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Role of protein kinase C in BSA-AGE-mediated inducible nitric oxide synthase expression in RAW 264.7 macrophages

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Abstract

In the present study, the roles of protein kinase C (PKC) in BSA-derived advanced glycosylation end products (BSA-AGEs)-induced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression were investigated. Treatment of RAW 264.7 cells with BSA-AGEs caused dose- and time-dependent increases in NO release and iNOS expression in RAW 264.7 cells, whereas BSA alone had no effect on iNOS induction. The tyrosine kinase inhibitor (genistein), the phosphatidylinositol-specific phospholipase C inhibitor (U-73122), the phosphatidylcholine-specific phospholipase C inhibitor (D-609), and the PKC inhibitors (staurosporine, Ro 31-8220, and Go 6976) all inhibited BSA-AGE-induced NO release and iNOS expression in RAW 264.7 cells. Stimulation of RAW 264.7 cells with BSA-AGEs resulted in the formation of inositol monophosphate; the response was attenuated by U-73122 and genistein. BSA-AGEs stimulated PKC-α, -βI, -δ, and -η but not -ζ translocation from the cytosol to the membrane. However, incubation of RAW 264.7 cells with BSA-AGEs increased phosphorylation of PKC-ζ at threonine-410, which reflects activation of PKC-ζ, indicating the possible involvement of these PKC isoforms in AGE-mediated effects. Pretreatment of RAW 264.7 cells with U-73122, D-609, and genistein reduced the AGE-stimulated translocation of PKC-α, -βI, -δ, and -η and activation of PKC-ζ. Taken together, these data suggest that BSA-AGEs might activate PKC and subsequently induce iNOS expression and NO release.

Keywords: Advanced glycosylation end products; Inducible nitric oxide synthase; Nitric oxide; Protein kinase C; RAW 264.7 macrophages

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. These complications have been related to the AGEs. AGEs are formed by the non-enzymatic ‘‘Maillard reaction’’, and have been considered to be an important factor in mediating diabetic sequelae [1]. AGEs are recognized by specific AGE receptors [2] and exert various biological effects. The selective presence of AGE receptors has been demonstrated in endothelium [3], mononuclear phagocytes [4], smooth muscle cells, mesangial cells, and certain neurons [5]. An AGE receptor complex can trigger signal transduction resulting in the production of tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) [6].

AGE-stimulated NO release has been implicated in the pathogenesis of diabetic sequelae [7,8]. NO is a diffusible gas that is generated enzymatically from L-arginine by NOS [9]. To date, three types of NOSs have been characterized. Two of them are continuously expressed and are called constitutive NOS:NOS-I (nNOS) is present essentially in neurons of the central and peripheral nervous systems, and NOS-III (eNOS) is originally localized in the cytoplasmic membrane of vascular endothelial cells; these two enzymes are calcium and calmodulin dependent.

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Abbreviations: AGEs, advanced glycosylation end products; FBS, fetal bovine serum; DAG, diacylglycerol; iNOS, inducible nitric oxide synthase; PC-PLC, phosphatidylcholine-specific phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; PKC, protein kinase C; PMA, phorbolmyristate acetate; TBST, Tris-buffered saline/Tween 20.

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On the other hand, the inducible type, NOS-II (iNOS) is calcium and calmodulin independent and is induced by a variety of signals in many cell lines [10]. NO plays important roles in both physiological and pathological conditions. Low concentrations of NO have been shown to serve as a neurotransmitter and vasodilator, while at high concentrations it is toxic and may be important in several neurodegenerative diseases [11]. AGEs induce iNOS expression in a variety of cell lines. Several lines of evidence suggest that different PKC isoforms are involved in the LPS- and cytokine-induced iNOS gene expression [12–14]. However, the role of PKC isoforms in AGE-stimulated iNOS expression in RAW 264.7 cells has not been delineated.

PKC was originally described as a Ca^{2+}- and phospholipid-dependent protein kinase activated by 1,2-diacylglycerol (DAG) and other lipids. The PKC pathway represents a major signal transduction system, and different tissues seem to have their own characteristic patterns of PKC isoform expression. To date, PKCs can be divided into three groups: (1) conventional PKCs (cPKCs), comprise the α, βI, βII, and γ isoforms (the PKC-β gene is alternatively spliced to produce two gene products which differ only in their extreme C-terminal ends). These PKC isotypes have Ca^{2+}- and DAG-dependent kinase activity. The cPKCs are targets of the tumor-promoting phorbol ester, PMA, which activates these enzymes by eliminating the requirement for DAG and decreasing the concentration of Ca^{2+} needed for activation; (2) novel PKCs (nPKCs), including the PKC-δ, -ε, -η, and -θ isoforms, are Ca^{2+} independent and regulated by DAG and phosphatidylserine, and (3) atypical PKCs (aPKCs), including PKC-ζ and -ι/λ isoforms, are Ca^{2+}- and DAG-independent kinases. In contrast to the conventional or novel PKCs, the aPKC isoforms do not respond to phorbol esters or DAG. aPKCs are targets of important lipid second messengers, such as ceramide, phosphatidic acid, and phosphatidylinositol-3-phosphate [15,16].

Many different mechanisms have bee proposed to explain how AGEs can damage various organs [17]. PKC activation is one of the sequelae of hyperglycemia and is thought to play a role in the development of diabetic complications [17]. In the present study, the role of PKC in the induction of iNOS in response to AGEs was studied. Our data reveal that AGEs might activate many signaling pathways, which result in PKC activation and subsequently iNOS induction.

2. Materials and methods

2.1. Materials

The antibodies specific for iNOS and PKC-ζ were obtained from Santa Cruz Biotechnology. Rat antibodies specific for phospho-PKC-ζ/ι (Thr 410/403) and antibodies specific for other PKC isoforms were purchased from Transduction Laboratories. Mouse or rabbit biotinylated immunoglobulins and 5-bromo-4-chloro- 3-indolyl-phosphate/4-nitro blue tetrazolium (BCIP/NBT) substrate was purchased from Kirkegaard & Perry Laboratories. Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim. Dulbecco's Modified Eagle's Medium (DMEM), FBS, glutamine, penicillin, and streptomycin were purchased from Life Technologies. Genistein, U-73122, D-609, staurosporine, Go 6976, and Ro 31-8220 were purchased from Calbiochem. All other chemicals were from Sigma.

