BJP BRITISH JOURNAL OF PHARMACOLOGY
BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00880.x
www.brjpharmacol.org

RESEARCH PAPER

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

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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

1×10^6 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 1×10^6 cells·mL^{-1} were incubated for 24 h. Cultured cells at 5×10^5 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 1×10^6 cells·mL^{-1} were used to analyse IFN-γ and TNF-α production, and monocytes at 1×10^6 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 1×10^6 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 1×10^6 cells·mL^{-1} were incubated at 37°C in a 5%CO_2/air mixture. After 1 h, cells at 2×10^5 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

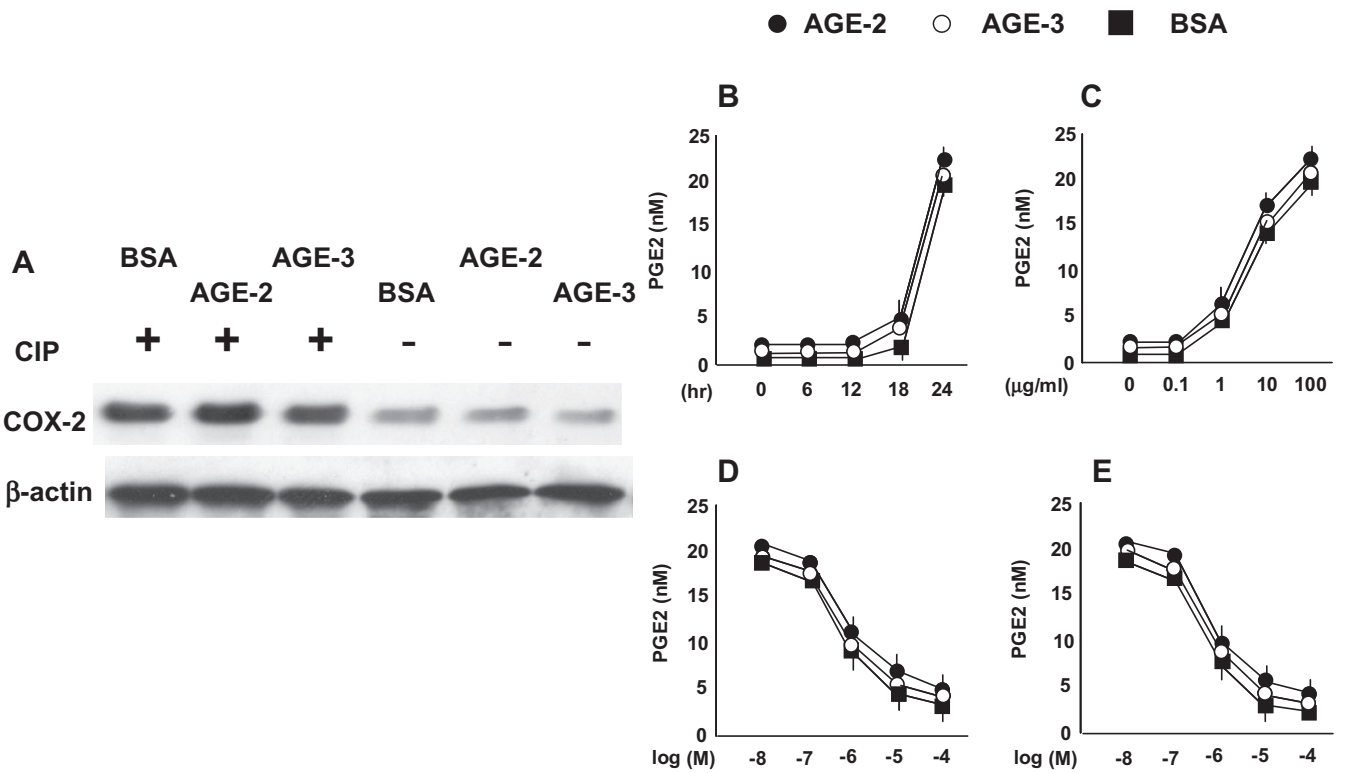

Figure 1

Effect of CIP on the expression of COX-2 and the production of prostaglandins E_2 (PGE_2) in monocytes. (A) Monocytes at 1×10^6 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 1×10^6 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

