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Metformin suppresses high glucose–induced poly(adenosine diphosphate–ribose) polymerase overactivation in aortic endothelial cells

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Abstract

Overactivation of poly(adenosine diphosphate–ribose) polymerase (PARP), an enzyme involved in cellular response to DNA injury resulting from oxidative and nitrosative stress, is considered to play a key role in the pathogenesis of diabetes complications by promoting numerous vascular dysfunctions. In this study, we examined the ability of metformin, which was reported to possess intrinsic vasculoprotective properties independently of its antihyperglycemic effects, to inhibit PARP activation induced by high glucose concentrations in bovine aortic endothelial cells; and we investigated the potential mechanisms involved in this inhibition. The PARP activity was measured by cellular enzyme-linked immuno-specific assay (CELISA) method; cell poly(ribosyl)ated protein polymer accumulation was evaluated by immunofluorescence. Peroxynitrite anion productions were determined using dihydrorhodamine 123 fluoroprobe; and expression of p47phox subunit of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase was analyzed by Western blot in the absence and presence of protein kinase C and NAD(P)H oxidase inhibitors (calphostin and diphenyleneiodonium chloride, respectively). Our data showed that a therapeutically relevant concentration of metformin (5.10^{-5} mol/L) was able to abolish PARP activation, to reduce poly (ribosyl)ated protein polymer accumulation, to decrease intracellular peroxynitrite anion level, and to reverse the overexpression of p47phox in bovine aortic endothelial cells stimulated by 25 mmol/L glucose in a similar manner to that of calphostin or diphenyleneiodonium chloride. Taken together, these results suggest that metformin could inhibit glucose-induced PARP activation through blockade of a protein kinase C–dependent NAD(P)H oxidase activation pathway. We propose that some of the beneficial effects of metformin on vascular endothelial cell functions in diabetes may be related to its inhibitory effect on PARP overactivation and its deleterious consequences.

1. Introduction

There is a compelling evidence indicating that oxidative stress [1] and nitrosative stress [2,3] are underlying factors in the pathogenesis of both microvascular and macrovascular complications of diabetes [4,5]. A causal link is now established between elevated glucose concentrations and hyperglycemic damage in vascular endothelial cells through an overproduction of superoxide anion ($O_2^{\bullet -}$) via multiple mechanisms, including increased mitochondrial electron transport chain activity and a protein kinase C (PKC)–dependent activation of membrane nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase [6,7]. Overproduction of $O_2^{\bullet -}$ and derived reactive oxygen species (ROS) affect many signal transduction pathways resulting in the activation of nuclear factors, such as the proinflammatory factor nuclear factor (NF)–κB. Furthermore, $O_2^{\bullet -}$ is able to rapidly react with nitric oxide radical ($^{\bullet}NO$) to form the strong oxidant peroxynitrite anion ($ONOO^-$). This latter is a potent initiator of DNA single-strand breakage that is a stimulus for the activation of the poly(adenosine diphosphate [ADP]–ribose) polymerase (PARP), an enzyme involved in

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maintaining chromatin structure and DNA repair. Once activated, PARP catalyzes the cleavage of its substrate nicotinamide adenine dinucleotide (NAD^+) into nicotinamide and ADP-ribose residues, which bind to nuclear proteins to form poly(ADP-ribosyl)ated protein polymers (PARs).

The involvement of PARP activation in the pathogenesis of diabetes and its complications was emphasized during the last years by both in vivo and in vitro studies [3,8-10]. Excessive activation of PARP in diabetic state induces cell NAD^+ and adenosine triphosphate depletions, changes of transcriptional regulation and gene expression [11], and inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase through its ADP-ribosylation. Inhibition of glyceraldehyde-3-phosphate dehydrogenase leads to an enhancement of mitochondrial superoxide production and favors the activation of the major pathways involved in the occurrence and development of diabetic complications [12,13]. Activation of these pathways implicated in vascular injury was abolished by blocking PARP activity [13], thus confirming the pivotal role of PARP activation in diabetic vascular dysfunction. Therefore, PARP inhibitors might have clinical efficacy in preventing the development and progression of diabetic complications [14].

The aim of the present study was to investigate whether the antihyperglycemic agent metformin (dimethylguanidine), widely used for the management of type 2 diabetes mellitus, is able to decrease glucose-induced PARP activation. The improvement of clinical outcomes observed with metformin in the UK Prospective Diabetes Study had suggested that this drug exerts additional cardiovascular protective effects beyond those expected from improved glycemic control [15]. Metformin was indeed reported to present numerous intrinsic vasculoprotective properties [16-20]. We and others have previously reported that metformin was able to reduce intracellular oxidative stress induced in vitro by pathophysiologically relevant stimuli of diabetic state such as high levels of glucose, angiotensin II [21], and advanced glycation end product [22,23], through inhibition of mitochondrial respiratory chain [24,25] and membrane NAD(P)H oxidase activation [21], and to modulate PKC activity [26,27].