2.1.1. Preparation of BSA-AGEs

BSA-AGEs were prepared by incubating 1 M glucose with 50 mg/mL BSA in PBS, pH 7.4, in the presence of PMSF (1.5 mM), EDTA (1 mM), and antibiotics (100 μg/mL penicillin and 40 μg/mL gentamicin) for at least 6 weeks. All incubations were performed under sterile conditions in the dark at 37°. After incubation, unreacted sugar was removed before the assay by extensive dialysis against PBS. The solution was separated into aliquots and stored frozen before use.

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

Cells from 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% FBS, and penicillin (100 U/mL)/streptomycin (100 mg/mL). Cultures were maintained in a humidified incubator in 5% CO_2 at 37°. Cells were plated at a concentration of 1×10^5 cells/mL and used for the experiment when they reached 80% confluency. Cells were cultured in 24-well plates for nitrite determination and in Petri dishes for protein expression. For preparation of cell lysates, cells were chilled on ice and washed three times with ice-cold PBS after incubation with AGEs and appropriate ligands. Subsequent procedures were conducted on ice unless otherwise specified. Cells were lysed in lysis buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM DTT, 0.1% mercaptoethanol, 0.5% Triton X-100, and the protease inhibitor cocktail (final concentration: 0.2 mM PMSF, 20 μg/mL aprotinin, 20 μg/mL leupeptin). Protein concentrations in cell lysates were determined by a BioRad protein assay following the manufacturer's recommendations. All cell lysates were stored at −70° until further measurements.

2.1.3. Assay for nitrite concentration

NO production in culture supernatant was assayed by measuring nitrite, its stable degradation product, using the Griess reagent. DMEM was changed to phenol red-free medium before the cells were treated with 300 μg/mL BSA-AGE. After 24 hr of incubation, the isolated supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 5% phosphoric acid) and incubated at room

temperature for 10 min. The absorbance was measured at 550 nm in a microplate reader. Sodium nitrite ($NaNO_2$) was used as a standard. In pretreatment experiments, cells were incubated with genistein (a tyrosine kinase inhibitor), U-73122 (a phosphatidylinositol-specific phospholipase C (PI-PLC) inhibitor), or staurosporine, Go 6976, and Ro 31-8220 (PKC inhibitors) for 30 min before the addition of AGEs.

2.1.4. Labeling of phospholipids and measurement of myo-[2-^{3}H]inositol phosphates

Levels of labeled inositol phosphates were measured as described previously [18]. In brief, cells were labeled for 24 hr in DMEM containing *myo*-[2-^{3}H]inositol (2.5 μCi/mL). At the end of the labeling period, LiCl (10 mM) and appropriate inhibitors were added and incubated at 37° for 20 min before stimulation with BSA-AGEs (300 μg/mL). After 1 hr of incubation, cells were washed with ice-cold PBS. Cells adhering to the plates were scraped off using a rubber policeman. The reaction was stopped with 1.2 mL of chloroform/methanol (1:2 v/v), and 0.5 mL of chloroform and 0.5 mL of 0.25 M HCl were added to form two phases. After centrifugation at 800 *g* for 10 min at 4°, 1-mL aliquots of the aqueous phases were neutralized with 1.5 M NH_4OH. Then, 5 mL of distilled water and 1 mL of Dowex 1 × 8 (100–200 mesh) resin slurry were added. The mixtures were then loaded onto columns after washing with 5 mL of water four times and 5 mL of 60 mM sodium formate/5 mM borax twice to remove [^{3}H]inositol and [^{3}H]glyceroylinositol. [^{3}H]Inositol monophosphate ([^{3}H]IP_1) was eluted with 2 × 5 mL of 0.2 M ammonium formate/0.1 M formic acid in scintillation counting vials. Scintillation counting cocktail was added, and the radioactivity was measured using a scintillation counter at 30% efficiency.

2.1.5. Fractionation of cellular extracts and analysis of the translocation of PKC isoforms

Cells were chilled on ice and washed three times with ice-cold PBS after incubation with AGEs and appropriate ligands. Cells adhering to the plates were scraped off using a rubber policeman. Cells were homogenized by adding homogenization buffer containing 20 mM Tris–HCl (pH 7.5), 5 mM EGTA, 2 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 1 mM DTT, and the protease inhibitor cocktail (final concentration: 0.2 mM PMSF, 20 μg/mL aprotinin, 20 μg/mL leupeptin). The cell suspension was chilled on ice for 30 min, and then centrifuged at 800 *g* for 10 min at 4°. The supernatant (containing cytosolic and membrane fractions) was then centrifuged at 25,000 *g* for 15 min at 4°. The supernatant represents the cytosolic fraction, and the pellet membrane fraction was resuspended in homogenization buffer containing 1% NP40. The protein levels of PKC isoforms (α, βI, δ, and η) in the cytosolic and membrane fractions were determined by Western blot analysis.

2.1.6. Polyacrylamide gel electrophoresis (PAGE) and Western blotting

Electrophoresis was ordinarily carried out on different percentages of SDS–PAGE. Following electrophoresis, proteins were then transferred to a PVDF membrane by electroblotting. After transfer, the PVDF membrane was blocked with blocking buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20, 5% skimmed milk, 0.1% sodium azide], at room temperature for 30 min or at 4° overnight. Then, the membrane was incubated at room temperature successively with primary antibody (antibodies specific for iNOS or PKC isoforms) for 2 hr, and with secondary antibody for 1 hr. After each incubation, the membrane was washed three times with TBST washing buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20]. Western blots were developed with BCIP/NBT as the substrate. The immunoreactive bands were scanned using a Microteck Scanner equipped with adapter and scanning software. Bands were quantitated using Image-Pro Plus software (Media Cybernetics). Western blot values were first corrected using their corresponding α-tubulin levels.

