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Metformin reduces angiotensin-mediated intracellular production of reactive oxygen species in endothelial cells through the inhibition of protein kinase C

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ABSTRACT

Oxidative stress plays a major role in the pathogenesis and in the onset of macrovascular complications of diabetes. We previously reported that the antihyperglycaemic drug metformin was able to decrease significantly intracellular reactive oxygen species (ROS) production of bovine aortic endothelial cells (BAEC) activated by high levels of glucose and angiotensin II (ANG). The aim of the present study was to investigate whether the antioxidant effect of metformin on BAEC could be mediated through a modulation of protein kinase C (PKC) activity, which plays a key role in the pathophysiology of diabetes. The effects of metformin on intracellular ROS production, PKC translocation and activity were studied on endothelial cells stimulated by PMA (a direct PKC activator), ANG or high levels of glucose as pathophysiological stimuli of endothelial dysfunction in diabetes. We showed that metformin decreased ROS production on PMA-, ANG- and glucose-stimulated BAEC in a similar manner to that obtained by PKC specific inhibitors (calphostin C, chelerythrine) alone. On the other hand, metformin reduced both PKC membrane translocation and kinase activity in ANG-stimulated cells. In PMA-activated cells, metformin reduced membrane PKC activity but we did not observe any alteration of PKC membrane translocation. Finally, in vitro incubation with purified PKC indicated that metformin had no direct effect on PKC activity. Taken together, our results suggest that metformin exerted intracellular antioxidant properties by decreasing ROS production through the inhibition of PKC activity.

1. Introduction

There is compelling evidence that the oxidative stress occurring in response to hyperglycemia is involved in the onset and development of micro and macrovascular complications in diabetes mellitus [1,2]. Hyperglycemia induces an overproduction of superoxide anion through several pathways including mitochondrial respiratory chain [3], advanced glycation end product formation [4], de novo synthesis of diacylglycerol (DAG) [5] and [6], and activation of NAD(P)H oxidase [7,8]. Such cellular oxidative stress can combine with nitrosative stress by inducing the formation of the highly reactive species peroxynitrite, which alter cell functions in the vascular wall (endothelium, smooth muscle cells, etc.), leading to cardiovascular dysfunction via multiple mechanisms [9–11].

The biguanide metformin (dimethylguanidine) is an antihyperglycemic agent used for the management of type 2 diabetes [12]. Its glucose-lowering effects are mainly the

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consequence of reduced hepatic glucose output through inhibition of gluconeogenesis and, to a lesser extent, of increased insulin-stimulated glucose uptake in skeletal muscle and adipocytes [13]. Several studies have shown reduced cardiovascular-related mortality rates in metformin users compared with sulfonylurea monotherapy users [14,15], indicating that metformin might have some additional cardiovascular protective effects beyond its antihyperglycaemic properties. In that way, our group and others have demonstrated that metformin possesses antioxidant properties that could participate to its cardiovascular protective effects [16,17]. Such antioxidant properties could explain some of the pharmacological actions of this drug through a modulation of redox-dependent transduction pathways. Indeed, it was recently shown that metformin activates the AMP-activated protein kinase pathway [18,19]. We postulated that this drug could also improve vascular cell functions through a modulation of PKC activity. PKC is a family of serine–threonine kinases that regulate many cellular functions by acting in multiple signal transduction mechanisms [20,21]. Activation of PKC was shown to participate to the development and progression of vascular complications of diabetes [22–25]. Previous studies have revealed that activation of PKC by high glucose levels was responsible for the enhancement in NAD(P)H oxidase activity especially in the vascular wall [26]; in return, the resulting overproduction of ROS may contribute to maintain PKC in an activate state [27,28]. Thus, vascular oxidative stress and activation of PKC involved in the cellular disorders observed in diabetic state (especially endothelial dysfunction) are closely related [29–31]. Another way for the activation of PKC is the enhanced activity of angiotensin (ANG), due to an alteration of renin–angiotensin system in the diabetic disease [32]. ANG stimulates vascular NAD(P)H oxidase, the major source of ROS in endothelial cells, through ANG receptor AT1R-PKC pathway activation [33–36].

Therefore, in this study we examined whether metformin exerts its antioxidant effect through a modulation of PKC activity in aortic endothelial cells. The effects of metformin on intracellular ROS production, PKC translocation and activity were evaluated in BAEC stimulated by three selected stimuli: phorbol myristate acetate (PMA) as a direct PKC activator, ANG as potent activator of NAD(P)H oxidase via the AT1R-PKC pathway, and high levels of glucose as pathophysiological stimuli of the diabetes state.

2. Materials and methods

2.1. Reagents

All cell culture materials, media and reagents were from Abcys (Paris, France). 2′,7′-Dichlorodihydrofluorescein diacetate probe (H_2DCF-DA) was purchased from Interchim (Montlucon, France). StressXpress® non-radioactive PKC Kinase Activity Assay kit was from Stressgen Bioreagents and monoclonal antibody against PKC (MC5) was purchased from Santa Cruz Biotechnology (Tebu-Bio, Le Perray en Yvelines, France). Peroxydase-conjugated anti-mouse IgG was from Amersham Biosciences (Orsay, France). Anti-actin (Ab-1) antibody and horseradish peroxydase-conjugated anti-mouse IgM was from Calbiochem Merck (Fontenay-sous-Bois, France). PepTag® non-radioactive PKC assay kit was obtained from Promega (Charbonnières les Bains, France). Super Signal West Pico chemiluminescence reagent was obtained from Pierce (Brebieres, France). Unless otherwise stated, all others chemicals were from Sigma–Aldrich (Saint Quentin Fallavier, France).

