 and BSA at 100 μg·$mL^{-1}$ had no effect at all (Wake *et al.*, 2009; Takahashi *et al.*, 2009a). Moreover, to determine the appropriate concentration of AGEs, the effects of AGE-2 and AGE-3 at concentrations ranging from 100 ng·$mL^{-1}$ to 100 μg·$mL^{-1}$ were examined after 24 h. AGE-2 and AGE-3, 10 and 100 μg·$mL^{-1}$, significantly enhanced the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α in PBMC and the proliferation in PBMC (Wake *et al.*, 2009; Takahashi *et al.*, 2009a). Therefore, the effects of CIP, at concentrations ranging from 0.1 to 100 μg·$mL^{-1}$, were determined on the responses induced by AGE-2 and AGE-3 100 μg·$mL^{-1}$ (Figure 3). CIP concentration-dependently inhibited the expressions of adhesion molecules, cytokine production and lymphocyte proliferation induced by AGE-2 and AGE-3 at




## Figure 1

Effect of CIP on the expression of COX-2 and the production of prostaglandins $E_2$ ($PGE_2$) in monocytes. (A) Monocytes at $1 \times 10^6$ cells·mL$^{-1}$ were incubated with CIP 100 μg·mL$^{-1}$ in the presence or absence of advanced glycation end product (AGE)-2, AGE-3 or bovine serum albumin (BSA) 100 μg·mL$^{-1}$ for 1 h. The expression of COX-2 protein was determined by Western immunoblotting as described in Methods. β-Actin was used as a control to correct for loading. (B) Time course for effect of CIP on $PGE_2$ production. Monocytes at $1 \times 10^6$ cells·mL$^{-1}$ were incubated with CIP 100 μg·mL$^{-1}$ in the presence of AGE-2, AGE-3 or BSA at 100 μg·mL$^{-1}$ for the indicated times. $PGE_2$ levels in the supernatant were determined by enzyme-linked immunosorbent assay. (C) Effect of CIP at increasing concentrations from 0.1 to 100 μg·mL$^{-1}$ was determined in the presence of AGE-2, AGE-3 or BSA 100 μg·mL$^{-1}$ for 24 h. The effects of a non-selective COX-2 inhibitor, indomethacin (D) and a selective COX-2 inhibitor, NS398 (E) on the actions of CIP at 100 μg·mL$^{-1}$ were determined in the presence of AGE-2, AGE-3 or BSA. The results are expressed as the means ± SEM of five donors with triplicate determinations. When an error bar was within a symbol, the bar was omitted.

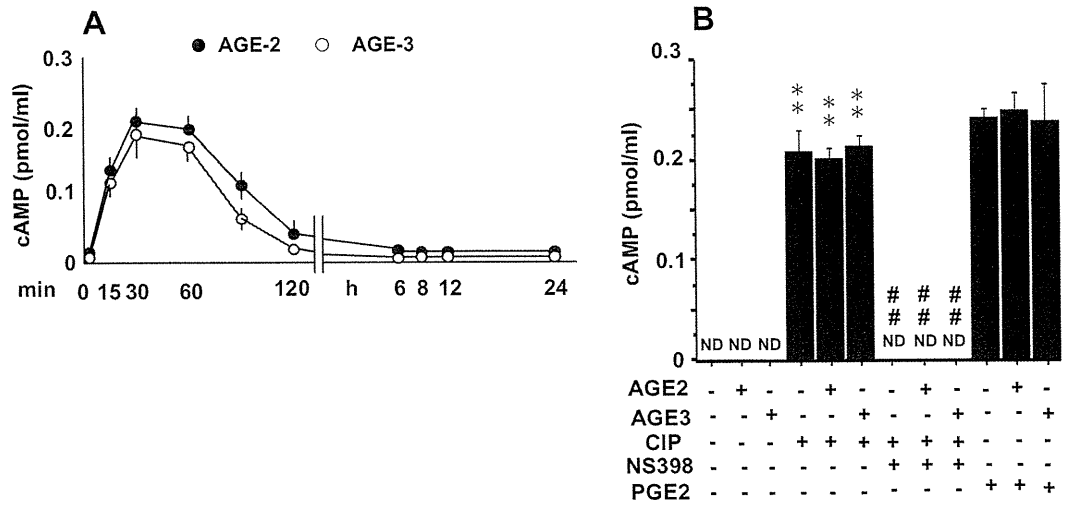
100 μg·mL$^{-1}$. The $IC_{50}$ values for the inhibitory effect of CIP on the expressions of ICAM-1, B7.1, B7.2 and CD40 in the presence of AGE-2 were estimated to be 4, 5, 4 and 5 μg·mL$^{-1}$, and those in the presence of AGE-3 were 3, 3, 5 and 4 μg·mL$^{-1}$, respectively. Moreover, the $IC_{50}$ values for the effect of CIP on cytokine production and lymphocyte proliferation in the presence of AGE-2 were estimated to be 3, 3 and 2 μg·mL$^{-1}$, and those in the presence of AGE-3 were 3, 3 and 2 μg·mL$^{-1}$, respectively. In the absence of AGEs, CIP at 10 and 100 μg·mL$^{-1}$ also inhibited the basal expression levels of adhesion molecule, but had no effect on cytokine production and lymphocyte proliferation.