2.1.7. Statistical analysis

Results are expressed as mean ± SEM from the number of independent experiments performed. One-way ANOVA and student's two-tailed *t* test were used to evaluate the statistical differences between means. A *P* value of less than 0.05 was taken as statistically significant.

3. Results

3.1. AGEs-stimulated NO release and iNOS expression in RAW 264.7 cells

Exposure to BSA-AGEs (30–1000 μg/mL) stimulated a dose-dependent increase in nitrite production (Fig. 1A) and 130-kDa iNOS expression in RAW 264.7 cells (Fig. 1B). The EC_{50} values of AGE-stimulated nitrite production and iNOS induction were both about 30 μg/mL, with the maximum at about 300 μg/mL of AGEs. Treatment with BSA-AGEs resulted in time-dependent nitrite accumulation and iNOS expression. The maximum nitrite accumulation in RAW 264.7 cells was seen after 36 hr of incubation with BSA-AGEs (Fig. 2A). The earliest induction of iNOS protein expression was seen at 2 hr, with a peak at 6 hr (Fig. 2B). As shown in Fig. 3A, BSA alone did not induce iNOS expression. To test whether BSA-AGE-induced iNOS expression was due to contaminated lipopolysaccharide (LPS), RAW 264.7 cells were pretreated with polymyxin B for 30 min before incubation with BSA-AGEs (300 μg/mL) or LPS (0.3 μg/mL) for 24 hr. Treatment of RAW 264.7 cells with polymyxin B, which binds and inactivates endotoxin, reduced LPS-induced iNOS expression, but did not alter BSA-AGE-induced iNOS expression (Fig. 3B).

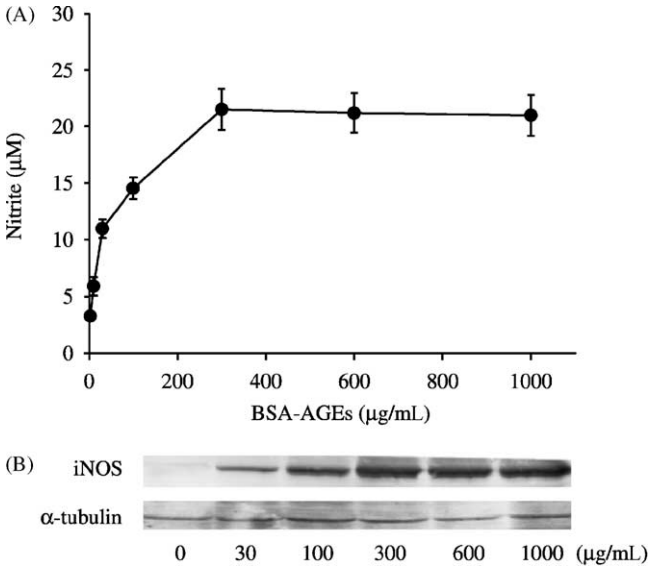

Fig. 1. Concentration-dependent BSA-AGE-induced nitrite release and iNOS expression in RAW 264.7 macrophages. In panel A, cells were incubated with various concentrations of BSA-AGEs for 24 hr, and then the culture medium was removed for nitrite measurement. Results are expressed as the mean ± SEM of five independent experiments performed in triplicate. In panel B, cells were incubated with the indicated concentrations of BSA-AGEs for 24 hr, and cell lysates were subjected to Western blotting using an iNOS-specific antibody for iNOS expression as described in "Section 2". Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Data represent a typical experiment that has been repeated three times.

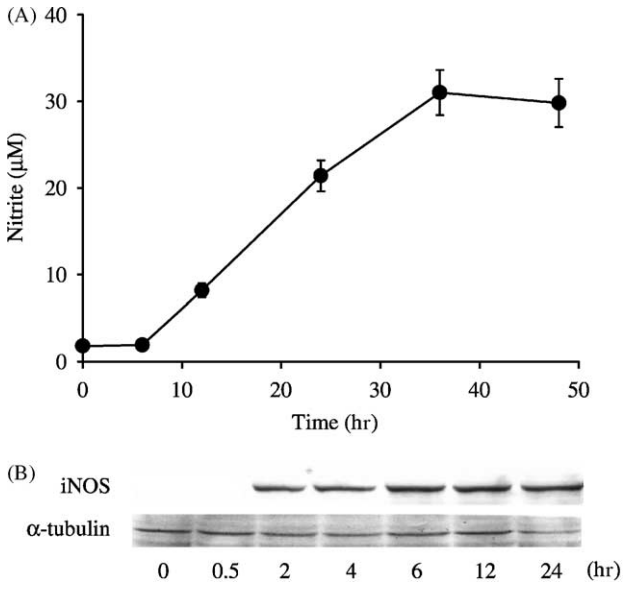

Fig. 2. Time-dependent BSA-AGE-induced nitrite release and iNOS expression in RAW 264.7 macrophages. In panel A, cells were incubated with 300 μg/mL BSA-AGEs for various time intervals, and then the culture medium was removed for nitrite measurement. Results are expressed as the means ± SEM of three independent experiments performed in triplicate. In panel B, cells were incubated with 300 μg/mL BSA-AGEs for the indicated time intervals, and cell lysates were subjected to Western blotting using an iNOS-specific antibody for iNOS expression as described in "Section 2". Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Data represent a typical experiment that has been repeated three times.

