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Activation of nicotinamide adenine dinucleotide phosphate (reduced form) oxidase by advanced glycation end products links oxidative stress to altered retinal vascular endothelial growth factor expression

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Abstract

Increasing evidence indicates that advanced glycation end products (AGEs) promote retinal alterations through oxidative stress. However, the pathways involved in AGE-induced generation of reactive oxygen species (ROS) in retinal cells are poorly defined. In the present study, we investigated the role of nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) oxidase in AGE-induced ROS intracellular generation and vascular endothelial growth factor (VEGF) expression in bovine retinal endothelial cells (BRECs). Incubation of BRECs with 100 μg/mL AGEs increased ROS generation and VEGF expression in these cells. Treatment of the cells with the NADPH oxidase inhibitors, apocynin and diphenylene iodonium, inhibited these effects. In retinal endothelial cells exposed to AGEs, translocation of protein kinase C (PKC)-β2 and p47phox was observed. Inhibition of PKC by treatment of the cells with calphostin C, GF10923X, and LY379196 totally suppressed AGE-mediated p47phox translocation and ROS generation. Incubation of BRECs with gliclazide inhibited AGE-induced PKC-β2 and p47phox translocation and totally abrogated AGE-mediated ROS generation and VEGF expression. Overall, these results demonstrate that AGEs induce intracellular ROS generation and VEGF expression in retinal endothelial cells through a PKC-dependent activation of NADPH oxidase. Inhibition of retinal NADPH oxidase expression and ROS generated by this system provides a new potential mechanism by which gliclazide may affect retinal VEGF expression and exert a beneficial effect on diabetic retinopathy.

1. Introduction

The pathogenesis of diabetic retinopathy is not completely understood, and, therefore, the options for effective therapeutic intervention early in the disease process remain extremely limited. Large-scale epidemiologic trials have established that hyperglycemia is a major risk factor for the development and progression of retinopathy. Pathogenic mechanisms linking hyperglycemia with diabetic retinopathy include increased polyol pathway flux, protein kinase C (PKC) activation, and increased advanced glycation end product (AGE) formation [1-6]. There is evidence suggesting that part of the deleterious effects of AGEs in diabetic retinopathy involves oxidative stress. Indeed, we and others have demonstrated that AGEs promote, through generation of oxidative stress, retinal leukostasis, cell death and apoptosis, vascular endothelial growth factor (VEGF) expression, and cell proliferation [7-12]. Moreover, 2 recent studies have shown that exposure of retinal cells in vitro to preformed AGEs induces accumulation of intracellular reactive oxygen species (ROS) in these cells [13,14].

Despite accumulating evidence pointing to a causal role of AGE-induced oxidative stress in the pathogenesis of diabetic retinopathy [6], the molecular mechanisms involved in the stimulation of ROS by AGEs in retinal cells are poorly defined. Major sources of ROS generation in vascular cells exposed to hyperglycemic conditions include mitochondria and NADPH oxidases. Consistent with a key role of these latter enzymes in diabetic vascular complications, NADPH oxidases have been identified as primary physiologic producers of superoxide in several animal models of vascular disease, including diabetes [15], and increased activity of these enzymes in micro- and macrovascular tissues of animal models and diabetic patients has

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been documented [16,17]. Recently, an important role of the NADPH oxidase system in mediating increased retinal oxidative stress and complications in diabetes has been proposed. In keeping with this, it has been shown that pericytes do express a functional phagocyte-type NADPH oxidase, which is up-regulated by high glucose levels, and a role of this enzyme in palmitate-induced apoptosis of pericytes has been suggested [18,19]. More importantly, a role of NADPH oxidase activity in hypoxia-induced increases in retinal VEGF expression and neovascularization has been established [20]. Based on these experimental observations, in the present study we sought to investigate the effect of AGEs on NADPH activation in retinal endothelial cells and the role of this enzyme in the stimulatory effect of AGEs on retinal VEGF expression [8]. Furthermore, on the basis of our previous data showing that gliclazide, a sulfonylurea with antioxidant properties [21], effectively inhibits AGE-induced retinal VEGF expression [8], we also evaluated the modulatory effect of this drug on NADPH activation and ROS generation in bovine retinal endothelial cells (BRECs) exposed to AGEs. Our results demonstrate that AGEs increase NADPH oxidase–driven ROS in BRECs and stimulate, through PKC-dependent NADPH oxidase activation, VEGF expression in these cells. They also provide evidence of an inhibitory effect of gliclazide on these parameters.