### *The involvement of prostanoid $EP_2$ and $EP_4$ receptors in the actions of CIP*

To determine the involvement of $PGE_2$ receptor subtypes in the effects of CIP on the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation, the effect of an $EP_2$ receptor antagonist, AH6809 (Takahashi *et al.*, 2009b) and an $EP_4$ receptor antagonist, AH23848 (Takahashi *et al.*, 2009b) at concentrations ranging from 0.01 to 100 μM were examined in the presence of CIP 100 μg·mL$^{-1}$ (Figure 4). AH6809 and AH23848 reversed the inhibitory effect of CIP on the increased expressions of adhesion molecules, cytokine production and lymphocyte proliferation induced by AGE-2 and AGE-3 in a concentration-dependent manner. AH6809 and AH23848 had no effect on the actions of AGE-2 and AGE-3 in the absence of CIP.

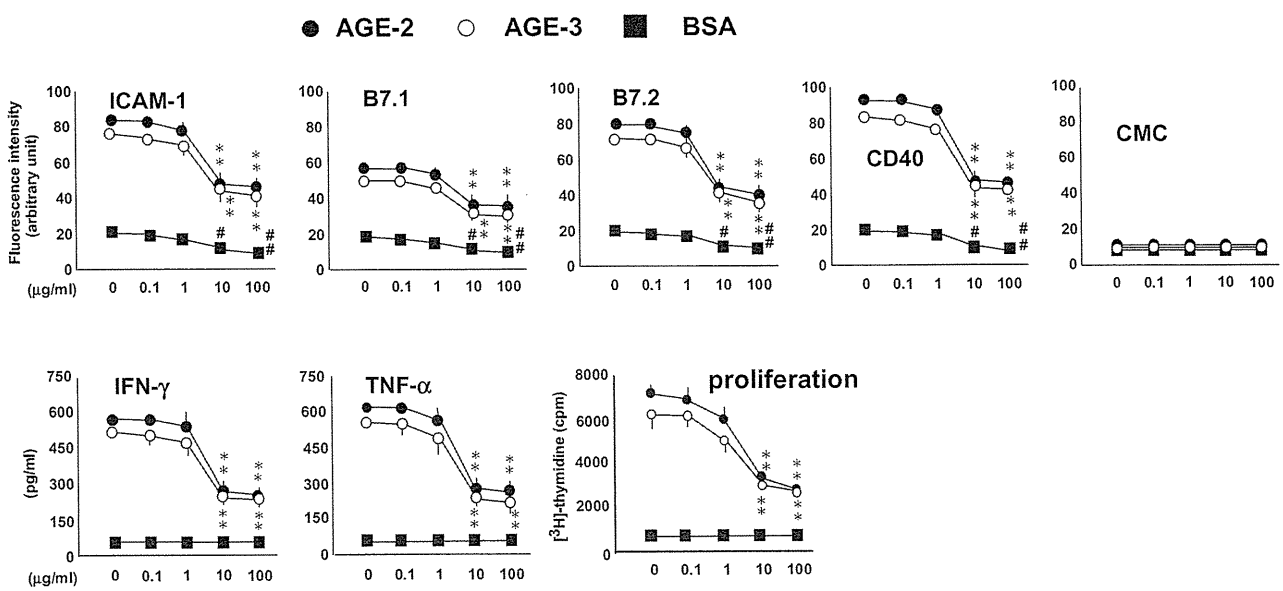
### *The effect of indomethacin, NS398 and H-89 on the actions of CIP*

The effects of indomethacin, NS398 and a PKA inhibitor, H-89 at concentrations ranging from 0.01



