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Involvement of p38 mitogen-activated protein kinase in PLL-AGE-induced cyclooxgenase-2 expression

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Abstract

In the present study, murine RAW 264.7 macrophages were incubated with poly-L-lysine-derived advanced glycosylation end products (PLL-AGEs) to examine cyclooxygenase-2 protein expression. Treatment of RAW 264.7 cells with PLL-AGEs caused the dose-dependent expression of cylooxygenase-2 but not cylooxygenase-1 and an increase in cylooxygenase activity. Increased cylooxygenase-2 expression was seen at 6 h and reached a maximum at 24 h. The tyrosine kinase inhibitor, genistein, and the p38 mitogen-activated protein kinase (MAPK) inhibitor, [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole] (SB 203580), inhibited PLL-AGE-induced cylooxygenase-2 expression, while the Ras inhibitor, FPT inhibitor II, and the MAP kinase kinase inhibitor, (2′ -amino-3′ -methoxyflavone) (PD 98059), had no effect on PLL-AGE-induced cylooxygenase-2 expression. Incubation of RAW 264.7 cells with PLL-AGEs resulted in activation of p38 MAPK, and this activation was suppressed by genistein and SB 203580. Taken together, our results suggest that activation of protein tyrosine kinase and p38 MAPK is involved in AGE-induced cyclooxygenase-2 expression in RAW 264.7 macrophages. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Glycosylation end product; Advanced; Cyclooxygenase-2; p38 MAP (mitogen activated protein) kinase; RAW 264.7 cell

1. Introduction

Aging or prolonged elevation of glucose levels in diabetic patients results in a number of complications including nephropathy, arteriosclerosis, retinopathy, neuropathy, and cataracts (Monnier et al., 1992). These complications have been related to advanced glycosylation end products (AGEs) (Brownlee et al., 1988). AGEs are fluorescent substances formed by the non-enzymatic "Maillard reaction" and are considered to be an important factor in mediating diabetic sequelae (Brownlee, 1991).

AGEs are recognized by specific AGE receptors (Brett et al., 1993) and exert various biological effects. The selective presence of AGE receptors has recently been demonstrated in the endothelium (Wautier et al., 1996), mononuclear phagocytes (Schmidt et al., 1993), smooth muscle cells, mesangial cells, and certain neurons (Hori et al., 1996). An AGE receptor complex can trigger signal transduction, resulting in the production of tumor necrosis factor and interleukin-1 (Hasegawa et al., 1991). Nevertheless, no prior studies have shown that AGEs stimulate cyclooxygenase-2 expression.

Cyclooxygenase, also known as prostaglandin endoperoxide H synthase, catalyzes the conversion of arachidonic acid to prostaglandin H_2, the precursor of prostanoids. The expression of cyclooxygenase has recently emerged as an important determinant of the cytotoxicity associated with inflammation (Seibert et al., 1995). Cyclooxygenase has two isoforms, designated cyclooxygenase-1 and cyclooxygenase-2. Cyclooxygenase-1 is constitutively expressed in many cell types. Cyclooxygenase-2 bears 60% overall identity with cyclooxygenase-1 at the amino acid sequence level. It is not present in most cells under normal conditions and is tightly regulated by a variety of stimuli including endotoxins, proinflammatory cytokines, and excitatory amino acids (O'Banion et al., 1991; Smith and Dewitt, 1996).

The signaling pathways of cyclooxygenase-2 gene expression have been studied in a number of cell systems. The expression of cyclooxygenase-2 seems to be closely

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related with the activation of mitogen-activated protein kinases (MAPKs) (Mestre et al., 2001). There are three important groups of MAPKs, including p44/42 MAPK, also known as extracellular signal-regulated kinase 1/2 (ERK 1/2), stress-activated protein kinase (SAPK)/c-*jun* N-terminal kinase (JNK), and p38 MAPK (Denhardt, 1996). The p44/42 MAPK pathway is preferentially activated by growth factors and mitogens, whereas the SAPK/JNK and p38 MAPK pathways are preferentially activated by inflammatory cytokines and various forms of stress (Denhardt, 1996). The p38 MAPK signaling pathway is involved in lipopolysaccharide-induced cyclooxygenase-2 expression in RAW 264.7 macrophages (Paul et al., 1999), J774 macrophages (Chen et al., 1999), and human monocytes (Dean et al., 1999). Activation of p38 MAPK is also involved in interleukin-1β-induced cylooxygenase-2 expression in human myometrial smooth muscle cells (Bartlett et al., 1999), cardiac myocytes (La-Pointe and Isenovic, 1999), HeLa cells (Ridley et al., 1998), rat renal mesangial cells (Guan et al., 1998b), and glomerular mesangial cells (Guan et al., 1997). Several studies using other stimuli have also linked activation of p38 MAPK to cyclooxygenase-2 expression in other types of cells (Matsuura et al., 1999; Pouliot et al., 1998; Subbaramaiah et al., 1998).

In the present study, we investigated the effect of PLL-derived AGEs on cyclooxygenase-2 expression in the murine macrophage cell line, RAW 264.7. We found that the PLL-AGEs stimulated a dose- and time-dependent up-regulation of both cyclooxygenase-2 protein expression and cyclooxygenase activity. These effects were blocked by pretreatment of RAW 264.7 cells with either the tyrosine kinase inhibitor, genistein, or the p38 MAP kinase inhibitor, [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole] (SB 203580), suggesting that the tyrosine kinase-p38 MAP kinase pathway is involved in this signaling event.