Given the pervading role of glucose-induced ROS generation in the biochemical processes leading to microvascular damage, these results suggest that use of pharmacologic compounds with joint hypoglycemic and antioxidant properties may be useful for the prevention and treatment of diabetic retinopathy.

2. Materials and methods

2.1. Reagents

BRECs were obtained from VEC Technologies (Rensselaer, NY). Endothelial basal medium was obtained from Clonetics (San Diego, CA). Bovine endothelial cell growth factor was purchased from Roche Molecular Biochemicals (Laval, Quebec, Canada). Plasma-derived horse serum, fibronectin, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), buthionine sulfoximine (BSO), vitamin E, immunoglobulin (Ig)-free bovine serum albumin (BSA), and phorbol myristate acetate (PMA, a PKC activator) were obtained from Sigma (St. Louis, MO). Penicillin-streptomycin and phosphate-buffered saline (PBS) were obtained from GIBCO BRL (Burlington, Ontario, Canada). Trypsin/EDTA was obtained from Wisent (St Bruno, Quebec, Canada). GF109203X, a selective inhibitor of classical PKC; calphostin C, a pan-specific PKC inhibitor; apocynin, a selective NADPH oxidase inhibitor; diphenylene iodonium (DPI) chloride, an inhibitor of flavoprotein-containing enzymes; and *N*-acetylcysteine (NAC) were obtained from Calbiochem (La Jolla, CA). Sodium salt gliclazide was supplied by Les Laboratoires Servier (Giay, France). Antibody against p47phox was obtained from R&D Systems (Minneapolis, MN). LY379196 was kindly provided by Eli Lilly (Indianapolis, IN). Polyclonal antibody to VEGF was purchased from Preprotech (Rock Hill, NJ). Antibody to PKC-β2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Preparation of AGEs

Immunoglobulin-free BSA was subjected to nonenzymatic glycation by incubation with 0.5 mol/L glucose in 0.4 mol/L PBS containing 0.5 mmol/L EDTA. The solution was sterile filtered by passage through a 0.2-μm Gelman filter and then incubated at 37°C for 4 weeks under aerobic conditions. Nonglycated albumin was obtained by incubating BSA in the same reaction mixture in the absence of glucose. At the end of the incubation period, samples were dialyzed extensively against 10 mmol/L PBS (pH 7.4) at 4°C to remove unreacted glucose. The presence of AGEs was confirmed by the typical absorption and fluorescent spectra patterns of these proteins [22]. Endotoxin content of the AGE preparations (100 μg/mL) was determined by the *Limulus* amebocyte lysate assay (Sigma) and was consistently found to be lower than 6 pg/mL.

2.3. BREC culture

BRECs were grown in endothelial basal medium supplemented with 10% plasma-derived horse serum, 3% FBS, 50 μg/mL heparin, 50 μg/mL bovine endothelial cell growth factor, and 1% (vol/vol) penicillin-streptomycin in fibronectin-coated flasks (25 cm^2) at 37°C in 5% carbon dioxide/95% air atmosphere. Confluent cells were trypsinized and subcultured in 24-well culture plates or 100 × 20-mm tissue culture dishes according to the appropriate assay conditions. Cells were used in all experiments at passages 3 to 6.

2.4. Determination of ROS production

Confluent BRECs were treated with appropriate agents for specific periods, with addition of the cell-permeable fluorogenic probe DCF-DA (20 μg/mL) during the last 20-minute incubation period. At the end of this incubation period, cells were washed and trypsinized. ROS production was monitored by measuring fluorescence in a LS50B luminescence spectrophotometer (Perkin Elmer, Wellesley, MA), using excitation and emission wavelengths of 498 and 522 nm, respectively.

2.5. Preparation of membrane and cytosolic fractions

Preparation of membrane and cytosolic fractions was performed as described previously [23]. Briefly, confluent BRECs were treated with appropriate reagents for specific periods, washed twice with Hanks balanced salt solution without calcium or magnesium and once with cold PBS, and then lysed in 600 μL of lysis buffer (20 mmol/L potassium phosphate [pH 7.0], 1 mmol/L EDTA, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and

0.5 mmol/L phenylmethylsulfonyl fluoride). To ensure complete rupture of the cells, lysates were subjected to 2 cycles of sonication (20 seconds each). Membrane and cytosolic fractions of the cell lysates were separated by centrifugation at 29 000*g* for 20 minutes at 4°C. After recovery of the supernatants, the cell pellet was rinsed with 600 μL of lysis buffer and centrifuged again. The final pellet was resuspended in 250 μL of oxidase assay buffer (50 mmol/L potassium phosphate buffer [pH 7.0], 1 mmol/L EDTA, 150 mmol/L sucrose, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.5 mmol/L phenylmethylsulfonyl fluoride).