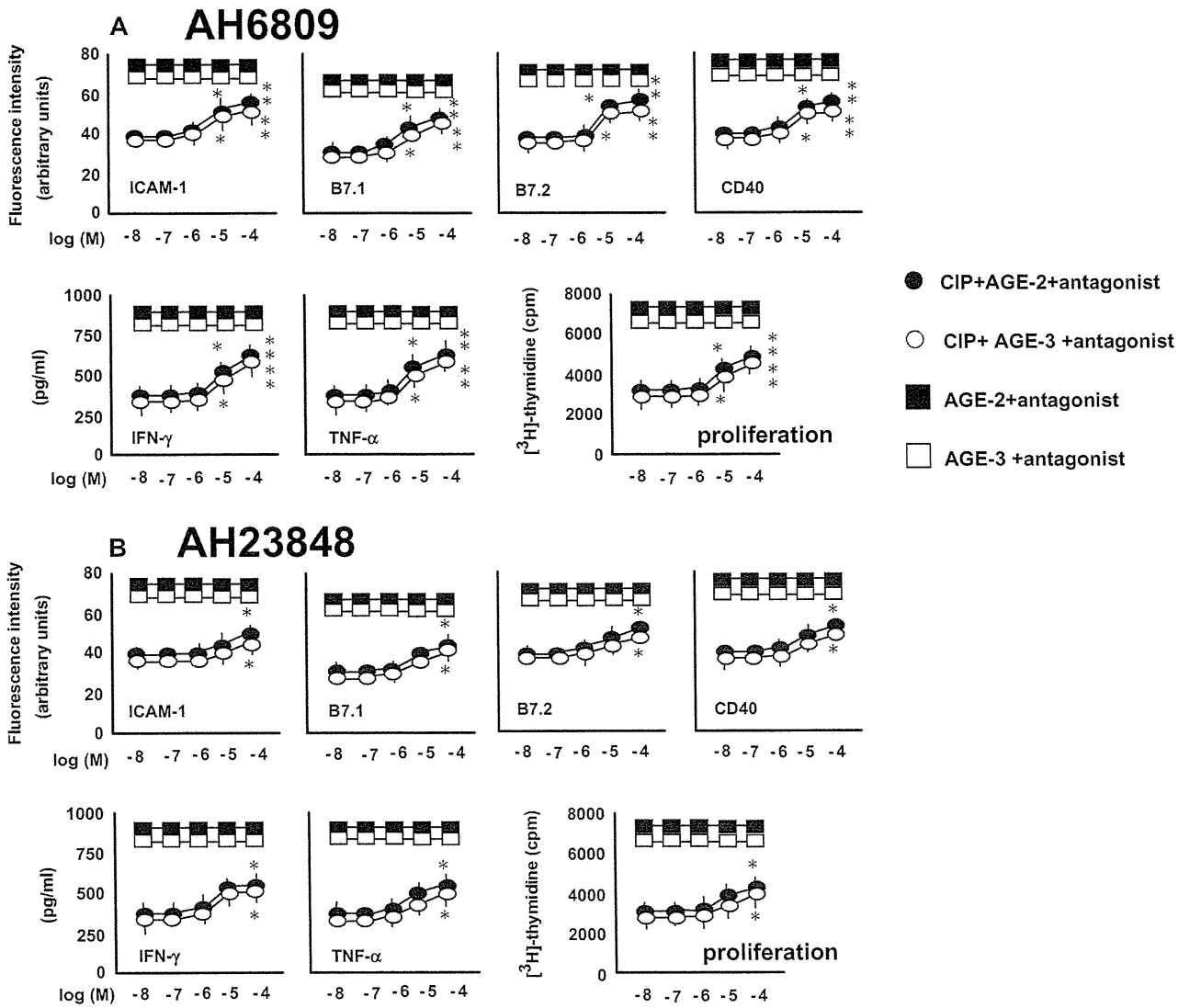

## Figure 2

Effect of CIP on the activation of cAMP in monocytes. (A) Time course for effect of CIP 100 μg·mL$^{-1}$ on cAMP production was determined by enzyme-linked immunosorbent assay. Monocytes at 1 × 10$^6$ cells·mL$^{-1}$ were incubated with CIP in the presence of advanced glycation end product (AGE)-2 and AGE-3 at 100 μg·mL$^{-1}$, and the time course changes in the levels of cAMP in monocytes were determined at the indicated time points. (B) Effect of COX-2 inhibitor, NS398 100 μM on CIP-induced production of cAMP in the presence or absence of AGE-2 and AGE-3. $^{**}P < 0.01$ compared with the value for bovine serum albumin alone. $^{\#\#}P < 0.01$ compared with the value for CIP. The results are the means ± SEM of triplicate findings from five donors. ND, not detected; $PGE_2$, prostaglandins $E_2$.