Here we examined the effects of metformin on PARP activity and PAR poly(ADP-ribose) accumulation, $ONOO^-$ formation, and expression of the p47phox NAD(P)H oxidase subunit in bovine aortic endothelial cells (BAEC) stimulated by high glucose levels.

2. Materials and methods

2.1. Reagents

Materials, media, and reagents for cell culture were from ABcys (Paris, France). Anti–mouse immunoglobulin G (IgG) Alexa Fluor 488 was purchased from Interchim (Montluçon, France). Monoclonal antibody against p47phox NAD(P)H oxidase subunit was purchased from Santa Cruz Biotechnology (Tebu-Bio, Le Perray en Yvelines, France). Peroxidase-conjugated anti–mouse IgG was from Amersham Biosciences (Orsay, France). Saponin, Mowiol, paraformaldehyde (PFA), 4′,6′-diamidino-2-phenylindole (DAPI), anti–actin antibody, and horseradish peroxidase–conjugated anti–mouse IgM were from Calbiochem (VWR, Fontenay-sous-Bois, France). Metformin, calphostin C, diphenyleneiodonium chloride (DPI), phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), leupeptin, and streptavidin-peroxidase polymer were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Monoclonal antibody against poly(ADP-ribose) (10H) and *N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N,N*-dimethylactamide (PJ-34) were from Alexis Biochemicals (Coger, Paris, France). Biotin-NAD and TACS-Sapphire were from Trevigen (Gentaur, Paris, France). Immobilon-P blotting membrane was from Millipore (Guyancourt, France).

2.2. Cell culture

Bovine aortic endothelial cells were grown at confluence in Dulbecco modified Eagle medium containing a basal level of glucose (5 mmol/L) and supplemented with 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C under a 5% CO_2 humidified atmosphere. Cells used in this study were between the sixth and 10th passages. Viability was assessed by the neutral red assay; a cell viability greater than 95% was constantly required for performing experiments.

2.3. Experimental procedure

The PARP activity assay, detection of PAR formation, and $ONOO^-$ measurement were performed in confluent BAEC treated as follows: pretreatment for 1 hour in basal glucose medium with or without metformin (10^{-7} to 5.10^{-5} mol/L) or/and the PKC inhibitor calphostin c (10^{-7} mol/L) or/and the NAD(P)H oxidase inhibitor DPI (5.10^{-8} mol/L), then incubation for 24 hours with or without these products in the presence of either normal (5 mmol/L) or high (25 mmol/L) glucose concentration. Previous data of our laboratory and others verified that incubation of endothelial cells with high D-mannitol levels, used as osmotic control, did not lead to a cellular oxidative response ([28] and unpublished results). On the other hand, it is noteworthy that the 5.10^{-8} mol/L DPI concentration used in our experiments was much lower than that reported to also partially inhibit mitochondrial complex [29]. For PARP activity measurement, cells were also pretreated for 1 hour then incubated with or without the PARP inhibitor PJ-34 (10^{-6} mol/L).

2.4. PARP activity measurement

The PARP activity was measured by a cellular enzyme-linked immuno-specific assay (CELISA) method using biotinylated NAD as previously reported [30]. Medium was replaced by the PARP reaction buffer (TRIS, 100 mmol/L [pH

8.0]; $MgCl_2$, 10 mmol/L; dithiothreitol, 1 mmol/L) containing 0.01% digitonin and 10 μmol/L biotinylated NAD^+. After a 30-minute incubation at 37°C, the buffer was removed; and cells were fixed by addition of prechilled 95% ethanol for 10 minutes at −20°C. Endogenous peroxidase activity was blocked by a 15-minute incubation with 0.5% hydrogen peroxide/methanol. Wells were washed once with phosphate-buffered saline (PBS) and blocked by BSA (1% in PBS) for 30 minutes at 37°C. After removing BSA solution, peroxidase-labeled streptavidin (diluted 1/500 in 1% BSA-PBS, 50 μL per well) was added for 30 minutes at 37°C; plates were then washed 3 times with PBS, and reaction was developed with TACS-Sapphire substrate (100 μL per well). Stop solution containing 0.2 mol/L HCl (100 μL per well) was added, and optical density was measured with a microplate spectrophotometer at 450 nm (IP400, Pasteur, France).