2.6. Measurement of PKC-β2, p47phox, and VEGF protein expression

Cytosol and membrane PKC-β2 expression, membrane p47phox, and VEGF expression were measured by Western blot analysis using specific antibodies.

2.7. Western blot analysis

Fifteen micrograms of proteins of membrane and cytosolic fractions of BRECs were applied to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane by using a Bio-Rad (Missisauga, ON, Canada) transfer blotting system at 100 V for 1 hour. Nonspecific binding was blocked with 5% BSA for 1 hour at room temperature. After washing with PBS/Tween (Fisher, Ottawa, ON, Canada) 0.1%, blots were incubated overnight at 4°C with appropriate antibodies in PBS/Tween. After further washing, membranes were incubated for 1 hour at room temperature with a horseradish peroxidase–conjugated goat anti–rabbit or anti–mouse IgG (1:5000). Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Density of the bands was measured by using Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

2.8. Measurement of superoxide dismutase, catalase, and glutathione peroxidase activities

Intracellular superoxide dismutase (SOD), catalase, and glutathione peroxidase activities were measured by using spectrophotometric analysis (BIOXYTECH SOD-525 and catalase-520 kits, Portland, OR) and colorimetic assay (BIOXYTECH GPx-340 kit), respectively.

2.9. Determination of cell viability

To exclude the possibility that experimental agents at maximal concentration used in the study may exert cytotoxic effects, cell viability was determined by trypan blue exclusion. It was consistently found to be 90% or higher (data not shown).

2.10. Statistical analysis

Statistical analysis of the results was performed by 1-way analysis of variance followed by the Student-Neuman-Keuls test. Differences were considered to be of statistical significance at $P < .05$. Results are expressed as the mean ± SEM.

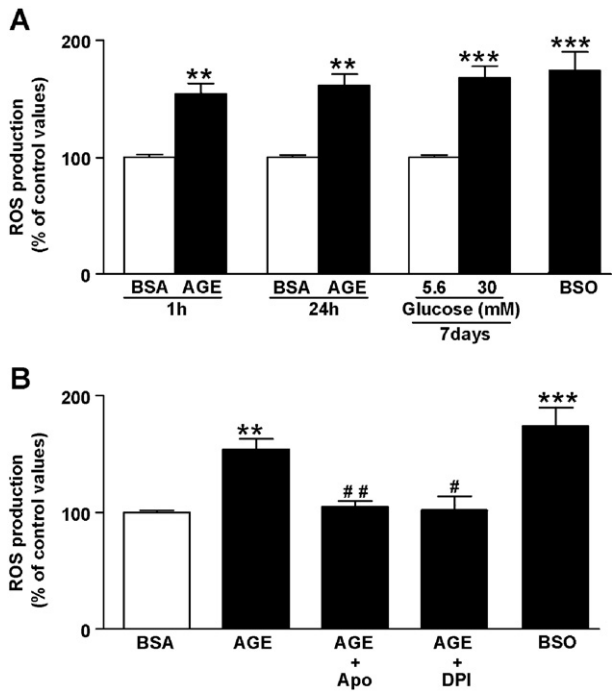

Fig. 1. Effect of AGEs on ROS generation in BRECs: role of NADPH oxidase. A, BRECs were treated for 1 or 24 hours with 100 μg/mL AGEs or for 7 days with 30 mmol/L glucose, with addition of 20 μg/mL DCF-DA during the last 20-minute incubation period. BSO was used as positive control. Intracellular ROS generation was quantified by measuring fluorescence. B, BRECs were pretreated or not for 1 hour with the NADPH oxidase inhibitors apocynin (Apo) (10 μM) or DPI (10 μM), then incubated in the presence of AGEs (100 μg/mL) for a further 24-hour period, with addition of 20 μg/mL DCF-DA during the last 20-minute incubation period. BSO was used as positive control. Intracellular ROS generation was quantified by measuring fluorescence. Data represent the mean ± SEM of 4 independent experiments. $^{**}P < .01$ vs BSA, $^{***}P < .001$ vs BSA or 5.6 mmol/L glucose, $^{\#}P < .05$ vs AGE, $^{\#\#}P < .01$ vs AGE.