2. Materials and methods

2.1. Materials

Affinity-purified mouse polyclonal antibodies to cyclooxygenase-2 and p38 MAPK were obtained from Transduction Laboratory (Lexington, KY). Antibodies specific for cyclooxygenase-1 and cytosolic phospholipase A_2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Genistein, FPT inhibitor II, (2′ -amino-3′ -methoxyflavone) (PD 98059), and [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole] (SB 203580) were purchased from Calbiochem (San Diego, CA). Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 nutrient mixture (1:1), fetal bovine serum, L-glutamine, sodium pyruvate, penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, MD). 5-Bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium substrate was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany). The prostaglandin E_2 enzyme immunoassay kit was purchased from Cayman Chemical (Ann Arbor, MI). The p38 MAP kinase assay kit was purchased from New England Biolabs (Beverly, MA). All other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Preparation of poly-L-lysine-derived advanced glycosylation end products

Poly-L-lysine-derived advanced glycosylation end products (PLL-AGEs) were prepared by incubating 1 M glucose with 50 mg/ml poly-L-lysine in phosphate-buffered saline (pH 7.4) for 6 months. All incubations were performed under sterile conditions in the dark at 37 °C. After incubation, unreacted sugars were removed before the assay by extensive dialysis against phosphate-buffered saline. These solutions were stored at −20 °C before use.

2.3. Culture of RAW 264.7 cells and preparation of cell lysates

Cells of the murine macrophage cell line, RAW 264.7, were cultured in DMEM/F-12 supplemented with 2.438 g/l $NaHCO_3$, 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum, and penicillin (100 U/ml)/streptomycin (100 mg/ml). Cultures were maintained in a humidified incubator in 5% CO_2 at 37 °C. Cells were plated at a concentration of 1×10^5 cells/ml and used for the experiment when they reached 80% confluency. Cells were harvested, chilled on ice, and washed three times with ice-cold PBS. Cells were lysed in lysis buffer containing 50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 100 μg/ml phenylmethylsulfonyl fluoride, 1% Nonindent P-40, and 4% protease inhibitor cocktails. Protein concentrations in the cell lysates were determined by a BioRad protein assay following the manufacturer's recommendations. All cell lysates were stored at −70 °C until further measurements.

2.4. Polyacrylamide gel electrophoresis and Western blotting

Electrophoresis was ordinarily carried out on different percentage sodium dodecyl sulphate polyacrylamide gels. Following electrophoresis, separated proteins on the gel were electrotransferred onto a polyvinyldifluoride membrane. Nonspecific bindings were blocked with blocking buffer containing 5% fat-free milk powder for 1 h at room temperature, followed by incubation with primary antibody in blocking buffer for 2 h. The polyvinyldifluoride membrane was then incubated with alkaline phosphatase-conjugated secondary antibody for 1 h. Subsequently, the Western blots were developed with 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium as substrate.

2.5. Measurements of prostaglandin E_2 release and cyclooxygenase activity in RAW 264.7 cell cultures

RAW 264.7 cells were cultured in 24-well culture plates and treated according to the experimental design when they reached 80% confluency. The culture medium was collected to measure the release of prostaglandin E_2 from endogenous arachidonic acid. Cyclooxygenase activity was quantified by providing cells with exogenous arachidonic acid, the substrate for cyclooxygenase, and measuring its conversion into prostaglandin E_2. For the latter experiment, cells were washed and fresh medium containing arachidonic acid (30 μM) was added for 15 min at 37 °C. The medium was then removed for the enzyme immunoassay of prostaglandin E_2. Prostaglandin E_2 was assayed by using a specific enzyme immunoassay system (Cayman Chemical). Briefly, alkaline phosphatase-conjugated prostaglandin E_2 (prostaglandin E_2 tracer), prostaglandin E_2 monoclonal antibody, and either medium of the RAW 264.7 cell culture or the prostaglandin E_2 standard were added to goat anti-mouse immunoglobulin-pre-coated 96-well plates and incubated for 1 h. Free prostaglandin E_2 and prostaglandin E_2 tracer competed for a limited amount of the prostaglandin E_2 monoclonal antibody. The amount of prostaglandin E_2 tracer bound to the prostaglandin E_2 monoclonal antibody was inversely proportional to the concentration of free prostaglandin E_2. The plate was then washed and developed with *para*-nitrophenyl phosphate. The resultant color was read at 405 nm on a microtiter plate photometer. The concentration of free prostaglandin E_2 in a sample was determined by interpolation from a standard curve, and data are expressed in picograms per ml (pg/ml) for each well.

2.6. Determination of p38 MAPK activity in RAW 264.7 cell cultures

RAW 264.7 cells were pretreated with inhibitors for 30 min before incubation with 300 μg/ml PLL-AGEs for another 30 min when cells in 10-cm Petri dishes reached 80% confluency. Cells were harvested and p38 MAPK activity was assayed using a commercial kinase assay kit (New England Biolabs). Briefly, cells were lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were then immunoprecipitated using immobilized phospho-p38 MAPK kinase (Thr180/Tyr182) monoclonal antibody. Pellets obtained by immunoprecipitation were suspended and incubated with kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$, supplemented with 200 μM ATP and 2 μg activating transcription factor 2 (ATF-2) fusion protein. The reaction was stopped by the addition of sample buffer and was subjected to 10% polyacrylamide gel electrophoresis. After transfer, the polyvinyldifluoride membrane was incubated with buffer containing anti-phospho-ATF-2 (Thr71) antibody, followed by buffer containing horseradish peroxidase-conjugated anti-rabbit antibody. The membrane was developed using LumiGLO chemiluminescent reagent, and the immunoreactive bands were visualized by autoradiography.