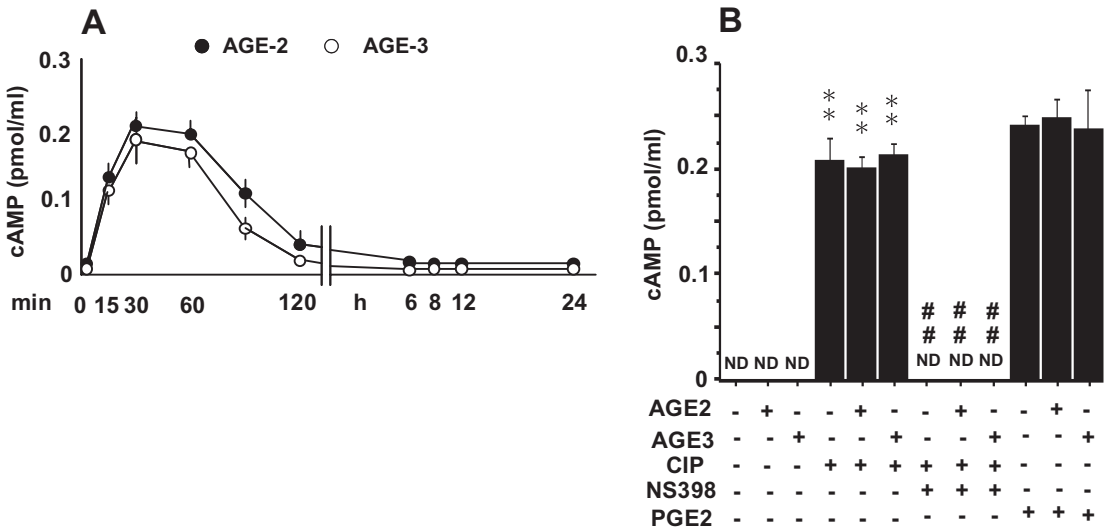

Figure 2

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.

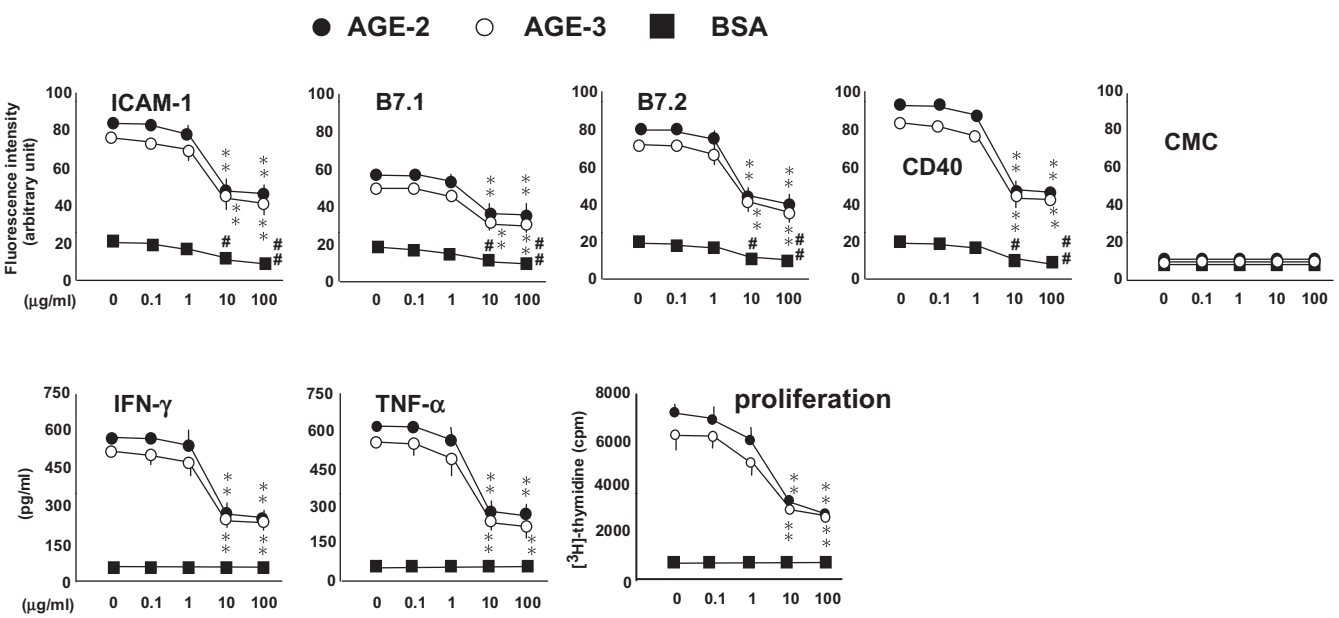

Figure 3

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.