3. Results

3.1. AGEs increase NADPH oxidase–driven ROS generation in BRECs

Treatment of BRECs with AGEs (100 μg/mL) for 1 or 24 hours enhanced ROS accumulation in these cells as compared with that observed with nonglycated albumin (BSA) (Fig. 1A) (ROS production [% of control values]: AGE, 1 hour, 150 ± 3, $P < .01$; AGE, 24 hours, 154 ± 3, $P < .01$). Similar effect was observed when cells were chronically exposed to high glucose levels (30 mmol/L) for 7 days or treated with the pro-oxidant compound BSO for 3 hours (Fig. 1A) (ROS production [% of control values]: glucose, 158 ± 4, $P < .001$; BSO, 171 ± 8, $P < .001$). The stimulatory effect of AGEs on ROS production was maintained up to 7 days (data not shown). Neither AGEs nor high glucose had any significant effects on antioxidant enzyme activities (SOD, catalase, and glutathione peroxidase) in BRECs (data not shown).

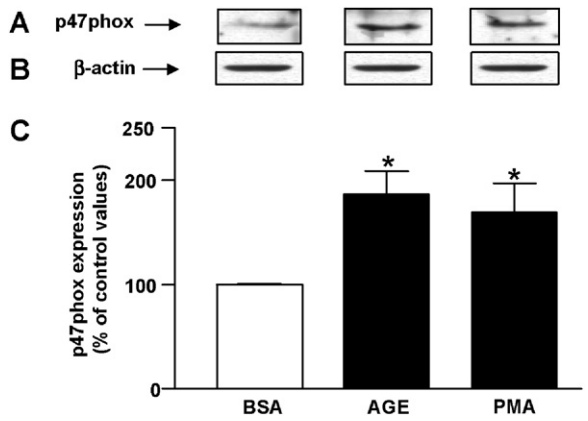

Fig. 2. Effect of AGEs on membrane p47phox protein levels in BRECs. BRECs were incubated with AGEs (100 μg/mL) or PMA (100 ng/mL, used as positive control) for 1 hour. Membrane p47phox (A) and β-actin (B) protein levels were measured by Western blot analysis. p47phox protein levels were normalized to the levels of β-actin protein expression (C). Data represent the mean ± SEM of 4 independent experiments. *P < .05 vs BSA.

Treatment of BRECs with the NADPH oxidase inhibitors, apocynin or DPI chloride, suppressed AGE-induced ROS production (Fig. 1B).

3.2. AGEs increase p47phox expression in BRECs

Because stimuli that activate NADPH oxidase cause translocation of the cytosolic p47phox NADPH subunit [24], levels of p47phox protein in the membrane fractions of AGE-stimulated BRECs were determined next. As shown in Fig. 2A, incubation of BRECs with AGEs (100 μg/mL) for 1 hour significantly increased the level of p47phox expression in the membrane fractions of these cells. Translocation of p47phox was also observed in PMA-treated BRECs (Fig. 2A), thus suggesting a role of PKC as an upstream kinase regulating p47phox in BRECs. Under these experimental conditions, no modulation of the β-actin protein levels, used as internal control, was observed (Fig. 2B). p47phox protein levels normalized to the levels of β-actin are shown in Fig. 2C.

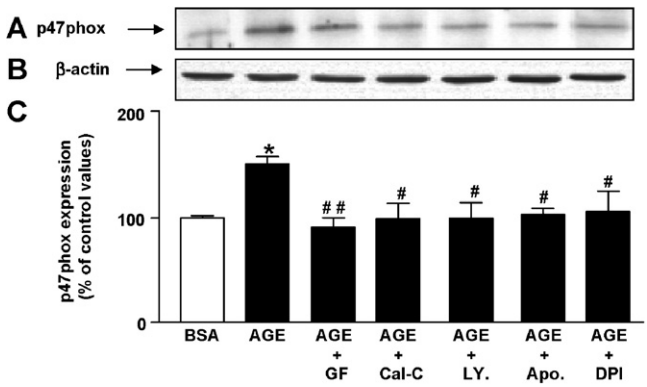

Fig. 3. Effect of PKC inhibitors on AGE-induced p47phox expression in BRECs. BRECs were preincubated for 1 hour with the PKC inhibitors GF10923X (GF) (20 nmol/L), calphostin C (Cal-C) (0.1 μg/mL), or LY379196 (LY) (30 nmol/L) or with the NADPH oxidase inhibitor apocynin (Apo) (10 μM) or DPI (10 μM) before exposure to AGEs (100 μg/mL) for a further 1-hour period. At the end of these incubation periods, membrane p47phox (A) and β-actin (B) protein expression were measured by Western blot analysis. p47phox protein levels were normalized to the levels of β-actin protein expression (C). Data represent the mean ± SEM of 4 independent experiments. *P < .05 vs BSA, $^{\#}P$ < .05 vs AGE, $^{\#\#}P$ < .01 vs AGE.