2.5. Immunofluorescence of PARs

Cells grown on glass coverslips and treated with high glucose levels for 24 hours with or without metformin (5.10^{-5} mol/L) were washed in PBS and fixed with 3% PFA for 15 minutes at room temperature. After washing, unreacted aldehyde groups were neutralized with 50 mmol/L NH_4Cl for 15 minutes; and cells were permeabilized with 0.1% saponin in PBS containing 0.2% BSA for 15 minutes. Cells were then incubated with mouse anti–poly(ADP-ribose) antibody 10H for 30 minutes at room temperature and washed with permeabilization buffer. Coverslips were incubated for 30 minutes at room temperature with anti–mouse IgG Alexa Fluor 488. The antibodies were diluted in PBS with 0.1% saponin and 3% BSA.

Cells were washed 3 times in PBS then incubated for 5 minutes in DAPI solution. The coverslips were finally washed in water, mounted with Mowiol, and examined with a fluorescence microscope (Zeiss, Jena, Germany).

2.6. $ONOO^-$ level determinations

Intracellular $ONOO^-$ formation was measured using the dihydrorhodamine 123 (DHR) probe. Dihydrorhodamine 123 passively diffuses across the membrane and forms the fluorescent product rhodamine 123 when oxidized by peroxides, especially $ONOO^-$ [31]. Cells were incubated with 10 μmol/L DHR at 37°C for 45 minutes, and $ONOO^-$ production was detected by measuring fluorescence of oxidized DHR (spectrofluorometer Fluostar, BMG, Champigny sur Marne, France; $\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 538 nm). Results were obtained as relative fluorescent units (microplate fluorescence analyzer Fluostar, BMG) and expressed as percentage of the fluorescence of treated cells compared with control cells that are grown in normal glucose level medium.

2.7. p47phox Western blot analysis

Cells were collected and washed twice in ice-cold PBS and resuspended in a TRIS-HCl 50-mmol/L buffer pH 7.5 containing EDTA (1 mmol/L), EGTA (2.5 mmol/L), NaCl (150 mmol/L), glycerol (10%), Triton X-100 (1%), dithiothreitol (1 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), leupeptin (10 μg/mL), and aprotinin (10 μg/mL) (lysis buffer). Cells were then briefly sonicated on ice (3 × 10 seconds at 80 W, Branson Sonicator, Technofix, Paris, France); lysates were centrifuged at 6000*g* for 5 minutes to remove nuclei and unbroken cells, and protein concentration in the supernatants (whole cells extracts) was determined using the Bio-Rad (Marnes-la-Coquette, France) protein assay kit. Forty micrograms of total proteins was migrated on 15% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and then transferred to Immobilon-P blotting membrane. The membrane was incubated first with blocking buffer (TRIS-HCl, 20 mmol/L; NaCl, 150 mmol/L; Tween 20, 0.05%; nonfat dry milk, 5% [pH 7.5]) for 1 hour at room temperature, then overnight in the presence of a mouse monoclonal antibody raised against NAD(P)H oxidase p47phox subunit (dilution, 1/300). Peroxidase-conjugated anti–mouse IgG was used for detection at a dilution of 1/4000, and immunoreactive proteins were visualized by chemiluminescence. Intensity of the individual bands was quantified by densitometry (Quantity One, Bio-Rad). Equal protein loading on the gel was checked using an anti–actin antibody.

2.8. Statistical analysis

Results are expressed as means ± SD of at least 3 separate and independent experiments. Statistical significance was