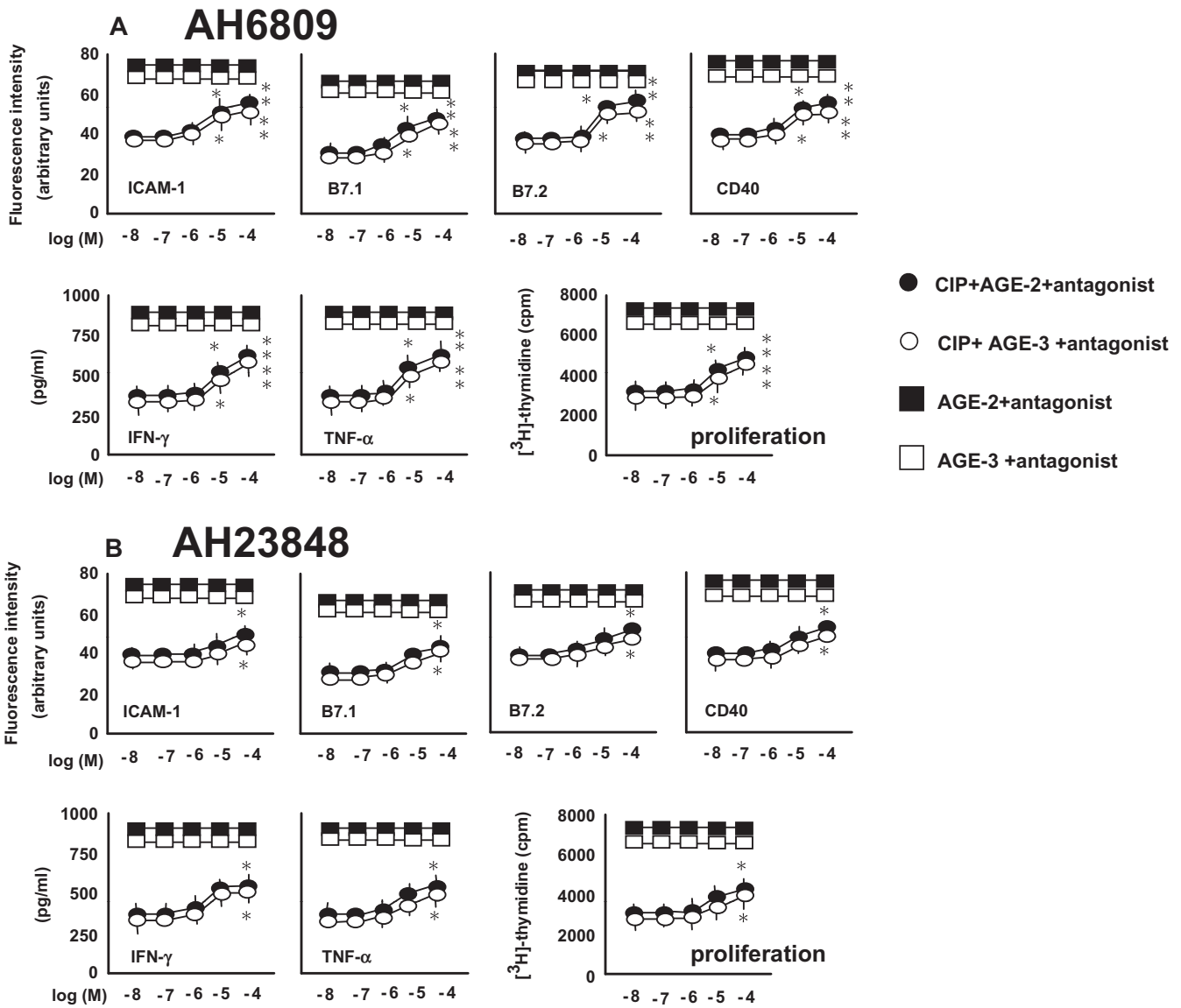

Figure 4

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 1×10^6 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).