3.3. Induction of p47phox expression in AGE-treated BRECs is PKC dependent

To further evaluate whether the NADPH oxidase activation documented in BRECs exposed to AGEs is PKC dependent, the effect of the pan-specific and selective classical PKC inhibitors, calphostin C and GF10923X, respectively, on AGE-induced p47phox translocation in BRECs was next assessed. Incubation of BRECs with these agents totally abolished AGE-induced p47phox translocation in these cells (Fig. 3A). A similar effect was observed

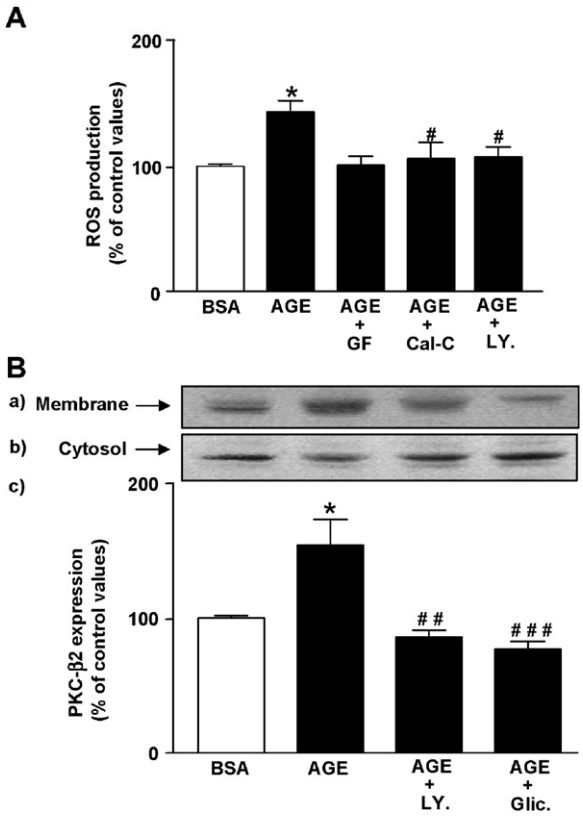

Fig. 4. Role of PKC-β in AGE-induced ROS generation in BRECs. A, BRECs were pretreated for 1 hour with the classical or pan-specific PKC inhibitors, GF10923X (GF) (20 nmol/L) and calphostin C (Cal-C) (0.1 μg/mL), or with the selective PKC-β inhibitor, LY379196 (LY) (30 nmol/L), before exposure to AGEs for a further 24-hour period, with addition of 20 μg/mL DCF-DA during the last 20-minute incubation period. Intracellular ROS generation was quantified by measuring fluorescence. Data represent the mean ± SEM of 4 independent experiments. *P < .05 vs BSA, $^{\#}P$ < .05 vs AGE. B, BRECs were pretreated for 1 hour with LY379196 (LY) (30 nmol/L) or gliclazide (10 μg/mL) then incubated with AGEs for 30 minutes. At the end of this incubation period, cytosol and membrane PKC-β2 activity was measured by Western blot analysis. Data represent the mean ± SEM of 3 to 5 independent experiments. *P < .05 vs BSA, $^{\#\#}P$ < .01 vs AGE, $^{\#\#\#}P$ < .001 vs AGE.