2.7. Statistical analysis

All data are expressed as the means ± S.E.M. Comparisons between groups were made by Student's *t*-test. A difference between groups of $P < 0.05$ was considered significant.

3. Results

3.1. PLL-AGEs increase cyclooxygenase activity and cyclooxygenase-2 expression

PGE_2 accumulation was measured 24 h after the addition of various concentrations (30–1000 μg/ml) of PLL-AGEs to RAW 264.7 macrophages. Treatment with PLL-AGEs had no significant effect on prostaglandin E_2 production. However, in the presence of exogenous arachidonic acid (30 μM), PLL-AGEs increased prostaglandin E_2 production in a concentration-dependent manner (Fig. 1A). These data indicate that cyclooxygenase activity is increased by PLL-AGEs. To determine whether the elevated prostaglandin E_2 production was related to upregulation of the 70-kDa cyclooxygenase-2 protein, Western blotting analysis was performed using a cyclooxygenase-2-specific antibody. As depicted in Fig. 1B, RAW 264.7 cells did not constitutively express the cyclooxygenae-2 protein. However, cyclooxygenase-2 was upregulated by PLL-AGEs at a concentration as low as 30 μg/ml and reached a maximum at 1000 μg/ml. Barely detectable levels of cyclooxygenase-1 protein were present in RAW 264.7 cells, and no significant changes in immunoreactivity could be detected following stimulation by PLL-AGEs. To confirm that the increased expression of cyclooxygenase-2 is responsible for the increased production of prostaglandin E_2, cells were washed and treated with the cyclooxygenae-2 specific inhibitor, [*N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide] (NS-398), for 30 min prior to the addition of exogenous arachidonic acid (30 μM). Treatment of the cells with NS-398 (1 μM) abolished the PLL-AGE-induced increase in prostaglandin E_2 production (data not shown). These results suggest that the enhancement of cyclooxygenase activity by PLL-AGEs in RAW 264.7 cells is mainly attributable to cyclooxygenase-2 and not to cyclooxygenase-1.

When cells were treated with a submaximal concentration (300 μg/ml) of PLL-AGEs for various time intervals, cyclooxygenase activity was increased in a time-dependent manner, as reflected by prostaglandin E_2 secretion (meas-

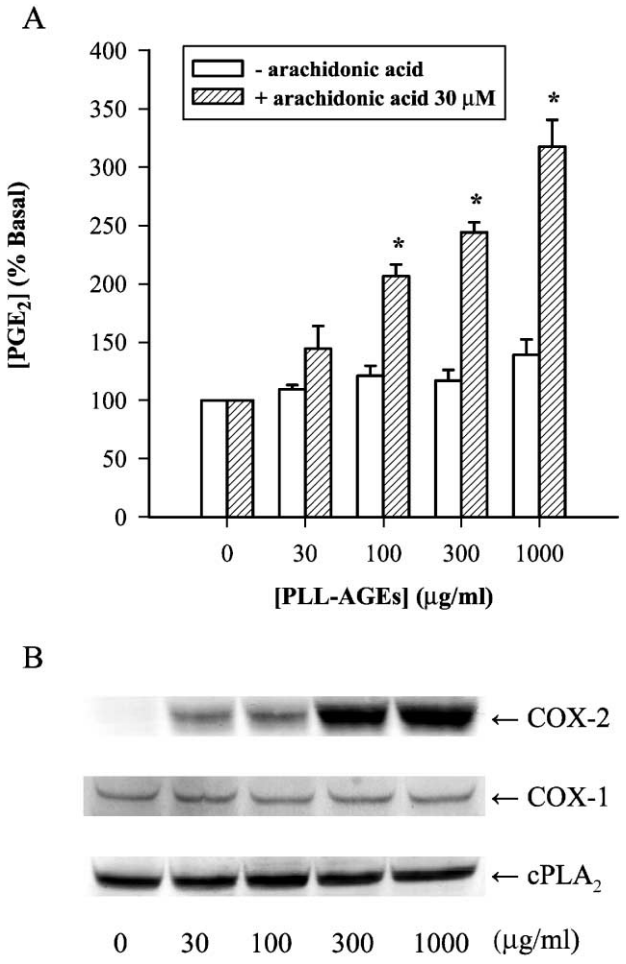

Fig. 1. Concentration-dependent increases in cyclooxygenase activity and cyclooxygenase-2 expression caused by PLL-AGEs in RAW 264.7 macrophages. In (A), cells were incubated with various concentrations of PLL-AGEs for 24 h. The supernatants were then collected and measured for prostaglandin E_2 release. Cyclooxygenase activity was quantified by measuring prostaglandin E_2 formation in the presence of exogenous arachidonic acid. Data represent means ± S.E.M. ($n=3$) of three independent experiments performed in duplicate. * $P<0.05$ as compared with the basal group. In (B), cells were incubated with the indicated concentrations of PLL-AGEs for 24 h. The extracted proteins were then immunodetected with cyclooxygenase-2-, cyclooxygenase-1- or cytosolic phodpholipase A_2 ($cPLA_2$)-specific antibody as described in Materials and methods. Equal loading of each lane was demonstrated by the similar intensity of $cPLA_2$.

ured in the presence of 30 μM exogenous arachidonic acid for 15 min) (Fig. 2A), concomitant with the upregulation of cyclooxygenase-2 protein, which began at 6 h and reached a maximum at 24 h (Fig. 2B). In the following experiments, cells were treated with 300 μg/ml of PLL-AGEs for 24 h. Since the AGEs used in these experiments were poly-L-lysine-derived, the effects of poly-L-lysine on the increase in cyclooxygenase activity and cyclooxygenase-2 expression in RAW 264.7 cells were checked. Under the same conditions, poly-L-lysine had no significant effect on either the increase in cyclooxygenase activity or cyclooxygenase-2 expression, whereas PLL-AGEs had a marked effect (data not shown), indicating the effects of poly-L-lysine can be ruled out in the following experiments.