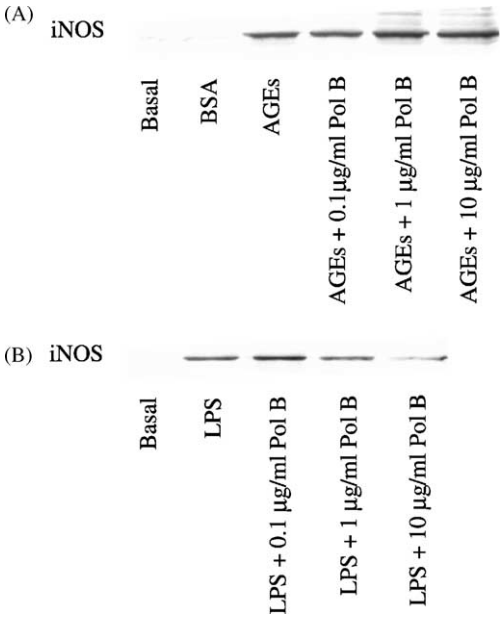

Fig. 3. Effects of polymyxin B on BSA-AGE-induced iNOS expression in RAW 264.7 macrophages. In panel A, cells were treated with 300 μg/mL BSA alone or pretreated with different concentrations of polymyxin B for 30 min, and then incubated with 300 μg/mL BSA-AGEs for 24 hr; cell lysates were subjected to Western blotting for iNOS expression. Data represent a typical experiment that has been repeated twice. In panel B, cells were pretreated with different concentrations of polymyxin B for 30 min, and then incubated with 0.3 μg/mL LPS for 24 hr, cell lysates were subjected to Western blotting for iNOS expression. Data represent a typical experiment that has been repeated twice.

3.2. Roles of tyrosine kinase, PI-PLC, and phosphatidylcholine-specific phospholipase C (PC-PLC) in AGE-induced NO production and iNOS expression in RAW 264.7 cells

Activation of the receptors of AGEs (RAGEs) may trigger a protein tyrosine kinase-related signal transduction cascade. To evaluate whether protein tyrosine kinase is involved in AGE-induced iNOS expression, the tyrosine kinase inhibitor, genistein, was used to pretreat the cells. Pretreatment of cells with genistein (10–50 μM) did not affect the basal level, while inhibited AGE-stimulated NO production in a dose-dependent manner (Fig. 4A). BSA-AGE-stimulated nitrite accumulation was inhibited by 32.7, 58.1, and 84.1% when cells were pretreated with 10, 30, and 50 μM genistein, respectively. Pretreatment of cells with the PI-PLC-specific inhibitor, U-73122, or the PC-PLC-specific inhibitor, D-609, caused a concentration-dependent inhibitory effect on AGE-stimulated nitrite production in RAW 264.7 cells (Fig. 4B and C). Consistently, AGE-induced iNOS expression was inhibited by pretreatment of cells with genistein (30 μM), U-73122 (10 μM), and D-609 (10 μM) (Fig. 4D). Thus, tyrosine kinase activation and PI-PLC and PC-PLC signaling cascades seem to be involved in AGE-stimulated NO release. Because the PI-PLC pathway may be involved in AGE-induced iNOS expression, AGE-stimulated PIP_2 turnover

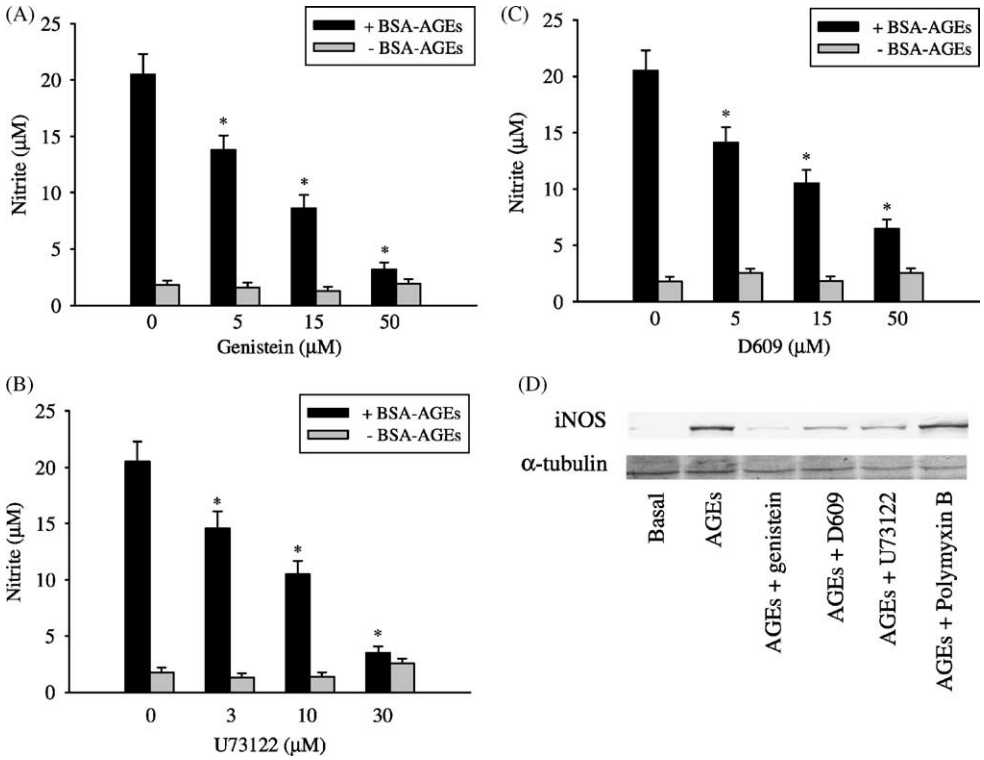

Fig. 4. Effects of genistein, U-73122, and D-609 on BSA-AGE-induced nitrite release and iNOS expression in RAW 264.7 macrophages. RAW 264.7 cells were pretreated with various concentrations of genistein (A), U-73122 (B), or D-609 (C) for 30 min, and then incubated without or with 300 mg/mL BSA-AGEs for 24 hr. The culture medium was removed for nitrite measurement. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with BSA-AGEs alone. In panel D, cells were pretreated with genistein (30 μM), U-73122 (10 μM), D-609 (10 μM), or polymyxin B (10 μg/mL), and then incubated with 300 mg/mL of BSA-AGEs for 24 hr. Cell lysates were subjected to Western blotting using an iNOS-specific antibody for iNOS expression as described in "Section 2". Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Data represent a typical experiment that has been repeated twice.

was investigated. Treatment of RAW 264.7 cells with AGEs (300 μg/mL) resulted in a time-dependent increase in the formation of inositol phosphate (Fig. 5A). AGEs stimulated a 2-fold increase in the formation of inositol phosphate after 1 hr of treatment of RAW 264.7 cells with AGEs (300 μg/mL). Pretreatment of RAW 264.7 cells with genistein (30 μM) or U-73122 (10 μM) inhibited the AGE-stimulated increase in the formation of inositol phosphate by 65 and 65%, respectively (Fig. 5B).