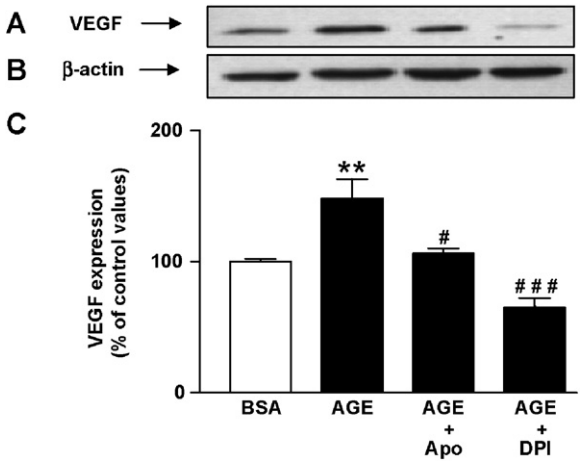

Fig. 5. Role of NADPH oxidase in mediating AGE-induced VEGF expression in BRECs. BRECs were preincubated for 1 hour with the NADPH oxidase inhibitors apocynin (Apo) (10 μM) or DPI (10 μM) before exposure to AGEs (100 μg/mL) for an additional 24-hour period. Total cell lysates were analyzed by Western blot analysis using antibodies to VEFG (A) and β-actin (B) proteins. VEGF protein levels were normalized to the levels of β-actin protein expression (C). Data represent the mean ± SEM of 4 independent experiments. $^{**}P < .01$ vs BSA, $^{\#}P < .05$ vs AGE, $^{\#\#\#}P < .001$ vs AGE.

when cells were treated with the selective PKC-β inhibitor, LY379196, or with the NADPH oxidase inhibitors, apocynin or DPI (Fig. 3A). Under these experimental conditions, no modulation of the β-actin protein levels, used as internal control, was observed (Fig. 3B). p47phox protein levels normalized to the levels of β-actin are shown in Fig. 3C.

3.4. Role of PKC-β in AGE-induced ROS generation in BRECs

To investigate whether activation of NADPH oxidase by PKC and more specifically by the PKC-β isoform is involved in AGE-induced ROS accumulation in retinal endothelial cells, BRECs were pretreated for 1 hour with GF10923X, calphostin C, or LY379196, before exposure to AGEs for a further 24-hour period, and ROS generation in these cells was determined. As shown in Fig. 4A, all these compounds totally suppressed the stimulatory effect of AGEs on intracellular ROS accumulation. LY379196 also totally suppressed AGE-induced membrane PKC-β2 expression in BRECs (Fig. 4B). Similar inhibitory effect was observed after treatment of the cells with gliclazide (Fig. 4B).

3.5. NADPH oxidase mediates AGE-induced VEGF protein expression in BRECs

We previously documented that AGEs up-regulate VEGF expression in cultured BRECs through PKC activation and increased oxidative stress [9]. On the basis of our present results showing PKC-dependent NADPH oxidase activation in AGE-treated BRECs, we next evaluated whether NADPH oxidase activation is required for AGE-induced VEGF expression in BRECs. Data presented in Fig. 5, which demonstrate that treatment of BRECs with the NADPH oxidase inhibitors, apocynin or DPI, totally suppresses AGE-induced VEGF expression in BRECs, confirm this hypothesis.

3.6. Inhibitory effect of gliclazide on AGE-induced ROS generation and p47phox translocation in BRECs

Treatment of BRECs with gliclazide (10 μg/mL) or with NAC (30 mmol/L) inhibited the stimulatory effect of AGEs (100 μg/mL) on intracellular ROS accumulation (Fig. 6A). In contrast, no effect of glyburide (2.5 μg/mL) on AGE-induced ROS production was observed (Fig. 6A). Gliclazide (10 μg/mL), but not glyburide (2.5 μg/mL), also totally abolished AGE-induced membrane p47phox protein expression (Fig. 6B-a) in BRECs. Under these experimental