3.2. PLL-AGE-stimulated cyclooxygenase-2 expression is not due to iNOS induction, reactive oxygen species, or lipopolysaccharide contamination

AGE-induced inducible nitric oxide synthase (iNOS) expression has been demonstrated in a variety of cell lines (Amore et al., 1997; Ido et al., 1996; Rojas et al., 1996). Since NO can stimulate cyclooxygenase-2 expression (Corbett et al., 1993; Marnett et al., 2000; Salvemini et al., 1993), RAW 264.7 cells were incubated with PLL-AGEs alone or in the presence of a nitric oxide synthase compet-

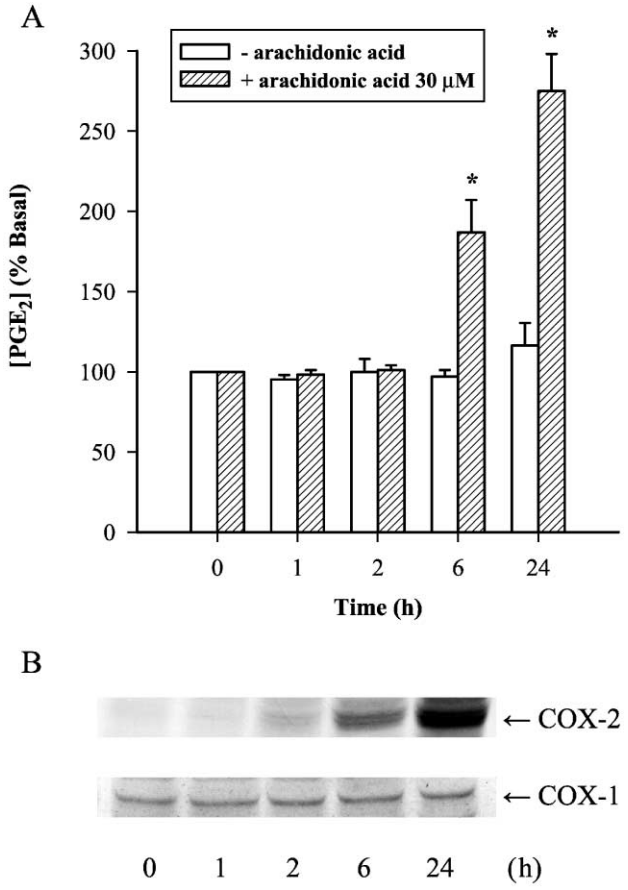

Fig. 2. Time-dependent increases in cyclooxygenase activity and cyclooxygenase-2 expression caused by PLL-AGEs in RAW 264.7 macrophages. In (A), cells were incubated with 300 μg/ml of PLL-AGEs for different time periods. The supernatants were then collected and measured for prostaglandin E_2 release. Cyclooxygenase-2 activity was quantified by measuring prostaglandin E_2 formation in the presence of exogenous arachidonic acid. Data represent means ± S.E.M. ($n=3$) of three independent experiments performed in duplicate. * $P<0.05$ as compared with the basal group. In (B), cells were incubated with 300 μg/ml of PLL-AGEs for the indicated time intervals. The extracted proteins were then immunodetected with cyclooxygenase-2- or cyclooxygenase-1-specific antibody as described in Materials and methods. Equal loading of each lane was demonstrated by the similar intensity of cyclooxygenase-1.

itive inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME). As shown in Fig. 3A, L-NAME did not inhibit PLL-AGE-induced cyclooxygenase-2 expression, suggesting that cyclooxygenase-2 expression is not secondary to iNOS induction.

It has been reported that superoxide will react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a reactive oxidant. In RAW 264.7 cells, the formation of peroxynitrite