3.3. Involvement of PKC in AGE-induced iNOS expression and NO production in RAW 264.7 cells

Because both PI-PLC and PC-PLC may increase DAG production, which subsequently leads to PKC activation, we next examined whether PKC inhibitors inhibit AGE-stimulated effects. The PKC inhibitors, staurosporine, Go 6976, and Ro 31-8220, were used to treat cells for 30 min prior to challenge by AGEs for 24 hr. Pretreatment of RAW 264.7 cells with staurosporine (30–1000 nM), Go 6976 (1–10 μM), or Ro 31-8220 (1–5 μM) inhibited AGE-stimulated nitrite accumulation in a dose-dependent manner (Fig. 6). AGE-induced-iNOS expression was also inhibited by staurosporine (100 nM), Go 6976 (3 μM), or Ro 31-8220 (3 μM). Previous studies have demonstrated that RAW 264.7 cells express the PKC-α, -βI, -δ, -η, and -ζ isoforms [19]. To examine which PKC isoform is involved in AGE-stimulated responses, the expression of each PKC isoform in cytosol and membrane fractions was examined. In resting cells, PKC-α, -βI, -δ, and -η were all detected in the cytosolic fraction, but were not detected in the particulate (membrane) fraction. Treatment of RAW 264.7 cells with AGEs (300 μg/mL) resulted in PKC-α, -βI, -δ, and -η translocation. Increases of PKC-α, -βI, -δ, and -η in the membrane fraction became evident at 10 min and persisted for 2 hr (PKC-δ and -η) (Fig. 7A). PKC down-regulation was not observed during a time course of 24 hr. PKC-ζ, a phorbol ester-independent aPKC, was neither translocated to the membrane fraction nor down-regulated by 24-hr exposure of cells to AGEs (300 μg/mL) (Fig. 7B). Because activation of PKC is accompanied by increased phosphorylation of threonine-410 at the activation loop, which reflects the activity state of the kinase [20], antibodies specific for threonine-410 phosphorylated PKC-ζ was used to detect the activation of PKC-ζ. Incubation of RAW 264.7 cells with AGEs (300 μg/mL) for 10 min increased phosphorylation of PKC-ζ at threonine-410 suggesting PKC-ζ was activated. A known PKC-ζ activator, TNF-α (100 nM) was used as a positive control (Fig. 7C). Protein levels of PKC did not change during the experiment shown in Fig. 7B.

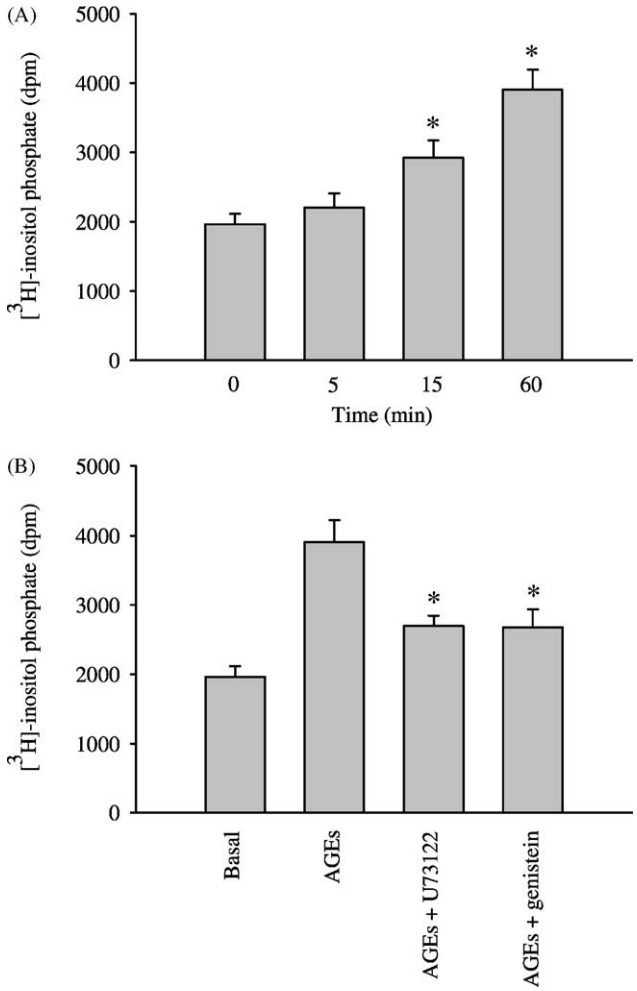

Fig. 5. Effects of U-73122 and genistein on BSA-AGE-induced [^{3}H]inositol phosphate formation in RAW 264.7 macrophages. In panel A, cells were labeled with *myo*-[^{3}H]inositol (2.5 μCi/mL) for 24 hr. LiCl (10 mM) was added before stimulation with BSA-AGEs (300 μg/mL) for different time periods, and inositol phosphate accumulation was measured as described in "Section 2". Results are expressed as the mean ± SEM of a typical experiment performed in triplicate. In panel B, cells were pretreated with 10 μM U-73122 or 30 μM genistein for 30 min before stimulation with 300 μg/mL BSA-AGEs for 1 hr. The results are expressed as the mean ± SEM of three independent experiments performed in triplicate. $^*P < 0.05$ compared with BSA-AGEs alone.