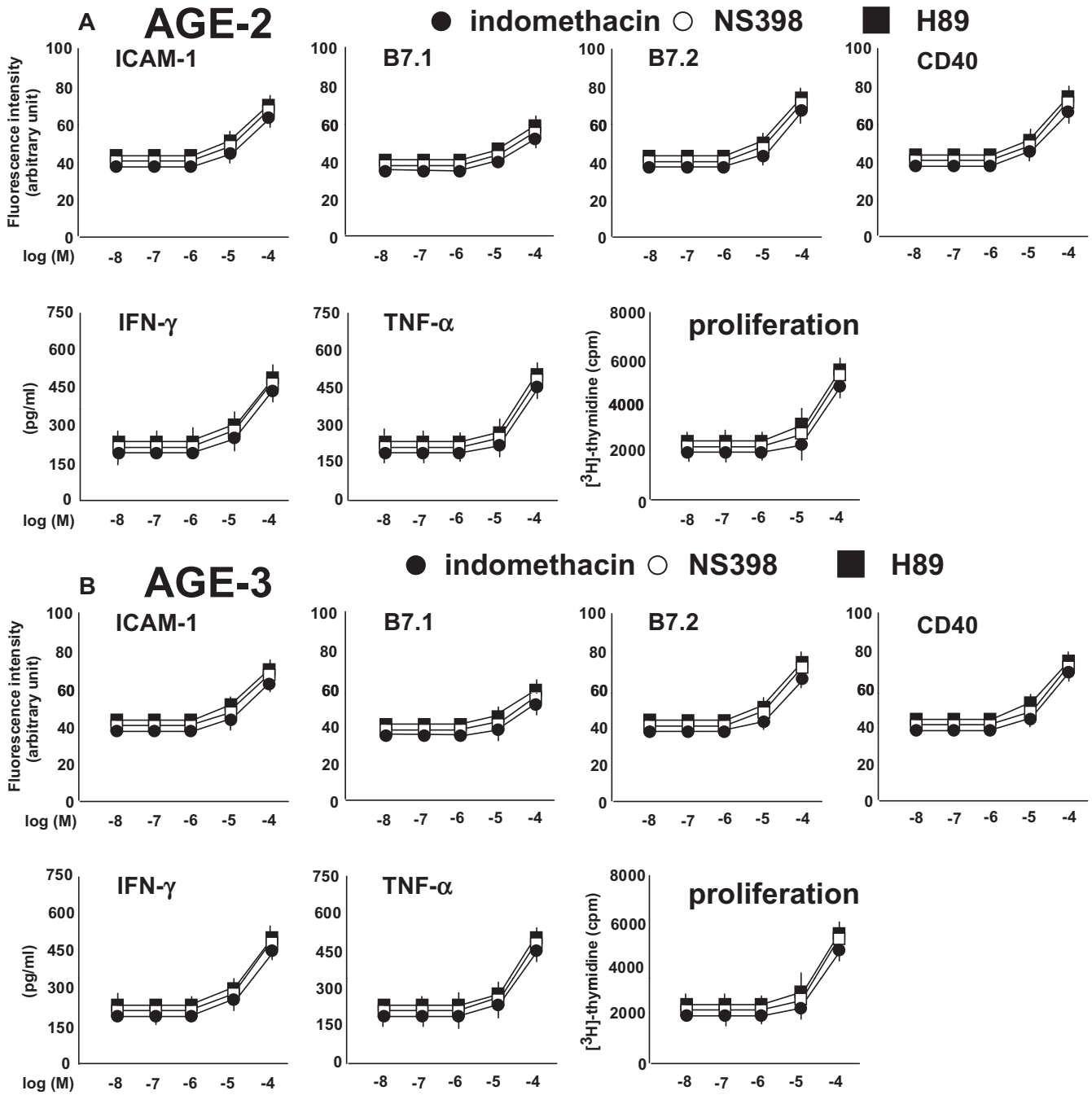

Figure 5

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 μg·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 μg·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 μg·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 μg·mL^{-1} (C max) or 14 μg·h^{-1}·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 μg·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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