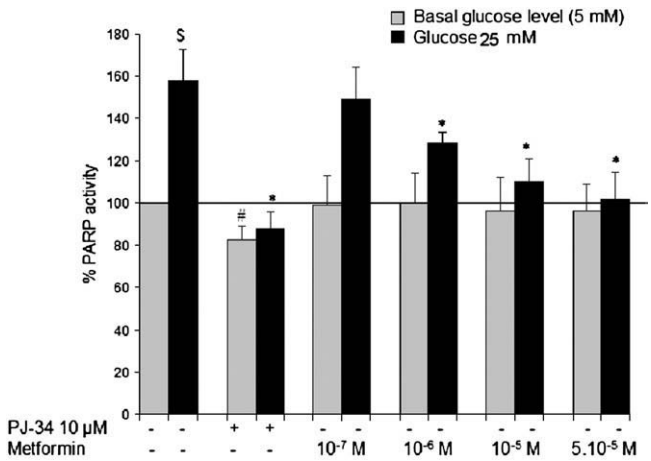

Fig. 1. Effect of metformin and PJ-34 on PARP activity induced by high glucose level (25 mmol/L). Confluent BAEC were treated for 24 hours with or without metformin (10^{-7} to 5.10^{-5} mol/L) or the PARP inhibitor PJ-34 in the presence of either normal (5 mmol/L) or high (25 mmol/L) glucose concentration. The PARP activity was measured by the CELISA method as described in "Materials and methods." $^{\$}P < .001$ vs cells exposed to basal glucose level (5 mmol/L); $^{\#}P < .001$ vs cells exposed to basal glucose level (5 mmol/L) without PJ-34; $^{*}P < .001$ vs cells exposed to high glucose level (25 mmol/L) without PJ-34 or metformin. These results are from at least 4 independent experiments.

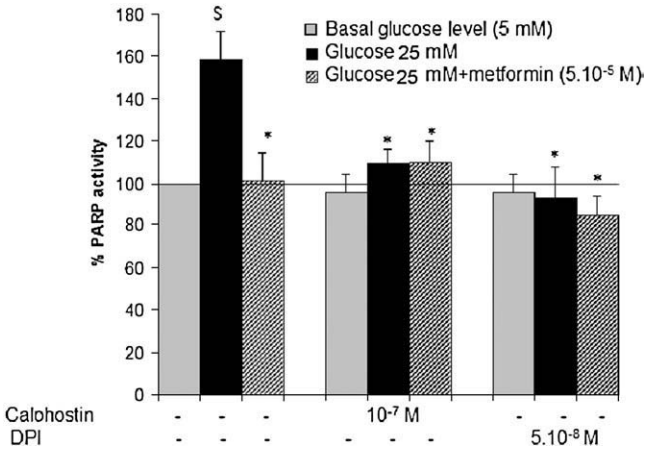

Fig. 2. Effect of metformin on PARP activity induced by high glucose level (25 mmol/L) in the presence or absence of PKC inhibitor (calphostin) and NAD(P)H oxidase inhibitor (DPI). Confluent BAEC were treated for 24 hours with or without metformin 5.10^{-5} mol/L or/and calphostin 10^{-7} mol/L or DPI 5.10^{-8} mol/L in the presence of either normal (5 mmol/L) or high (25 mmol/L) glucose. The PARP activity was then measured by the CELISA method as described in "Materials and methods." $^{\$}P < .001$ vs cells exposed to basal glucose level (5 mmol/L); $^{*}P < .001$ vs cells exposed to high glucose level (25 mmol/L) without metformin. These results are from 4 independent experiments.

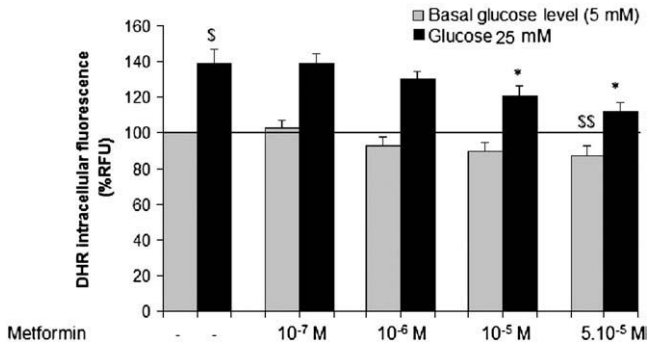

Fig. 4. Effects of metformin on the intracellular $ONOO^{-}$ production. Confluent cells were treated with metformin (10^{-7} to 5.10^{-5} mol/L) in the presence of either normal (5 mmol/L) or high (25 mmol/L) glucose for 24 hours, and then intracellular fluorescence of the probe (DHR) was measured. Results are expressed taking basal fluorescence of cells exposed to basal glucose level (5 mmol/L) as the 100% reference. $^{\$}P < .001$, $^{\$\$}P < .01$ vs cells exposed to basal glucose level (5 mmol/L); $^{*}P < .001$ vs cells exposed to high glucose level (25 mmol/L) without metformin. Results are from 6 independent experiments.

determined by the Student *t* test. *P* values less than .05 were considered statistically significant.