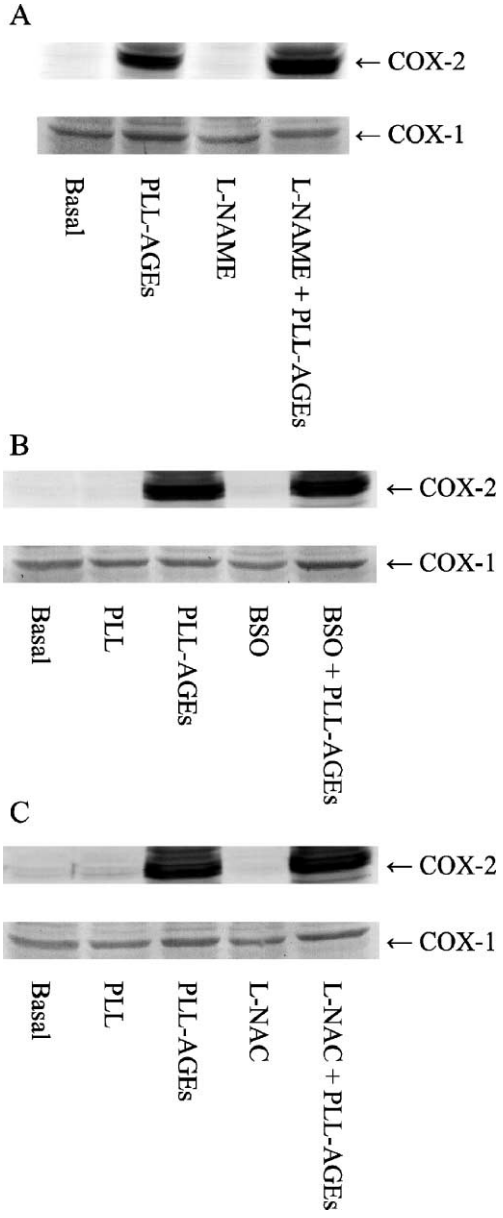

Fig. 3. Effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME), L-buthionine-[*S*,*R*]-sulfoximine (BSO), and L-nitro-acetyl-cysteine (L-NAC) on COX-2 expression induced by PLL-AGEs in RAW 264.7 macrophages. Cells were pretreated with 10 μM L-NAME (A), 100 μg/ml BSO (B), or 10 μM L-NAC (C) for 30 min before incubation with PLL-AGEs (300 μg/ml) for 24 h. The extracted proteins were then immunodetected with cyclooxygenase-2- or cyclooxygenase-1-specific antibody as described in Materials and methods. Equal loading of each lane was demonstrated by the similar intensity of cyclooxygenase-1.

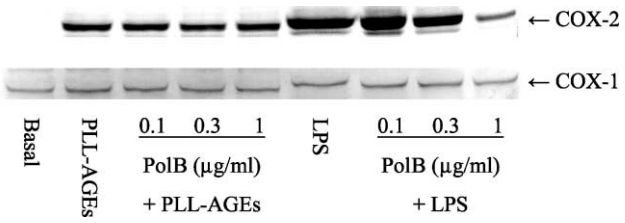

Fig. 4. Comparison between the effects of polymyxin B (PolB) on cyclooxygenase-2 expression induced by lipopolysaccharide (LPS) and PLL-AGEs in RAW 264.7 macrophages. Cells were pretreated with various concentrations of PolB for 30 min before incubation with PLL-AGEs (300 μg/ml) or LPS (0.3 μg/ml) for 24 h. The extracted proteins were then immunodetected with cyclooxygenase-2- or cyclooxygenase-1-specific antibody as described in Materials and methods. Equal loading of each lane was demonstrated by the similar intensity of cyclooxygenase-1.

can enhance both basal and lipopolysaccharide-stimulated prostanoid production by upregulating cyclooxygenase-2 (Guastadisegni et al., 1997). Reactive oxygen species per se, such as superoxide (O_2^-), also play an important role in the pathways that result in arachidonate release and prostaglandin E_2 formation by cyclooxygenase-2 in murine peritoneal macrophages stimulated by lipopolysaccharide (Martinez et al., 2000). To elucidate the role of reactive oxygen species on PLL-AGE-induced cyclooxygenase-2 expression in RAW 264.7 cells, L-buthionine-[*S*,*R*]-sulfoximine, a γ-glutamylcysteine synthetase inhibitor, and L-nitro-acetyl-cysteine, a known glutathione precursor, were used to pretreat RAW 264.7 cells, respectively, followed by a 24-h incubation with PLL-AGEs. As shown in Fig. 3B, L-buthionine-[*S*,*R*]-sulfoximine did not enhance PLL-AGE-induced cyclooxygenase-2 expression. The antioxidant L-nitro-acetyl-cysteine, which possesses both direct reducing activity and increases cellular glutathione (Moldeus and Cotgreave, 1994), also did not affect PLL-AGE-induced cyclooxygenase-2 expression (Fig. 3C). Thus, reactive oxygen species are not involved in PLL-AGE-induced cyclooxygenase-2 expression.

There is a possibility that lipopolysaccharide may be involved in PLL-AGE-induced cyclooxygenase-2 expression through contamination of lipopolysaccharide during the preparation of PLL-AGEs. To rule out this possibility, RAW 264.7 cells were pretreated with different concentrations of polymyxin B, which binds and inactivates endotoxin, before incubation with PLL-AGEs or lipopolysaccharide (0.3 μg/ml). PLL-AGE-induced cyclooxygenase-2 expression was not affected by polymyxin B, whereas lipopolysaccharide-induced cyclooxygenase-2. expression was suppressed dose-dependently (Fig. 4). Thus, it is clear that lipopolysaccharide is not involved in these reactions.