3.4. Effects of genistein, U-73122, and D-609 on PKC isoform translocation in RAW 264.7 cells

Because tyrosine kinase activation, and PI-PLC and PC-PLC signaling cascades seem to be involved in PKC-α, -βI, -δ, and -η activation, which subsequently leads to iNOS expression and NO release, we next examined whether AGE-stimulated PKC-α, -βI, -δ, and -η translocation is inhibited by genistein, U-731222, or D-609. As shown in Fig. 8, exposure of cells to AGEs (300 μg/mL) for 10 min increases PKC-α, -βI, -δ, and -η immunoreactivities in the membranes by 1.8-, 1.5-, 2.7-, and 1.3-fold, respectively. Pretreatment of cells for 30 min with genistein (30 μM) reduced the BSA-AGE-stimulated increase in PKC-α, -βI, -δ, and -η immunoreactivities in the membranes to 0.7-, 0.2-, 0.4-, and 0.05-fold, respectively (Fig. 8). Similar results were seen when cells were pretreated with U-73122 (10 μM), or D-609 (10 μM) for 30 min. The combination of D-609 and U-73122 resulted in more marked inhibition of PKC translocation in the membranes. Pretreatment of the cells for 30 min with U-73122 (10 μM) and D-609 (10 μM) reduced the increase in membrane PKC-α, -βI, -δ, and -η immunoreactivities to 0.6-, 0.2-, 0.9-, and 0.1-fold, respectively. Pretreatment of the cells for 30 min with genistein (30 μM), U-73122 (10 μM), or D-609 (10 μM) also reduced the increase of PKC-ζ phosphorylation by AGEs (Fig. 8B). Therefore, BSA-AGEs may stimulate PKC activation and finally induce iNOS expression in RAW 264.7 cells.

4. Discussion

AGEs bind to their receptors, i.e. RAGEs, resulting in alteration of a variety of gene expressions. They may increase the production of proinflammatory substances and lead to the development of diabetic complications. AGEs stimulate iNOS expression in a variety of cell lines [7,21], which in turn stimulate NO production. However, the signal transduction pathway of AGE-induced iNOS expression has not been elucidated. We previously demonstrated that p38 MAP kinase is involved in AGE-induced iNOS expression and NO accumulation in C6 glioma cells [22]. In this report, we present evidence that protein tyrosine kinase, PC-PLC, PI-PLC, and PKC are involved in the signal transduction pathway leading to the expression of iNOS.

Several mechanisms may contribute to the induction of iNOS in RAW 264.7 macrophages. One possibility is that AGEs stimulate cytokine release. Indeed, previous reports have shown that AGEs may increase the release of cytokines including IL-1β, TNF-α, and IGF-1 [6,23,24]. These cytokines may induce iNOS expression and NO production. However, whether AGEs directly induce iNOS expression or indirectly mediate its expression through cytokine release remains to be determined. The other possibility is that LPS contamination in the BSA-AGEs preparation causes activation of the signaling cascade. Preparation of BSA-AGEs requires long-term incubation of BSA with a high concentration of glucose, which may lead to bacterial contamination. Commercially available BSA itself may contain endotoxin that may induce iNOS expression as well. These possibilities were excluded by the facts that (1) BSA alone did not cause iNOS expression; (2) while LPS-induced iNOS expression was blocked by polymyxin B, the AGE-stimulated response was not; and (3) LPS-induced iNOS expression is dependent on the presence of serum in the culture medium [20], while AGE-induced iNOS expression is performed in serum-free medium. Furthermore, we previously demonstrated that BSA-AGE-stimulated nitrite accumulation was attenuated

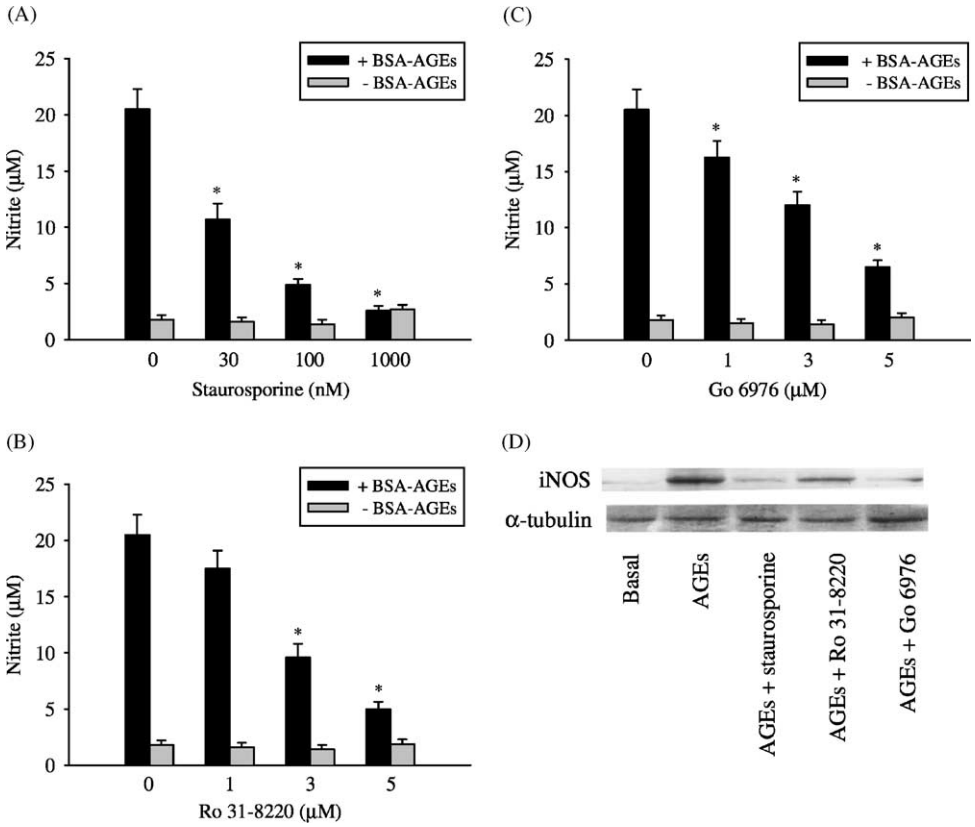

Fig. 6. Effects of PKC inhibitors on BSA-AGE-induced nitrite release and iNOS expression in RAW 264.7 macrophages. RAW 264.7 cells were pretreated with different concentrations of staurosporine (A), Ro 31-8220 (B), or Go 6976 (C) for 30 min, and then incubated without or with 300 μg/mL BSA-AGEs for 24 hr. After incubation, the culture medium was removed for nitrite measurement. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with BSA-AGEs alone. In panel D, cells were pretreated with staurosporine (100 nM), Ro 31-8220, (3 μM), or Go 6976 (3 μM) for 30 min, and then incubated with 300 μg/mL BSA-AGEs for 24 hr. Cell lysates were subjected to Western blotting for iNOS expression as described in "Section 2". Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Data represent a typical experiment that has been repeated three times.

by pretreatment of C6 glioma cells with RNase-AGE-specific antibodies [22].