3. Results

3.1. Effect of metformin on PARP activity and PAR formation

As expected, exposure of BAEC to high glucose concentrations (25 mmol/L) for 24 hours resulted in a significant enhancement of PARP activity (+57%, $P < .001$), which was abolished by treatment of cells by the specific PARP inhibitor PJ-34 (10 μmol/L) (Fig. 1). Treatment of cells by metformin (10^{-7} to 5.10^{-5} mol/L) led to a dose-dependent decrease of glucose-induced PARP overactivation that was significant from 10^{-6} mol/L. The pharmacologic metformin concentration 5.10^{-5} mol/L completely inhibited glucose-induced PARP activation, which returned to the level of nonstimulated cells. This concentration of metformin was therefore used for following experiments. As indicated in Fig. 2, PKC inhibitor calphostin (10^{-7} mol/L) and NAD(P)H oxidase inhibitor DPI (5.10^{-8} mol/L) abolished enhancement of PARP activity in cells exposed to 25 mmol/L glucose. Metformin 5.10^{-5} mol/L was as effective as calphostin or DPI alone to prevent PARP overactivation, and no additive effect was observed when it was tested in the presence of each of them. By contrast, metformin, calphostin, and DPI had no effect on PARP activity in cells incubated with 5 mmol/L glucose (Fig. 2).

Exposure of cells to 25-mmol/L glucose concentrations also induced PAR accumulation, as shown by immunofluorescence assay (Fig. 3). Treatment of cells by metformin 5.10^{-5} mol/L decreased this glucose-induced

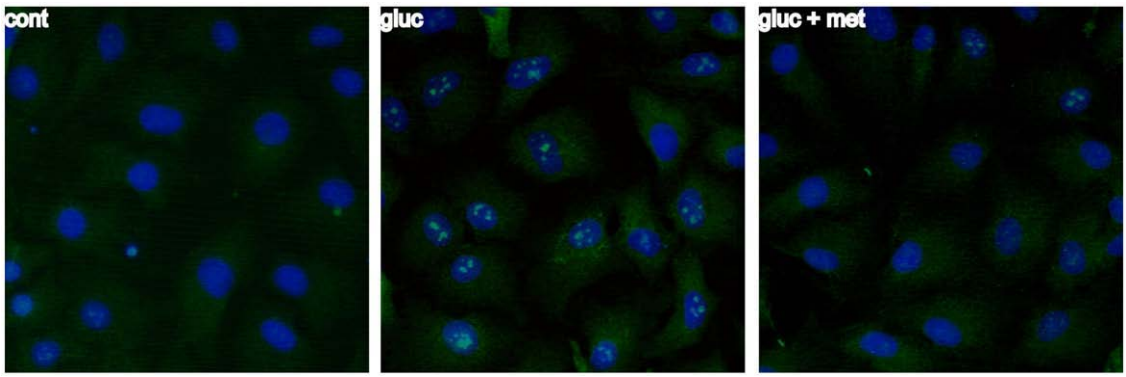

Fig. 3. Effect of metformin on high glucose–induced PAR formation. Confluent cells were exposed to 5 mmol/L glucose (cont) or 25 mmol/L glucose in the absence (gluc) or the presence of metformin 5.10^{-5} mol/L (gluc + met). Cells were fixed with PFA and immunostained for PARs (green). Nuclei were counterstained with DAPI (blue). Merged pictures are shown and representative of 3 independent experiments.

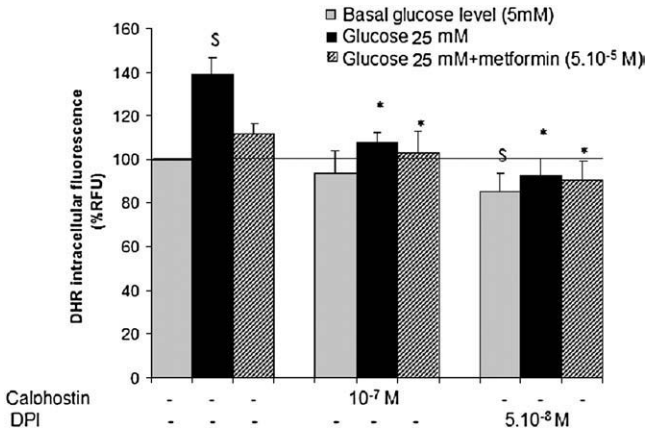

Fig. 5. Effects of metformin in the presence or absence of the PKC inhibitor (calphostin) or NAD(P)H oxidase inhibitor (DPI) on the intracellular $ONOO^-$ production. Confluent cells were treated with calphostin or DPI, with or without metformin (5.10^{-5} mol/L), in the presence of either normal (5 mmol/L) or high (25 mmol/L) glucose as described in "Materials and methods." Intracellular fluorescence of the probe (DHR) was measured. Results are expressed taking cells exposed to basal glucose level (5 mmol/L) as the 100% reference. $^{\$}P < .001$ vs cells exposed to basal glucose level (5 mmol/L); $^{*}P < .001$ vs cells exposed to high glucose level (25 mmol/L) without metformin. These results are from at least 6 independent experiments.