## Figure 3

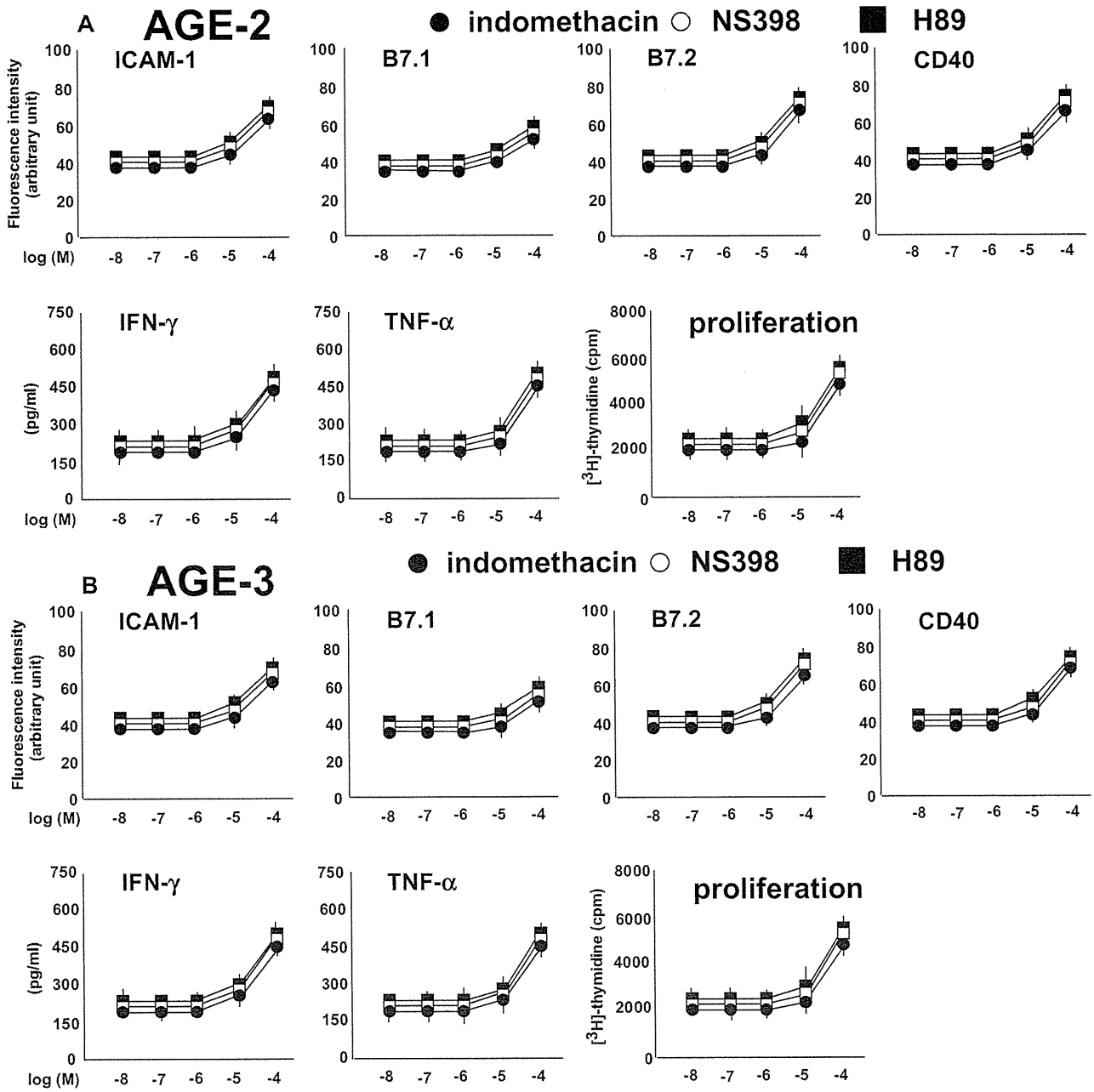
Effects of CIP on advanced glycation end product (AGE)-2- and AGE-3-induced expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes, production of interferon (IFN)-γ and tumour necrosis factor (TNF)-α in peripheral blood mononuclear cells (PBMC) and lymphocyte proliferation in PBMC. PBMC at 1 × 10$^6$ cells mL$^{-1}$ were incubated with AGE-2, AGE-3 and bovine serum albumin (BSA) at 100 μg·mL$^{-1}$ in the presence of CIP at increasing concentrations from 0.1 to 100 μg·mL$^{-1}$ for 24 h. Expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. Fluorescein isothiocyanate-conjugated $IgG_1$ was used as an isotype-matched control Ab. IFN-γ and TNF-α concentrations in conditioned media were determined by enzyme-linked immunosorbent assay. Lymphocyte proliferation was determined by [$^3$H]-thymidine uptake as described in Methods. The results are expressed as the means ± SEM of five donors with triplicate determinations. $^{**}P < 0.01$ compared with the value in the presence of AGE-2 and AGE-3 alone. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the value for medium alone. When an error bar was within a symbol, the bar was omitted.