3.3. Mechanisms by which PLL-AGEs increase cyclooxygenase activity and cyclooxygenase-2 expression

To elucidate the mechanisms responsible for changes in cyclooxygenase activity and cyclooxygenase-2 protein

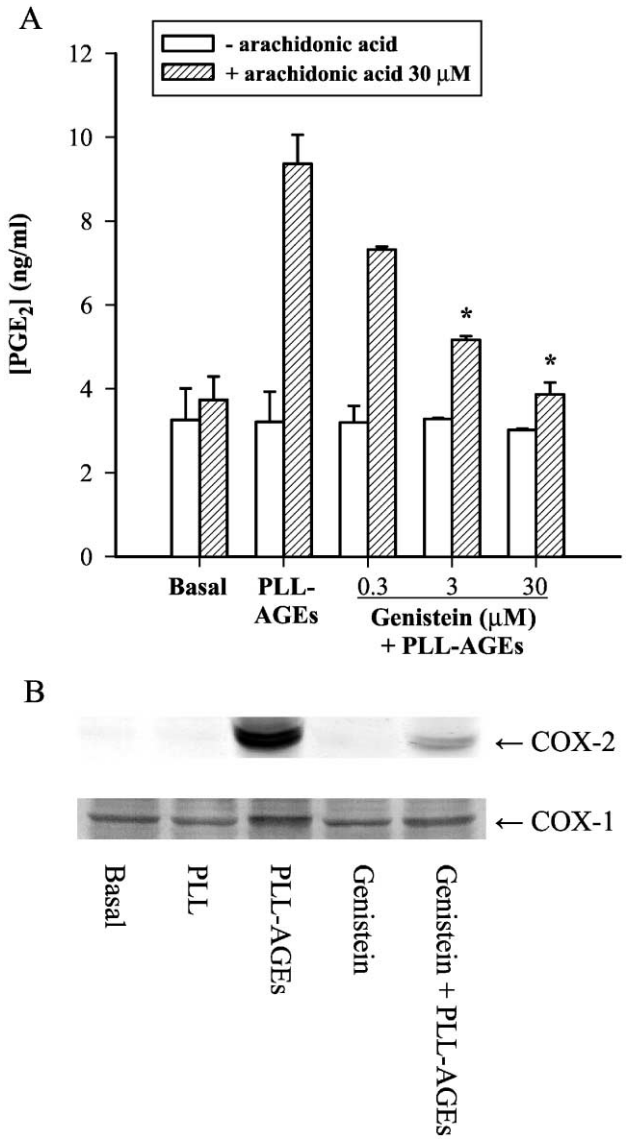

Fig. 5. Effects of genistein on the increases in cyclooxygenase activity and cyclooxygenase-2 expression induced by PLL-AGEs in RAW 264.7 macrophages. In (A), cells were pretreated with various concentrations of genistein for 30 min before incubation with PLL-AGEs (300 μg/ml) for 24 h. The supernatants were then collected and measured for prostaglandin E_2 release. COX activity was quantified by measuring PGE_2 formation in the presence of exogenous arachidonic acid. Data represent means ± S.E.M. ($n=3$) of three independent experiments performed in duplicate. * $P<0.05$ as compared with PLL-AGEs alone. In (B), cells were pretreated with 30 μM genistein for 30 min before incubation with PLL-AGEs (300 μg/ml) for 24 h. The extracted proteins were then immunodetected with cyclooxygenase-2- or cyclooxygenase-1-specific antibody as described in Materials and methods. Equal loading of each lane was demonstrated by the similar intensity of cyclooxygenase-1.

expression, RAW 264.7 cells were incubated with PLL-AGEs alone or in the presence of various inhibitors. Genistein (0.3–30 μM), a protein tyrosine kinase inhibitor, suppressed the activity of cyclooxygenase in RAW 264.7 cells incubated with PLL-AGEs in a concentration-dependent manner (Fig. 5A). The upregulation of cyclooxygenase-2 was also inhibited by 30 μM of genistein (Fig. 5B). SB 203580 (0.1–10 μM), a selective inhibitor of p38 MAPK, also inhibited both PLL-AGEs-induced cyclooxygenase activity and cyclooxygenase-2 expression in RAW 264.7 cells in a dose-dependent manner (Fig. 6). However, pretreatment of RAW 264.7 cells with a Ras farnesylation inhibitor II (10–30 μM) (Fig. 7A), or PD 98059 (10–30 μM), a MAP kinase kinase selective inhibitor (Fig. 7B), did not affect PLL-AGE-induced cyclooxygenase-2 expression. These data suggest that protein tyrosine kinase and p38 MAPK are important for mediating the induction of cyclooxygenase activity and cyclooxygenase-2 expression by PLL-AGEs.