It has been shown that overexpression of PKC increases iNOS expression [25]. Consistent with this finding, three PKC inhibitors, Go 6976, Ro 31-8220, and staurosporine, dose-dependently inhibited AGE-stimulated iNOS expression and NO release, indicating that PKC activation is involved in the AGE-mediated regulation of NO release and iNOS expression in RAW 264.7 cells. The role of PKC in AGE-induced iNOS expression was further confirmed by AGE-stimulated PKC translocation. DAG is a well-established activator of PKC [26] that may be generated directly by PI-PLC-catalyzed PI breakdown or PC-PLC-catalyzed phosphatidylcholine breakdown [27]. We have demonstrated that treatment of RAW 264.7 cells with AGEs for 1 hr may double the inositol phosphate production (Fig. 5). On the other hand, in NIH 3T3 cells, elevated levels of PC-PLC-derived DAG activate PKC isoform λ [28]. Thus, AGE activation of PI-PLC and PC-PLC may increase DAG production, which may activate PKC. The fact that genistein attenuated AGE-induced PI hydrolysis, indicating that the PI-PLC involved might be PLC γ, since PLC γ is an SH_2 domain-containing protein that utilizes this module to link phosphortyrosine-containing sequences in a receptor protein or cytoplasmic protein tyrosine kinase to PI hydrolysis. However, genistein at 50 μM inhibits AGE-stimulated nitrite production by 84%, yet only inhibits AGE-stimulated inositol phosphate accumulation by 65%. Given protein tyrosine kinase may mediate many signaling pathways, the interpretation that inhibition of AGE-stimulated protein tyrosine kinase activation always involves PI-PLC activation may not be correct.

Recent studies have revealed that many diabetic complications are associated with the activation of PKC [8,29]. Among the various PKC isoforms, PKC-β appears to be important in vascular dysfuntion [8], PKC-α and -ε have been related to the development of insulin resistance [30], many other PKC isotypes are also shown to play important role in diabetic complications [16]. In agreement with this, we demonstrate that treatment of RAW 264.7 cells with AGEs results in PKC-α, -βI, -δ, and -η translocation. Additionally, the AGE-stimulated effect was inhibited by Go 6976, an inhibitor of the cPKC isoforms that inhibits the Ca^{2+}-dependent isozymes PKC-α and -βI, but has no effect on the kinase activity of Ca^{2+}-independent subtypes PKC-δ, -ε, and -ζ. Another PKC inhibitor, Ro 31-8220,

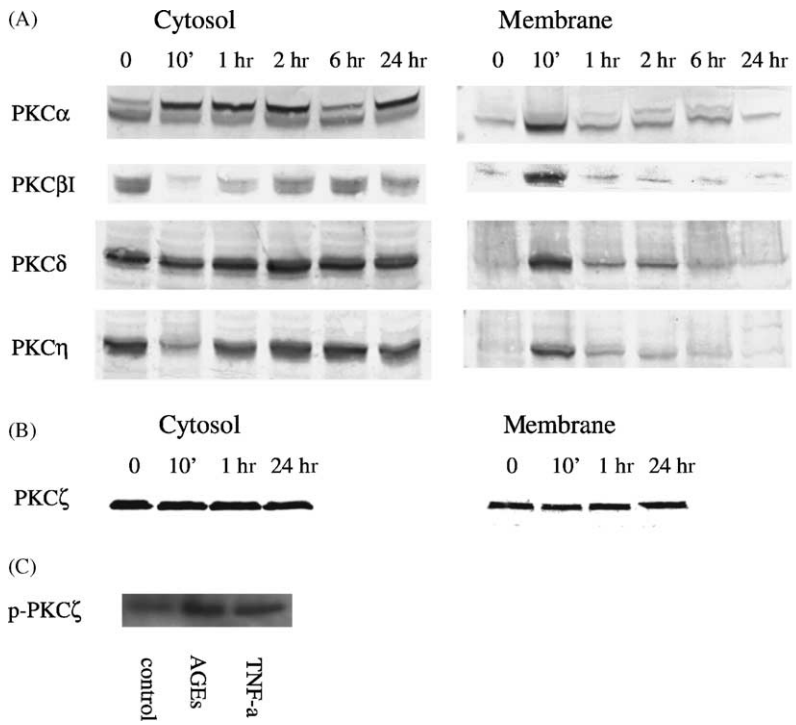

Fig. 7. Translocation of PKC isoforms in response to BSA-AGEs in RAW 264.7 macrophages. Confluent cells were treated with 300 μg/mL BSA-AGEs for 10 min, or 1, 2, 6, or 24 hr, and then separated into cytosol and membrane fractions. Proteins in the cytosol and membrane fractions were subjected to Western blot analysis using specific PKC-α, -βI, -δ, and -η antibodies (A) or PKC-ζ specific antibodies (B) as described in "Section 2". In panel C, cells were incubated with 300 μg/mL BSA-AGEs (lane 2) or TNF-α (lane 3) for 10 min. Cell lysates were subjected to Western blotting and immunoblotted with anti-phospho-PKC-ζ/ι (Thr 410/403) antibodies as described in "Section 2". Data represent a typical experiment that has been repeated three times.

which inhibits PKC-α, -βI, -γ, and -ε, also inhibited AGE-stimulated iNOS expression and nitrite accumulation in RAW 264.7 cells [16]. Our inhibitor data support the concept that PKC-α and -βI are important in AGE-induced iNOS expression, but these results do not distinguish relative importance of these isotypes. Moreover, most of the PKC inhibitors have potential limitations. For example, staurosporine and Ro 31-8220 have some PKC-independent effects, whereas Go 6976 may inhibit PKC-μ at high concentrations. Because few pharmacological agents that show good selectivity for individual PKC isoforms, strategies such as expression of overexpression or dominant negative mutants or treatment with antisense oligonucleotides are required to delineate the relative importance of these specific isotypes. However, three different PKC inhibitors were unable to completely block the AGEs response (Fig. 5), indicating that other AGE-activated components might also be involved in NO production.