## Figure 4

Effects of prostanoid receptor antagonists on the inhibitory effects of CIP on the expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40, the production of interferon (IFN)-γ and tumour necrosis factor (TNF)-α and lymphocyte proliferation. PBMC at $1 \times 10^6$ cells·mL$^{-1}$ treated with an E-prostanoid $(EP)_2$ receptor antagonist, 6-isopropoxy-9-oxaxanthene-2-carboxylic acid (A) or an $EP_4$ receptor antagonist, (4Z)-7-[(rel-1S,2S,5R)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid (B), at increasing concentrations from 0.01 to 100 μM were incubated with CIP 100 μg·mL$^{-1}$ in the presence of advanced glycation end product (AGE)-2 and AGE-3 at 100 μg·mL$^{-1}$. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. IFN-γ and TNF-α concentrations in conditioned media were determined by enzyme-linked immunosorbent assay. Lymphocyte proliferation was determined by [$^3$H]-thymidine uptake as described in Methods. The results are expressed as the means ± SEM of five donors with triplicate determinations. *$P$ < 0.05, **$P$ < 0.01 compared with the value for CIP in the presence of AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

to 100 μM on CIP-inhibited expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and lymphocyte proliferation in PBMC were determined in the presence of AGE-2 and AGE-3, 100 μg·mL$^{-1}$, at 24 h (Figure 5). NS398, indomethacin and H-89 reversed the inhibitory effect of CIP on adhesion molecule expressions, cytokine production and lymphocyte proliferation. In the absence of CIP, these inhibitors did not affect the actions of AGE-2 and AGE-3 (data not shown).




## Figure 5

Effects of indomethacin, NS398 and N-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-89) on the actions of CIP. The effect of indomethacin, NS398 and H-89 at increasing concentrations ranging from 0.01 to 100 μM on the actions of CIP 100 μg·mL$^{-1}$ were examined in the presence of , advanced glycation end product (AGE)-2 (A) and AGE-3 (B) at 100 μg·mL$^{-1}$. Expressions of intercellular adhesion molecule (ICAM)-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. Interferon (IFN)-γ and tumour necrosis factor (TNF)-α concentrations in conditioned media were determined by enzyme-linked immunosorbent assay. Lymphocyte proliferation was determined by [$^3$H]-thymidine uptake as described in Methods. The results are expressed as the means ± SEM of triplicate findings from five donors. When an error bar was within a symbol, the bar was omitted.

## Discussion and conclusions

The level of AGE-2 in the serum of patients with diabetes has been reported to be 17 μg·mL$^{-1}$ (Enomoto *et al.*, 2006; Nakamura *et al.*, 2007). AGEs at concentrations ranging from 50 to 200 μg·mL$^{-1}$ have been shown to significantly increase human monocyte adhesion to bovine retinal endothelial

cells (Mamputu and Renier, 2004). AGEs at 200 $\mu g \cdot mL^{-1}$ increase the expressions of CD40, CD80 and CD86 and the production of IFN-γ in dendritic cells (Ge *et al.*, 2005). In a previous study, we found that AGE-2 and AGE-3 at 10 and 100 $\mu g \cdot mL^{-1}$ significantly up-regulate the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α in PBMC and lymphocyte proliferation (Wake *et al.*, 2009; Takahashi *et al.*, 2009a). Therefore, the concentration of 100 $\mu g \cdot mL^{-1}$ used in the present study covers the pathological concentration of AGEs in the serum of patients with diabetes reported in previous studies (Enomoto *et al.*, 2006; Nakamura *et al.*, 2007).

We found that AGE-2 and AGE-3 increased the production of IFN-γ and TNF-α in monocytes isolated from PBMC, exhibiting 20% of the amount obtained in PBMC (Takahashi *et al.*, 2009a). AGE-2 and AGE-3 had no effect on the production of IFN-γ and TNF-α in T-cells isolated from PBMC. Anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs inhibited the AGE-2- and AGE-3-induced production of IFN-γ and TNF-α in PBMC, indicating that an enhancement of the cell-to-cell interaction between monocytes and T-cells, through an increase in plural adhesion molecule expression on monocytes, is required for the effects of AGE-2- and AGE-3 on cytokine production. Together with these results, we examined the effect of CIP on the increased expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α in PBMC and lymphocyte proliferation in PBMC induced by AGE-2 and AGE-3.