PAR formation, in accordance with the decrease in PARP overactivation (Fig. 3).

3.2. Effect of metformin on $ONOO^-$ formation

Measurement of intracellular level of $ONOO^-$ was performed using the DHR probe. Although commonly used to detect $ONOO^-$, DHR also can react with other products [32]. As expected, exposure of BAEC to 25 mmol/L glucose for 24 hours significantly increased $ONOO^-$ productions (+39%, $P < .001$) when compared with exposure to 5 mmol/L glucose (Fig. 4). Metformin attenuated this increase of $ONOO^-$ production in a dose-dependent manner, and the concentration of 5.10^{-5} mol/L had a marked and significant effect (+12% vs 39%, $P < .001$). Fig. 5 showed that calphostin (10^{-7} mol/L) and DPI (5.10^{-8} mol/L) abolished $ONOO^-$ generation induced by 25 mmol/L glucose. Interestingly, no additive effect was observed when metformin 5.10^{-5} mol/L was tested in the presence of the inhibitors.

Metformin 5.10^{-5} mol/L also decreased $ONOO^-$ production in cells exposed to 5 mmol/L glucose (−13%, $P < .01$) (Fig. 4), in a similar manner to that of calphostin (−7%) or DPI (−15%, $P < .001$) (Fig. 5).

3.3. Effect of metformin on expression of the NAD(P)H oxidase subunit p47phox

Western blot on Fig. 6 showed that exposure of BAEC to high glucose levels for 24 hours led to a significant increase of p47phox expression. Treatment of cells by metformin 5.10^{-5} mol/L significantly decreased the glucose-induced p47phox overexpression and restored it to the level in cells exposed to 5 mmol/L glucose. This effect appeared similar to that of calphostin (10^{-7} mol/L).

4. Discussion

The principal finding of this study is that metformin was able to blunt the overactivation of PARP in cells exposed to high glucose concentration, and our data contribute in part to elucidate the molecular mechanisms involved in this effect.

Recent studies have pointed out the involvement of PARP activation in the pathogenesis of diabetes and diabetic complications, including cardiovascular dysfunction [14,33,34], atherosclerosis [35], nephropathy [10], neuropathy [36-39], and retinopathy [40]. In accordance with data from experimental animals supporting such role of PARP, there is significant direct experimental evidence showing the role of PARP activation in human diabetic microvasculature. Early activation of PARP leads to impaired vasorelaxant function not only in diabetes but also in healthy subjects at risk of developing of diabetes [41,42].

High glucose levels also may favor an increased expression of inducible nitric oxide synthase through the activation of NF-κB, thus resulting in the increase in nitric oxide generation. The latter interacts with $O_2^{\bullet -}$ to form the strong oxidant $ONOO^-$ [43,44]. There is accumulating evidence supporting the key role of peroxynitrite in the pathogenesis of diabetes and diabetic complications in

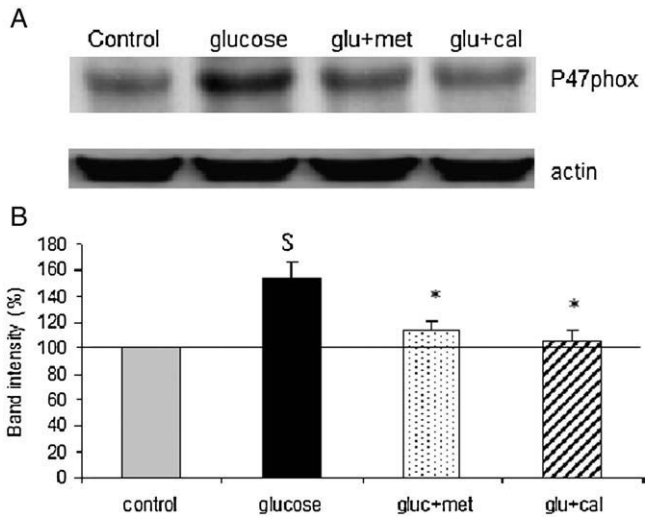

Fig. 6. Effects of metformin (5.10^{-5} mol/L) or the PKC inhibitor (calphostin) on P47phox expression. Western blot was performed using the specific antibody against the P47phox subunit of NAD(P)H oxidase. Confluent cells were treated with metformin or calphostin in the presence of high (25 mmol/L) glucose for 24 hours. Cell extracts were prepared as described in "Materials and methods." The extracts were analyzed on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (A), A representative blot is shown. (B), Intensity of the individual bands was quantified by densitometry (Quantity One, Bio-Rad). Results are representative of 3 independent experiments. $^{\$}P < .05$ vs cells exposed to basal glucose level (5 mmol/L); $^{*}P < .05$ vs cells exposed to high glucose level (30 mmol/L).