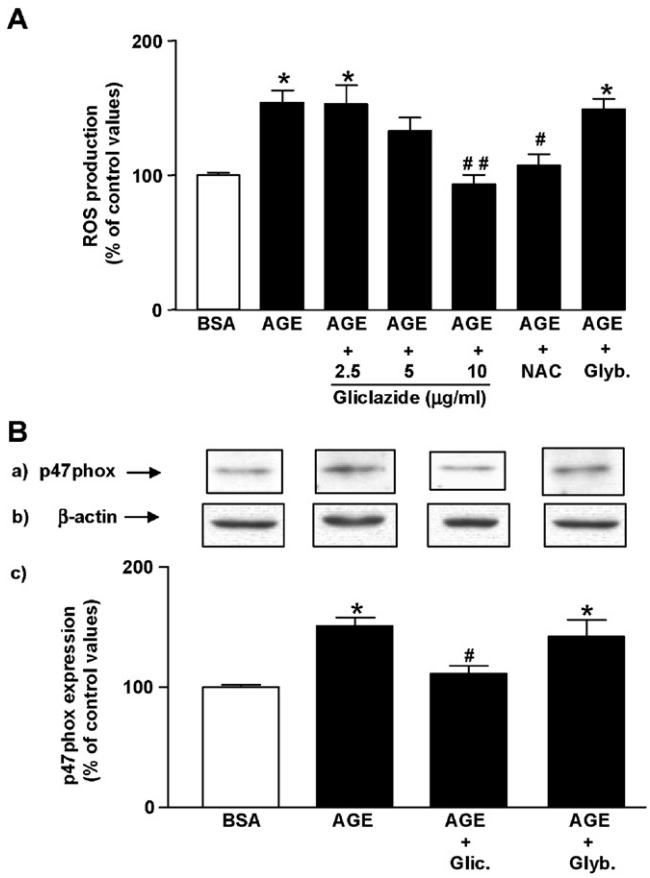

Fig. 6. Inhibitory effect of gliclazide on AGE-induced ROS production and p47phox translocation in BRECs. A, BRECs were preincubated for 1 hour with gliclazide (2.5-10 μg/mL), NAC (30 mmol/L), or glyburide (Glyb) (2.5 μg/mL) before exposure to AGEs (100 μg/mL) for a further 24-hour period, with addition of 20 μg/mL DCF-DA during the last 20-minute incubation period. Intracellular ROS generation was quantified by measuring fluorescence. Data represent the mean ± SEM of 4 independent experiments. $^{*}P < .05$ vs BSA, $^{\#}P < .05$ vs AGE, $^{\#\#}P < .01$ vs AGE. B, BRECs were preincubated for 1 hour with gliclazide (Glic) (10 μg/mL), or glyburide (Glyb) (2.5 μg/mL), then exposed to AGEs (100 μg/mL) for 1 hour. At the end of this incubation period, membrane p47phox (a) and β-actin (b) protein levels were measured by Western blot analysis. p47phox protein levels were normalized to the levels of β-actin protein expression (c). Data represent the mean ± SEM of 4 independent experiments. $^{*}P < .05$ vs BSA, $^{\#}P < .05$ vs AGE.

conditions, no modulation of the β-actin protein levels (used as internal control) was observed (Fig. 6B-b). p47phox protein levels normalized to the levels of β-actin are illustrated in Fig. 6B-c.

4. Discussion

Intracellular ROS accumulation has been proposed as the common element linking major signaling pathways implicated in hyperglycemia-induced vascular damage. Among the cellular sources of ROS in the diabetic vascular system, attention has been increasingly paid to NADPH oxidase as the most important one [15]. Supporting the involvement of this multicomponent enzyme in the pathogenesis of diabetic vasculopathies, increased NADPH oxidase activity and subunit protein expression has been documented in vessels [16,17,25], kidney [26-29], and retina [20] from diabetic animals and patients. Consistent with an important role of NADPH oxidase–driven ROS production in the pathogenesis of diabetic retinopathy, upregulation of NADPH oxidase in the ischemic retina has been associated with VEGF expression and neovascularization [20], and increased expression of this enzyme has been documented in cultured retinal cells exposed to a diabetic environment [6,18,20]. Recent findings have underscored the central role of NADPH oxidase in AGE-RAGE–mediated (RAGE = receptor for AGE) generation of ROS in human umbilical vein endothelial cells [30]. In line with these results, data generated in the present study demonstrate that activation of NADPH oxidase mediates AGE-induced enhancing oxidative stress in retinal endothelial cells.