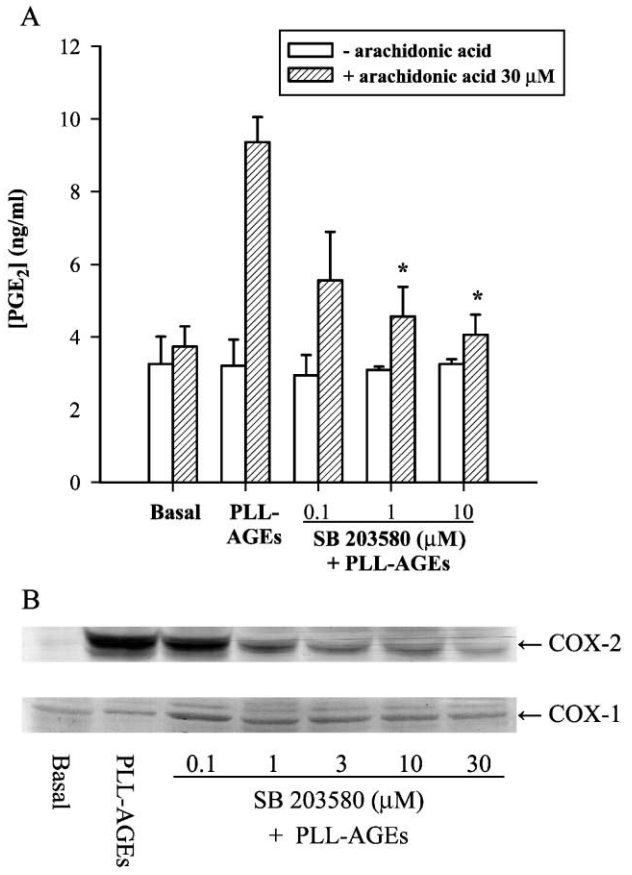

Fig. 6. Effects of SB 203580 on the increases in cyclooxygenase activity and cyclooxygenase-2 expression induced by PLL-AGEs in RAW 264.7 macrophages. In (A), cells were pretreated with various concentrations of SB 203580 for 30 min before incubation with PLL-AGEs (300 μg/ml) for 24 h. The supernatants were then collected and measured for PGE_2 release. Cyclooxygenase activity was quantified by measuring PGE_2 formation in the presence of exogenous arachidonic acid. Data represent means± S.E.M. ($n=3$) of three independent experiments performed in duplicate. * $P<0.05$ as compared with PLL-AGEs alone. In (B), cells were pretreated with various concentrations of SB 203580 for 30 min before incubation with PLL-AGEs (300 μg/ml) for 24 h. The extracted proteins were subjected to Western blotting with cyclooxygenase-2- or cyclooxygenase-1-specific antibody as described in Materials and methods. Equal loading of each lane was demonstrated by the similar intensity of cyclooxygenase-1.

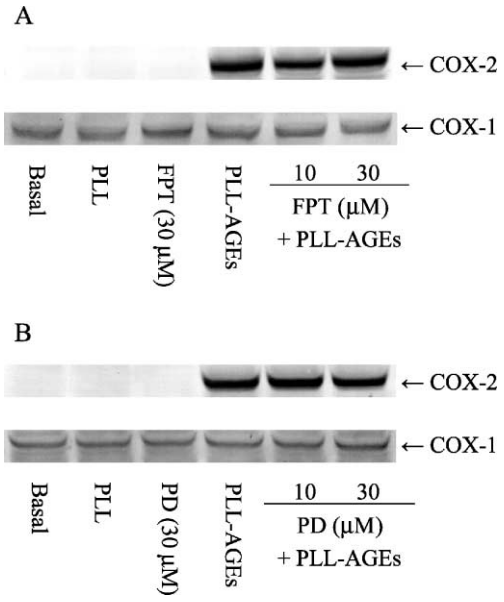

Fig. 7. Effects of FPT inhibitor II (FPT) and PD 98059 (PD) on cyclooxygenase-2 expression induced by PLL-AGEs in RAW 264.7 macrophages. Cells were pretreated with different concentrations of FPT-II inhibitor (A) or PD 98059 (B) for 30 min before incubation with PLL-AGEs (300 μg/ml) for 24 h. The extracted proteins were then immunodetected with cyclooxygenase-2- or cyclooxygenase-1-specific antibody as described in Materials and methods. Equal loading of each lane was demonstrated by the similar intensity of cyclooxygenase-1.

3.4. PLL-AGES activate p38 mitogen-activated protein kinase

To further investigate whether protein tyrosine kinase is the upstream activator of p38 MAPK, the p38 MAPK assay was performed in the presence or absence of the protein tyrosine kinase inhibitor, genistein. Pretreatment of cells for 30 min with genistein (30 μM) and SB 203580 (10 μM) inhibited the PLL-AGE-induced increase in p38 MAPK activity (Fig. 8). Consistent with the effects of PLL-AGEs on cyclooxygenase activity and cyclooxygenase-2 expression, poly-L-lysine alone had no effect on p38 MAPK activity. These results suggest that PLL-AGEs may activate p38 MAPK through upstream tyrosine phosphorylation and finally induce cyclooxygenase-2 expression in RAW 264.7 cells.

4. Discussion

AGEs are present in lesions, possess a wide range of biological properties potentially occurring during the pathogenesis of diabetic complications, and are believed to play an important role in the pathogenesis of diabetic complications (Brownlee, 1991). In the present study, we demonstrated that PLL-AGEs increased cyclooxygenase activity by inducing cyclooxygenase-2 expression in murine RAW 264.7 macrophages. The upregulation was restricted to cyclooxygenase-2 and did not involve cyclooxygenase-1. The AGE-induced response is a consequence of the activation of p38 MAP kinase. To our knowledge, this is the first report that demonstrates that AGEs can stimulate cyclooxygenase-2 expression. There is growing evidence that cyclooxygenase-2 is widely expressed in arteriosclerotic lesions (Baker et al., 1999; Yan et al., 2000). The induction of cyclooxygenase-2 may be a critical mechanism in AGE-induced diabetic complications, especially diabetic arteriosclerosis.

AGEs have been shown to activate protein tyrosine kinase (Khechai et al., 1997), p21 Ras (Lander et al., 1997), protein kinase C-βII (Scivittaro et al., 2000), p42 MAPK (Simm et al., 1997), and nuclear factor-κB (Huttunen et al., 1999; Mohamed et al., 1999). In this study, we demonstrated that the tyrosine kinase inhibitor, genistein, and the p38 mitogen-activated protein kinase inhibitor, SB 203580, inhibited PLL-AGE-induced cyclooxygenase-2 expression. However, the Ras inhibitor, FPT inhibitor II, and the MAP kinase kinase inhibitor, PD 98059, had no effect on PLL-AGE-induced cyclooxygenase-2 expression. These results suggest that AGE-induced cyclooxygenase-2 expression may occur via upstream tyrosine phosphorylation and p38 MAP kinase activation, whereas p21 Ras and the p44/42 MAPK are not involved in the signaling cascades in RAW 264.7 cells. Consistently, we showed that AGEs increased p38 MAP kinase activity, an effect which was inhibited by the tyrosine kinase inhibitor, genistein. The cyclooxygenase-2 enzyme can be induced by different mitogen-activated protein kinase cascades such as p38 MAPK (Chen et al., 1999), p44/42 MAPK (Paul et al., 1999), mitogen- and stress-activated protein kinases-1 and -2 (MSK1, MSK2) and their putative substrates, the transcription factors cyclic AMP response element-binding protein (CREB) and activating transcription