humans [44,45]. Increased nitrotyrosine levels are detectable in plasma and platelets of diabetic patients, and nitrotyrosine is directly harmful to endothelial cells [44]. High glucose levels were shown to increase nitrotyrosine formation in human endothelial cells [46] and in diabetic patients during the postprandial period [47,48]. The degree of cell death and/or dysfunction correlates with levels of nitrotyrosine in endothelial cells, cardiomyocytes, and fibroblasts from myocardial biopsies of diabetic patients [45]. In addition, nitrotyrosine immunoreactivity is increased in the microvasculature of patients with type 2 diabetes mellitus and correlates with fasting blood glucose, hemoglobin A_{1c}, intracellular adhesion molecule, vascular cellular adhesion molecule, and endothelial dysfunction [41].

Overactivation of PARP, which results from excessive DNA damage mainly induced by $ONOO^-$, causes NAD^+ depletion, energy failure, and inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. This promotes the enhancement of mitochondrial ROS production and activation of pathways believed to be the main molecular mechanisms of diabetic complications—increased hexosamine pathway flux, advanced glycation end product formation, polyol pathway flux, activation of PKC via de novo synthesis of diacylglycerol, and subsequently NAD(P)H oxidase [13]—that contribute to worsen endothelial oxidative stress [49]. The PARP activity was also shown to be associated to DNA binding affinity of the NF-κB in diabetic patients [42]. Therefore, PARP could represent a potential drug target for an early therapeutic intervention in diabetes. Data from studies in animal models support this hypothesis because PARP-deficient mice were shown to be completely protected from streptozotocin-induced diabetes [50]. Similarly, treatment of streptozotocin-diabetic animals with PARP inhibitor exerted beneficial effects on their endothelial dysfunction [51] and prevented the development of neuropathy [36]. In addition, PARP inhibition by PJ-34 blocked NF-κB activation [40].

In the present report, we investigated the effect of metformin on PARP activation in BAEC stimulated by high glucose concentration. As expected, we observed that 25 mmol/L glucose promoted significant intracellular increases of PARP activity, PAR formation, and $ONOO^-$ production. These alterations were abolished in the presence of the highly specific inhibitor of PKC calphostin or the NAD(P)H oxidase inhibitor DPI. By contrast, these inhibitors had little effect on the production of $ONOO^-$ and no effect on PARP activity in cells exposed to 5 mmol/L glucose. Taken together, these data suggested that PKC-dependent activation of NAD(P)H oxidase constituted a major pathway of $O_2^{\bullet-}$ production, as reported for other vascular cells stimulated by glucose [6,7,52], and subsequently of $ONOO^-$ generation responsible for PARP activation in our endothelial cell model. In the same way, an increased expression of the p47phox NAD(P)H oxidase subunit was observed in renal vasculature of diabetic rats [53]; and a PKC-dependent activation of NAD(P)H oxidase was demonstrated to be required for oxidative stress in diabetic glomeruli [54].

Our results showed that metformin was able to reduce enhancements of PARP activity, PAR accumulation, and $ONOO^-$ production in a dose-dependent manner in BAEC exposed to high glucose. The pharmacologically relevant metformin concentration 5.10^{-5} mol/L was as potent as calphostin and DPI in suppressing PARP activation and in preventing $ONOO^-$ production in stimulated cells. By contrast, it was ineffective in modulating basal PARP activity in cells exposed to glucose 5 mmol/L; but it slightly reduced $ONOO^-$ formation in a similar manner to that of DPI. Taking into account the fact that DPI is known to inhibit all flavoenzymes including those of the respiratory chain [55], this result was in agreement with our previous report showing that metformin reduced by a low extent intracellular ROS in nonstimulated BAEC through an inhibition of both mitochondrial respiratory chain production and constitutive NAD(P)H oxidase activity [21].