In a randomized crossover study, the concentration of ciprofloxacin was found to be 3 $\mu g \cdot mL^{-1}$ (C max) or 14 $\mu g \cdot h^{-1} \cdot mL$ (area under the serum concentration time curve from 0 to 12 h) in the serum of a patient who had been given a single oral dose of 500 mg (Issa *et al.*, 2007; van Zanten *et al.*, 2008), which is within the range of the concentrations used in the present study. Recently, we reported that CIP increased the expression of COX-2 and the production of $PGE_2$ in human monocytes (Takahashi *et al.*, 2005). In the present study, we examined, for the first time, the effects of CIP on the immune response of monocytes treated with AGE-2 and AGE-3. CIP increased the expression of COX-2 and the production of $PGE_2$ in the presence or absence of AGE-2 and AGE-3 (Figure 1); 20 nM $PGE_2$ was detected in the medium of monocytes treated with 100 $\mu g \cdot mL^{-1}$ CIP in the absence or presence of these AGEs. We also determined the levels of other COX-2 metabolites, including $PGE_1$, $PGD_2$, $PGF_2$, $PGI_2$, $PGJ_2$ and thromboxane, in the medium of monocytes treated with CIP in the presence or absence of AGE-2 and AGE-3, but all were under the detection limits (data not shown). This increase in endogenous $PGE_2$ production induced by CIP was inhibited by the non-selective COX-2 inhibitor, indomethacin, and the selective COX-2 inhibitor, NS398 (Figure 1C,D), indicating that this increase in endogenous $PGE_2$ production might depend on the enhancement of COX-2 expression. CIP also elevated the intracellular level of cAMP in monocytes irrespective of the presence of AGE-2 and AGE-3 (Figure 2) and this effect was abolished by NS398 (Figure 2C). These results suggest that the endogenously produced $PGE_2$ and elevation of cAMP are associated with the CIP-induced enhancement of COX-2 expression.

In a previous study, we found that $PGE_2$ inhibited the enhanced expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and lymphocyte proliferation in human PBMC induced by AGE-2 and AGE-3 (Takahashi *et al.*, 2009b). An $EP_2$ receptor agonist, ONO-AE1-259-01, an $EP_4$ receptor agonist, ONO-AE1-329, and a mixed $EP_2/EP_4$ receptor agonist, 11-deoxy-$PGE_1$, mimicked the effects of $PGE_2$ on adhesion molecule expression, cytokine production and lymphocyte proliferation. Moreover, an $EP_2$ receptor antagonist, AH6809, and an $EP_4$ receptor antagonist, AH23848, inhibited the actions of $PGE_2$. Therefore, it was suggested that the inhibitory effect of $PGE_2$ was mediated by the stimulation of $EP_2$ and $EP_4$ receptors. $PGE_2$, $EP_2$ and $EP_4$ receptor agonists induced the production of cAMP in monocytes irrespective of the presence of AGE-2 and AGE-3. A PKA inhibitor, H89, inhibited the actions of $PGE_2$. A cAMP analogue, dibutyryl cAMP, and an adenylate cyclase activator, forskolin, mimicked the effect of $PGE_2$. These results suggested the involvement of $EP_2/EP_4$ receptor and the cAMP/PKA pathway in the actions of $PGE_2$.

As shown in Figure 3, CIP suppressed the increased expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and lymphocyte proliferation induced by AGE-2 and AGE-3. The $IC_{50}$ values for these effects of CIP in the presence of AGE-2 and AGE-3 were consistent with those in the presence of IL-18 (Takahashi *et al.*, 2005). While CIP on its own inhibited the basal expression levels of adhesion molecule, it had no effect on cytokine production and lymphocyte proliferation. On the other hand, $PGE_2$ by itself had no effect on the basal levels of adhesion molecule expressions, cytokine production and lymphocyte proliferation (Takahashi *et al.*, 2009a). The COX-2 inhibitors, but not the PKA inhibitor, abolished the effect of CIP on adhesion molecule expressions in the absence of AGE-2 and AGE-3 (Takahashi *et al.*, 2005), suggesting that endogenous $PGE_2$ is not

involved in the effects of CIP in the absence of AGE-2 and AGE-3.

The $EP_2$ receptor antagonist, AH6809, and the $EP_4$ receptor antagonist, AH23848, partially inhibited the actions of CIP (Figure 4). Moreover, inhibitors of COX-2 and PKA partially reversed the inhibitory effect of CIP on the enhanced expressions of adhesion molecule, cytokine production and lymphocyte proliferation induced by AGE-2 and AGE-3 (Figure 5). We observed a similar pattern for the inhibitory effects of CIP on IL-18-induced activation of monocytes in human PBMC via $EP_2/EP_4$ receptor (Takahashi *et al.*, 2005). Therefore, endogenous mechanisms that are both $PGE_2$ dependent and $PGE_2$ independent may be associated with the actions of CIP.

In a previous study, using an *in vitro* binding assay, we found that AGE-2 and AGE-3 had a higher affinity for RAGE than AGE-4 and AGE-5 (Takahashi *et al.*, 2009a). AGE-2 and AGE-3, but not AGE-4 and AGE-5, up-regulated the expression of the RAGE receptor on the cell surface of monocytes. We found that $PGE_2$ had no effect on the expression of RAGE in the presence and absence of AGE-2 and AGE-3 (Takahashi *et al.*, 2009b). In the present study, we found that CIP also had no effect on the expression of RAGE (data not shown), suggesting that there might be distinct signal transduction pathways for the regulation of expression of RAGE and adhesion molecules, leading to enhanced expression of adhesion molecules and RAGE, which are differentially regulated by the cAMP-PKA system.