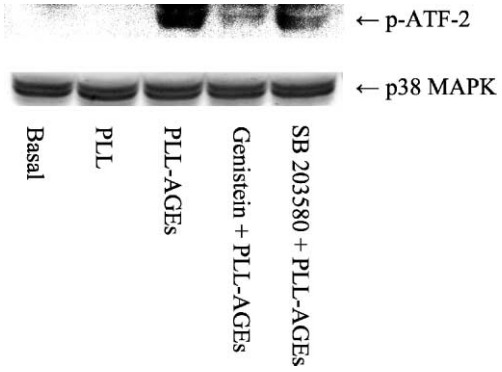

Fig. 8. Effects of genistein and SB 203580 on the increase in p38 mitogen-activated protein kinase (MAPK) activity induced by PLL-AGEs in RAW 264.7 macrophages. RAW 264.7 cells were pretreated with genistein (30 μM) or (SB 203580) (10 μM) before incubation with PLL-AGEs (300 μg/ml) for 30 min. The extracted protein was then subjected to the immunoprecipitate-kinase assay as described in Materials and methods. p38 MAPK activity was measured by phosphorylation of activating transcription Factor 2. Equal loading of each lane was demonstrated by the similar intensity of p38 MAPK.

factor-1 (ATF-1) (Caivano and Cohen, 2000). Taken together, our data suggest that AGEs may activate tyrosine kinase, which in turn stimulates p38 MAPK and results in cyclooxygenase-2 induction.

The murine cyclooxygenase gene promoter contains a NF-κB binding site. Given that the NF-κB signaling pathway regulates cyclooxygenase-2 expression (Lim et al., 2001), the transcription factor NF-κB may play an important role in AGE-induced cyclooxygenase-2 expression. Indeed, we have found that AGE-induced cyclooxygenase-2 expression is blocked by the NF-κB inhibitor, pyrrolidone dithiocarbamate, suggesting that NF-κB is involved in this signaling event (unpublished data).

Several mechanisms may be responsible for the induction of cyclooxygenase-2 in RAW 264.7 macrophages. One of the factors is lipopolysaccharide contaminating the PLL-AGEs preparation. Preparation of PLL-AGEs requires long-term incubation of poly-L-lysine with high concentrations of glucose, which may lead to bacterial contamination. Commercially available poly-L-lysine may contain endotoxins, which may induce cyclooxygenase-2 expression as well. These possibilities were excluded by the finding that poly-L-lysine alone did not cause cyclooxygenase-2 expression and that polymyxin B inhibited lipopolysaccharide-induced but not AGE-induced cyclooxygenase-2 expression in RAW 264.7 cells. Further, AGE-induced cyclooxygenase-2 expression was attenuated by anti-RNAse-AGEs antibodies (data not shown).

Oxidative stress has been implicated in cyclooxygenase-2 induction (Martinez et al., 2000) and linked to the development of diabetic complications (Ceriello, 1999). AGEs may increase oxidative stress in many cells (Loske et al., 1998; Schmidt et al., 1996; Scivittaro et al., 2000). To test whether AGE-induced cyclooxygenase-2 expression is due to an increase in oxidative stress, we adjusted the endogenous antioxidant concentrations prior to AGEs treatment. Glutathione is one of the major intracellular antioxidants. Increasing glutathione concentrations by the glutathione precursor, L-nitro-acetyl-cysteine, or decreasing glutathione by the gamma-glutamylcysteine synthetase inhibitor, L-buthionine-[*S*,*R*]-sulfoximine, did not alter AGE-induced cyclooxygenase-2 expression, suggesting that AGE-induced cyclooxygenase-2 expression is not due to reactive oxygen species. AGEs have been shown to induce iNOS expression in many cell types (Amore et al., 1997; Ido et al., 1996; Rojas et al., 1996). Induction of iNOS leads to the accumulation of NO, which may react with H_2O_2 to form peroxynitrite, a potent inducer of the cyclooxygenase-2 gene. Co-induction of cyclooxygenase-2 and iNOS genes has been reported in many cytokine-mediated inflammatory effects (Guan et al., 1998a; Misko et al., 1995; Serou et al., 1999). In RAW 264.7 cells, pretreatment with the non-selective NOS inhibitor, L-NAME, did not affect AGE-induced cyclooxygenase-2 expression, suggesting that there is no cross-regulation among iNOS and cyclooxygenase-2, although both of them are induced after AGE stimulation. The importance of these findings is that AGEs may mediate their deleterious effects through cyclooxygenase-2 induction, which is secondary to neither oxidative stress nor NO accumulation.

In conclusion, this study provides evidence for a novel role of AGEs in the regulation of cyclooxygenase-2 expression. Our results raise the possibility that diabetic complications are mediated by AGE-induced cyclooxygenase-2 expression. It will be of interest to determine whether cyclooxygenase-2 inhibitors can be used to control diabetic squealae.

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