Consequently, our data suggested that the potent effect of metformin on $ONOO^-$ production and PARP activation in cells stimulated by high glucose concentration was linked to a marked reduction of the NAD(P)H oxidase activation. Because no additional effect was observed when metformin was coincubated with PKC or NAD(P)H oxidase inhibitors in stimulated cells, the effect of metformin on PARP activity probably resulted, at least in part, from the inhibition of a PKC-dependent activation of the NAD(P)H oxidase pathway. Supporting these findings, we observed on Western blot that metformin suppressed glucose-induced overexpression of the NAD(P)H oxidase p47phox subunit in a similar manner to that of the PKC inhibitor calphostin. A potential limitation of this result is that the effect of metformin was examined on the p47phox expression of total cell lysate alone, which did not allow the study of the subunit membrane translocation. Nevertheless, recent reports indicated that high glucose–induced NAD(P)H oxidase activation in vascular cells involves a stimulation by PKC of both expression and phosphorylation of its p47phox subunit [56]. In addition, we and others have previously shown that metformin was able to inhibit PKC activation in high glucose–stimulated endothelial cells [26,27] and to sharply reduce $O_2^{\bullet-}$ generated by angiotensin II-induced NAD(P)H oxidase activation [21] that involves phosphorylation of p47phox by PKC [57,58]. Therefore, the present findings strongly suggest that metformin diminishes NAD(P)H oxidase p47phox overexpression by acting on PKC. On the other hand, a possible effect of metformin on nitric oxide synthase expression/activity was not studied in this work. Few data are available on the capability of metformin to modulate nitric oxide synthase activity [59,60]; we cannot exclude such effect of metformin in the observed results of this study.

Taken together, our results showed that metformin was able to reduce PARP activation and PAR formation induced

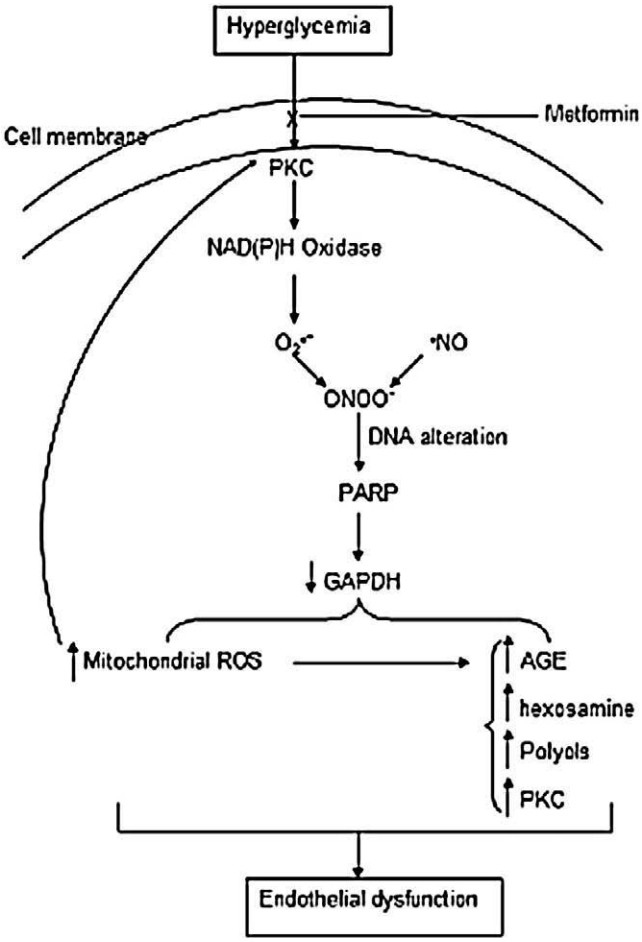

Fig. 7. Proposed unified mechanism by which metformin improves vascular functions.

in BAEC by high levels of glucose, probably by inhibiting activation of PKC and subsequently that of NAD(P)H oxidase. By this way, metformin could interrupt the downstream event cascade leading to PARP activation with subsequent enhancement of mitochondrial ROS production and activation of deleterious metabolic pathways, and thus could mitigate the vicious circle that maintains diabetic vascular endothelial dysfunction (Fig. 7). In this regard, it is of interest to note that (*a*) we demonstrated that metformin had no effect on PKC activity in a cell-free system [27] and (*b*) the effect of the drug on the respiratory chain function was previously presumed to involve an unknown cell-signaling pathway [61] with obligatory plasmatic membrane events [25]. We therefore propose that the inhibitory effect of metformin on PKC activation at membrane level might be a pivotal mechanism to explain its vasculoprotective properties.

In summary, the control by metformin of the intracellular oxidative stress involved in the pathogenesis of diabetic vascular complications is probably mediated through an inhibition of a PKC-dependent activation of NAD(P)H oxidase that appears to be a major pathway in the ROS and $ONOO^-$ generation responsible for PARP overactivation.

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