Skin ulceration is a very common complication in diabetic patients and is often associated with cutaneous microangiopathy and neuropathy in these patients (Ngo *et al.*, 2005). In addition, AGEs have been shown to accumulate in the skin of diabetic patients (Liao *et al.*, 2009) and bacterial infections frequently occur in the feet of patients with diabetes mellitus and can cause serious complications (Peterson *et al.*, 1989). CIP is the antibiotic that is most frequently used to treat these foot infections (Peterson *et al.*, 1989) and the concentrations of CIP reached at the target site are several-fold higher than those in the serum (Licitra *et al.*, 1987). In addition, $PGE_2$, which is induced by monocytes, inhibits procollagen secretion by human vascular smooth muscle cells, leading to extracellular matrix remodelling and resistance to rupture during atherosclerosis (Fitzsimmons *et al.*, 1999). An elevation of cAMP in endothelial cells inhibits proliferation, leading to the inhibition of atherosclerosis in patients with diabetes (Lorenowicz *et al.*, 2007). The present data are consistent with the finding that the elevation of cAMP prevents the production of TNF-α in monocytes of diabetic patients (Jain *et al.*, 2002). These findings together with our results indicate that an elevation of intracellular cAMP production may regulate the activation of vascular smooth muscle cells, endothelial cells and monocytes. In conclusion, we found that the anti-microbial agent CIP is able to regulate monocyte responses and that an increased production of $PGE_2$ is involved in this effect. Hence, the present results suggest that CIP has therapeutic potential for the treatment of the systemic inflammatory response associated with diabetes. However, ciprofloxacin also has the ability to increase blood glucose levels; therefore, this should be taken into consideration when assessing its therapeutic value.

## Acknowledgements

The authors also thank Ms Miyuki Shiotani and Mr Yukinari Isomoto for technical assistance. This work was supported in part by grants from the Japanese Society for the Promotion of Science [Grants 18590509, 20590539, 17659159, 19659061, 21659141, 21390071, 215905694], from the Scientific Research from Ministry of Health, Labour and Welfare of Japan and from the Takeda Science Foundation.

## Statement of conflicts of interest

None.

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Acta Med. Okayama, 2009
Vol. 63, No. 1, pp. 65-69


Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

*Short Communication*

# Specific Removal of Monocytes from Peripheral Blood of Septic Patients by Polymyxin B-immobilized Filter Column

Masahiro Nishibori[a]*, Hideo K Takahashi[a], Hiroshi Katayama[b], Shuji Mori, Shinya Saito[c], Hiromi Iwagaki[c], Noriaki Tanaka[c], Kiyoshi Morita[b], and Aiji Ohtsuka[d]

*Departments of [a]Pharmacology, [b]Anesthesiology and Resuscitology, [c]Surgical Oncology, and [d]Human Morphology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan*

**Lipopolysaccharide (LPS) is one of the major causes of septic shock. The polymyxin B-immobilized filter column (PMX) was developed for the adsorption of endotoxin by direct hemoperfusion and has been used for the treatment of LPS-induced septic shock. In this study, we demonstrated that PMX also specifically bound monocytes from the peripheral blood leukocytes of septic patients by mean of an analysis of bound cells using immunocytochemical and electron microscopic techniques. The specific removal of monocytes from septic patients may produce beneficial effects by reducing the interaction between monocytes and functionally associated cells including vascular endothelial cells.**

**Key words**: septic shock, polymixin B-immobilized column, monocyte, adsorptive removal

Sepsis and septic shock, often associated with multiple organ failure, still remain important causes of morbidity and mortality in intensive care units. Many types of therapeutic trials for the treatment of septic shock have failed however, recent phase III studies using recombinant activated protein C demonstrated the effectiveness of this therapy [1, 2]. Lipopolysaccharide (LPS), one of the major causes of septic shock, together with LPS binding protein, binds to CD14 on the surfaces of monocytes/macrophages, leading to the activation of the signaling molecule complex of Toll-like receptor-4 (TLR-4) and MD2. Polymyxin B can bind LPS and neutralize its biological activity therefore, the polymyxin B-immobilized filter (PMX) column was developed for the adsorption of endotoxin by hemoperfusion [3]. There is now increasing evidence supporting the usefulness of this treatment, showing improvement of the survival rate in LPS-induced circulatory disorders and systemic inflammatory response syndrome. Moreover, the effectiveness of hemoperfusion with this column for treating septic shock beyond LPS endotoxemia [4] prompted us to investigate additional mechanisms. Since it is well known that different populations of leukocytes are activated during septic shock and change their adhesive phenotype, we hypothesized that some population of leukocytes may be adsorbed in the column and removed from the blood circulation after treatment. To examine this hypothesis, we investigated the cellular components in the PMX columns after direct hemofiltration in 4 septic patients.