PKC-ζ, an aPKC, can be activated by TNF-α [31] and IL-1β [32] and is involved in AGE-induced NO production in RAW 264.7 cells [25]. Incubation of mesangial cells with IL-1β causes translocation of PKC-ζ from the cytosolic fraction to the particulate (membrane) compartment [32]. However, incubation of RAW 264.7 cell with AGEs does not result in translocation of PKC-ζ from cytosol to membranes. Because PKC-ζ lacks the Ca^{2+} binding (C2) domain and has only one instead of two cysteine-rich zinc finger motifs in the C1 domain responsible for phorbol ester binding [15,16], PKC-ζ does not appear to bind phorbol esters and is neither translocated to the membrane fraction nor down-regulated in response to phorbol ester [16]. In an attempt to determine whether PKC-ζ is activated by AGEs or not, we carried out an experiment to detect the phosphorylation of threonine-410 at the activation loop of PKC-ζ, which reflects the activity state of PKC-ζ [20]. Incubation of RAW 264.7 cells with AGEs increases phosphorylation of PKC-ζ at threonine-410 suggesting PKC-ζ is activated by AGEs stimulation. Inhibition of AGE-stimulated PKC-ζ phosphorylation by genistein, U-73122, and D-609 deserve further discussion. Although PKC-ζ is considered as an aPKC, which does not respond to phorbol esters or diacyglycerol, substantial evidence emerged that activation of PC-PLC and generation of diacyglycerol by PLC may lead to activation of PKC-ζ [33–36]. Although there are evidences showing PC-PLC is coupled to acidic sphingomyelinase, the mechanisms that couple PLC and PKC are not completely clear.

In conclusion, AGEs may activate PI-PLC and PC-PLC through an upstream protein tyrosine kinase to elicit PKC activation and subsequently induce iNOS expression. Of the PKC isoforms present in RAW 264.7 cells, PKC-α, -βI, -δ, -η, and -ζ activations may be involved in AGE-induced iNOS expression. This study supports the notion that PKC activation plays an important role in the development of diabetic complications, and the isoform-specific inhibitors may be useful for the treatment of diabetic complications.

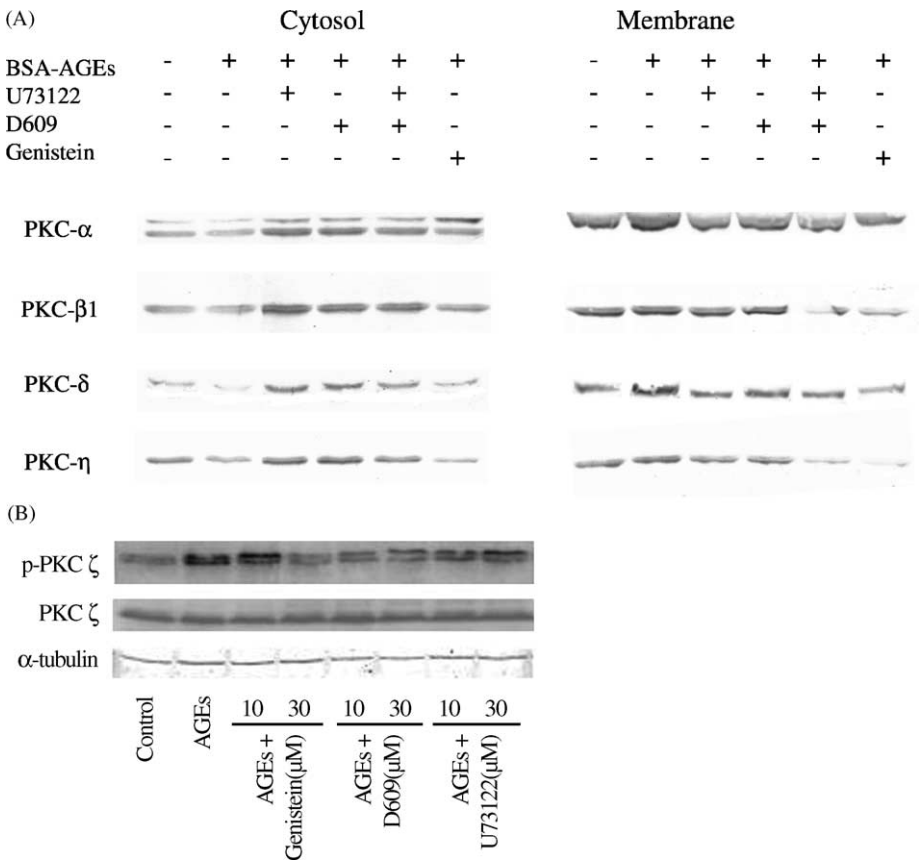

Fig. 8. Effects of genistein, U-73122, and D-609 on BSA-AGE-induced PKC isoform translocation in RAW 264.7 macrophages. Confluent cells were pretreated with 30 μM genistein, 10 μM U-73122, or 10 μM D-609 for 30 min and incubated with 300 μg/mL BSA-AGEs for 10 min; and then separated into cytosol and membrane fractions. Extracted proteins in the cytosol and membrane fractions were subjected to Western blot analysis using specific PKC-α, -βI, -δ, and -η antibodies as described in "Section 2". Data represent a typical experiment that has been repeated three times. In panel B, cells were pretreated with the indicated concentration of genistein, D-609, or U-73122 for 30 min and incubated with 300 μg/mL BSA-AGEs (lane 2) for 10 min. Cell lysates were subjected to Western blotting and immunoblotted with anti-phospho-PKC-ζ and anti-phospho-PKC-ζ/ι (Thr 410/403) antibodies as described in "Section 2". Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Data represent a typical experiment that has been repeated five times.

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