PKC activation is a key biochemical event in diabetes that is strongly implicated in the pathogenesis of diabetic vasculopathies, including retinopathy [4]. Activation of PKC, especially the β isoform, is enhanced in the diabetic retina and, in early experimental diabetes, mediates retinal blood flow abnormalities [31-34]. Activation of this signal transduction event also plays an important role in regulating endothelial cell permeability [35] and proliferation [7] and represents an important signaling component for VEGF [8] and retinal leukostasis [9]. Treatment with selective PKC-β inhibitors significantly reduced PKC activity in the retina of diabetic animals and concomitantly reduced diabetes-induced increase in retinal mean circulation time [32,36]. Factors that contribute to PKC activation in vascular cells are mainly metabolic, with hyperglycemia and AGEs being probably main elements. In vascular cells exposed to high glucose levels, a major consequence of PKC activation is the activation of NADPH oxidase with subsequent ROS production [37]. In diabetic vascular tissues, PKC-dependent activation of NADPH oxidase can be amplified by various factors, including high levels of glucose [18,38,39], fatty acid [19], and angiotensin II [18,40]. Studies in endothelial cells have demonstrated that one major mechanism underlying the activation of NADPH by PKC is the PKC-dependent phosphorylation of the p47phox regulatory subunit and its translocation to the Nox2/p22phox heterodimer [24]. Consistent with this notion, the present study shows that exposure of BRECs to AGEs induces p47phox translocation and that PKC inhibitors abolish this effect. These data, along with the observation that both PKC and NADPH inhibitors totally suppress AGE-induced ROS accumulation in BRECs, indicate that generation of oxidative stress in these cells occurs through PKC-dependent NADPH oxidase activation. We have previously reported that treatment of BRECs with AGEs induces the translocation of PKC-β in BRECs [7], and a causal relationship has been established between phosphorylation and translocation of p47phox by PKC-β and enhanced oxidative stress in diabetic vascular cells [27,41]. That PKC-β contributes to the NADPH oxidase–driven oxidative stress in AGE-treated BRECs is supported by our results showing that pretreatment of the cells with the PKC-β inhibitor, LY379196, completely abolished AGE-induced p47phox translocation and ROS production. These data, together with previous ones showing that translocation of p47phox by PKC-β activation is required for ROS generation in diabetic glomeruli [27], suggest a critical role of this signaling event in causing oxidative stress in diabetic vascular cells.

The idea that oxidative stress might be a contributing factor in diabetic retinal alterations carries within it the notion that use of antioxidants may be beneficial in reducing diabetic retinal changes. Previous studies have shown that administration of antioxidants to diabetic animals prevents the development of retinopathy [42,43] and ameliorates metabolic, hemodynamic, biochemical, and histologic retinal abnormalities that might be relevant to diabetic retinopathy [44-48]. In accordance with these results, we and others have previously documented, in small human and animal studies, that gliclazide, a sulfonylurea with antioxidant properties, inhibits key biologic events involved in diabetic retinopathy [7-9] and prevents the retinal complications of diabetes [49-51]. Our present data, which demonstrate that gliclazide, at concentrations in the therapeutic range (5 to 10 μg/mL), effectively inhibits intracellular production of ROS and p47phox expression in retinal endothelial cells, identify NADPH oxidase as a new key molecular target of this drug in retinal cells. These results along with previous ones showing a similar effect of gliclazide on renal expression of NADPH oxidase in diabetic rats [52] suggest a novel mechanism by which gliclazide may be effective in preventing diabetic vascular complications. Because gliclazide inhibits PKC activation [7] and PKC-β2 expression in AGE-treated retinal cells, it seems reasonable to postulate that the suppressive action of gliclazide on NADPH oxidase–driven ROS production that we documented in the present study may be achieved through inhibition of PKC-dependent phosphorylation and translocation of p47phox. Consistent with this possibility, PKC inhibitors have been reported to inhibit the

phosphorylation and translocation of p47phox in vascular cells, and inhibition of these molecular events have been correlated with a decrease in ROS production [53,54].

VEGF is an important determinant of AGE-induced retinal vascular alterations. In vitro and in vivo studies have shown that AGEs induce retinal VEGF expression, and convincing evidence has been provided for a role of oxidative stress in this effect [8,10]. Our present data showing that AGEs induce NADPH oxidase activation in cultured retinal endothelial cells and that NADPH oxidase inhibitors abolish the stimulatory effect of AGEs on VEGF expression in these cells demonstrate that activation of NADPH oxidase by AGEs links oxidant stress to retinal VEGF induction. These data, along with the observation that inhibition of NADPH oxidase activity blocks VEGF overexpression during ischemic retinopathy [20], clearly identify this enzyme as one potential key target for the inhibition of the deleterious effect of AGEs in diabetic retinopathy. Because neural cells primarily express VEGF in diabetic retinopathy [55,56] and show increased VEGF expression after acute exposure to exogenous AGEs [10], substantiation of the physiologic relevance of the present work awaits future studies using these cells.

In summary, the results of the present study demonstrate a central role of NADPH oxidase in AGE-mediated ROS generation and VEGF induction in BRECs. They also show that gliclazide inhibits this effect. Whether gliclazide, through inhibiting retinal NADPH oxidase–driven ROS production, may prevent the retinal complications of diabetes remains to be investigated.

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