The original diseases of the 4 patients were ileus of sigmoid colon (case 1; 69 ys male), embolism of superior mesenteric artery (case 2; 76 ys male), pye-

Received September 8, 2008; accepted October 28, 2008.
*Corresponding author. Phone:+81-86-235-7140; Fax:+81-86-235-7140
E-mail:mbori@md.okayama-u.ac.jp (M. Nishibori)

lonephritis (case 3; 67 ys female) and liver abscess (case 4; 58 ys female). The patients were admitted to the university hospital or educational training hospitals in Okayama city from 2003 to 2004. The systolic and diastolic blood pressures before and after the treatment were $87.5 \pm 20.2$ and $48.3 \pm 7.7$ mmHg vs. $105.8 \pm 29.2$ and $56.3 \pm 15.3$ mmHg, respectively (mean $\pm$ SEM). The investigations were carried out with the approval of the ethical review committee of Okayama University Medical School. Written informed consent was obtained from the families of all patients.

The used PMX columns were washed with 500 ml of saline in the ICU. Then, the columns were cooled on ice and moved into a cold room at 4℃. The columns were successively washed with 500 ml of ice-cold saline at a flow rate of 16 ml/min 3 times, and the cells in each 500 ml fraction were analyzed. The columns were then opened and the cells attached to the filters were collected by shaking the filter in ice-cold saline. The collected cells from the fiber were smeared on the slide glass and subjected to May-Grunwald-Gimsa or immunocytochemical staining with PE-conjugated anti-CD14 and FITC-conjugated anti-CD68 antibodies. For scanning electron microscopic and transmission electron microscopic examinations, the PMX and the collected cells from the PMX column were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer. FACS analysis was performed on the collected cells as well as peripheral blood mononuclear cells as described previously [5].

We found huge amounts of cellular components in the hemoperfused PMX columns from 4 patients (Fig. 1A). Fig. 1C shows the nuclear staining of leukocytes on the filter by hematoxylin when the filter was fixed immediately after opening the column. Fig. 1A is the corresponding picture made by scanning electron microscopy in which the cells include leukocytes and erythrocytes. About 70 to 80% of the total cells were recovered from the filter by gentle shaking in ice-cold saline (Figs. 1B and D). Fig. 2B clearly shows by May-Grunwald-Gimsa staining that the recovered leukocytes have the typical nuclear shape of monocytes with slight basophilic cytoplasm. Immunocytochemical staining confirmed that almost all leukocytes were immunoreactive for both CD14 and CD68 (Figs. 2C–F). The transmission electron microscopy also revealed that most of the cells recovered from the PMX filter have monocyte features polymorphic nuclei, few specific granules and many vacuoles (Fig. 3). Apoptotic changes such as nuclear condensation were not observed. These findings were common to four cases. Fig. 4 summarizes the leukocyte populations in the washings and in the PMX columns from four patients. Even in the washing fraction more than 75% cells were monocytes, although the cell numbers in the washings were less than 1% of those attached to the filter. The CD68 localization on the plasma membrane as well as within the cytoplasm (Fig. 2E) and the ultrastructural appearance of monocytes with irregularly shaped processes (Fig. 3) and numerous phagocytic vesicles strongly suggested that the monocytes trapped in the column had been activated through the pathological processes of septic shock. These results as a whole indicated that the PMX column specifically adsorbed monocytes (98.5% purity) among leukocytes from the peripheral blood of septic patients.

Analysis of the expression of adhesion molecules in monocytes from the peripheral blood as well as those recovered from PMX columns revealed that the expression levels of all adhesion molecules examined in patients were lower than those in normal volunteers (Fig. 5). The comparison of the expression levels of each adhesion molecule among monocytes from pre-treatment PBMC and those from PMX columns showed that the expression levels of CD11b in monocytes from PMX columns were lower than those in pre- and post-treatment PBMC. Also, CD62L expression in monocytes from PMX columns was lower than that in post-treatment PBMC. CD62L and CD11b, an $\alpha$ chain of the Mac-1 molecule, were considered to be involved in the cellular interaction therefore, the reduced levels of CD11b and 62L in monocytes in PMX columns may represent the shedding of these molecules after the stimulation of monocytes. Although the detailed phenotype features of bound monocytes are not known at present, it is quite likely that specific populations of monocytes show an increased affinity to PMX. It is not clear what enabled the monocytes with low levels of CD11b and CD62L to bind selectively to PMX. However, the removal of particular populations of monocytes from blood may provide a new strategy for the treatment of septic shock. Further research is necessary to confirm the causative relationship between the removal of specific