2.2. Cell culture

Bovine Aortic Endothelial cells (BAEC) were grown at confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 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 tenth passages. Viability was assessed by the neutral red assay; a cell viability >95% was constantly required for performing experiments.

2.3. ROS determination

Intracellular ROS production was measured using the H_2DCF-DA probe as previously described [37] with a slight modification. The cell-permeant form H_2DCF-DA diffuses through cell membrane and the acetate moiety of the probe is hydrolyzed by intracellular esterases to produce an oxidative-sensitive form, dichlorodihydrofluorescein (H_2DCF), which is polar and trapped within the cells. The H_2DCF oxidation by ROS (mainly by endogenous H_2O_2) results in the formation of the fluorescent compound, dihydrofluorescein (DCF). Cells were incubated with 10 μM DCFDA at 37 °C for 45 min and ROS production was detected by measuring fluorescence of oxidized DCF (spectrofluorometer Fluostar, BMG; $\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 530 nm).

ROS production was measured in confluent BAEC grown in 96-well plates and stimulated by PMA (3×10^{-7} M, 1 h), ANG (10^{-6} M, 1 h) or glucose-supplemented medium (25 mM, 72 h). The effects of metformin (10^{-5} M, 1 h) and/or the specific PKC inhibitors, calphostin C and chelerythrin (10^{-7} and 10^{-8} M, respectively, 1 h) were analyzed. Results were obtained as Relative Fluorescent Units (RFU—microplate fluorescence analyzer Fluostar-BMG) and expressed as percentage of the fluorescence of treated cells compared to control cells, which are stimulated with PMA, ANG or glucose but without metformin and PKC inhibitors.

2.4. Intracellular PKC location and activity

2.4.1. Cell treatment

For PMA- and ANG-stimulated cells, confluent BAEC in 75-mm culture dishes were incubated with metformin for 1 h then treated by PMA (3×10^{-7} M) for 1 h or by ANG (10^{-6} M) for 20 min. For glucose-stimulated cells, confluent BAEC in 75-mm culture dishes were incubated with glucose-supplemented medium (25 mM) for 72 h then treated at the last 2 h by metformin.

2.4.2. Cell fractionation

Cytosolic and membrane fractions were prepared as previously reported [38] with several modifications. Cells treated as described above were collected and washed twice in

ice-cold PBS and resuspended in a Tris–HCl 20 mM buffer pH 7.5 containing EDTA (5 mM), EGTA (5 mM), β-mercaptoethanol (50 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM), leupeptin (40 μg/mL) (lysis buffer). Cells were then lysed by sonication (4× 10 s at 80 W, Brand Sonicator); homogenates were centrifuged at 6000 × *g* for 5 min to remove nuclei and unbroken cells, then at 100,000 × *g* for 1 h. The supernatant (cytosolic fraction) was separated and the pellet (cell membranes) was resuspended in lysis buffer containing 1% Triton X-100, mixed and sonicated on ice until complete homogenized. All preparation steps were performed at +4 °C. Protein concentration of the supernatant and membrane fractions was determined using the Bio-Rad protein assay (Bio-Rad, Marnes la coquette, France).

2.4.3. *Western blot analyses*

Cytosolic and membrane fractions (40–60 μg total proteins) were analyzed on 10% SDS-polyacrylamide electrophoresis gel then transferred to nitrocellulose membrane (Bio-Rad). Membrane was incubated first with blocking buffer (Tris–HCl 20 mM, NaCl 150 mM, Tween 20 0.05%, nonfat dry milk 5%, pH 7.5) for 1 h at room temperature and then overnight in the presence of a mouse monoclonal antibody raised against residues 292–317 of PKC isoforms α, β and γ (dilution: 1/500). Peroxydase-conjugated anti-mouse IgG was used for detection at a dilution of 1:5000 and the immunoreactive proteins were visualized by chemiluminescence.

2.5. *PKC activity assay*

PKC activity in both cytosolic and membrane fractions was measured with the non-radioactive PepTag® assay (Promega, France) according to the manufacturer's instructions. Briefly, after incubation of cell extract samples with a fluorescent peptide substrate that is specifically phosphorylated by PKC, the phosphorylated peptides were separated from the non-phosphorylated ones by electrophoresis on a 1% agarose gel. To estimate the amount of phosphorylated peptide the corresponding bands were excised from the gel under UV light and solubilized. Fluorescence of the solubilized solution was measured spectrophotometrically at 570 nm. Specific PKC activity was reported as nmol of PKC per mg of total protein.

2.6. *Assay for direct effect of metformin on PKC activity*

A direct effect of metformin on PKC activity was tested using the StressXpress® non-radioactive PKC kinase activity ELISA assay (Stressgen), according to the manufacturer's instructions. Briefly, active recombinant PKC was incubated with increasing concentrations of metformin (10^{-7} to 10^{-4} M) or staurosporine (10^{-5} M) as a positive control, then deposited in wells of a 96-well plate coated with PKC substrate. An ATP solution was added to initiate the reaction; after 90 min of incubation at 30 °C the kinase reaction was stopped and a phosphospecific substrate antibody was added. Specific fixation of this antibody was revealed by a peroxydase-conjugated secondary antibody followed by tetramethylbenzidine substrate (TMB) to develop the assay. The color development was measured spectrophotometrically at 450 nm (IP400, Pasteur, France).

2.7. *Statistical analysis*

All the experiments were performed at least in triplicate. The results are expressed as the mean ± S.E.M. of at least three separate and independent experiments. Statistical significance was determined by the non-parametric Mann–Whitney test. p values <0.05 were considered statistically significant.

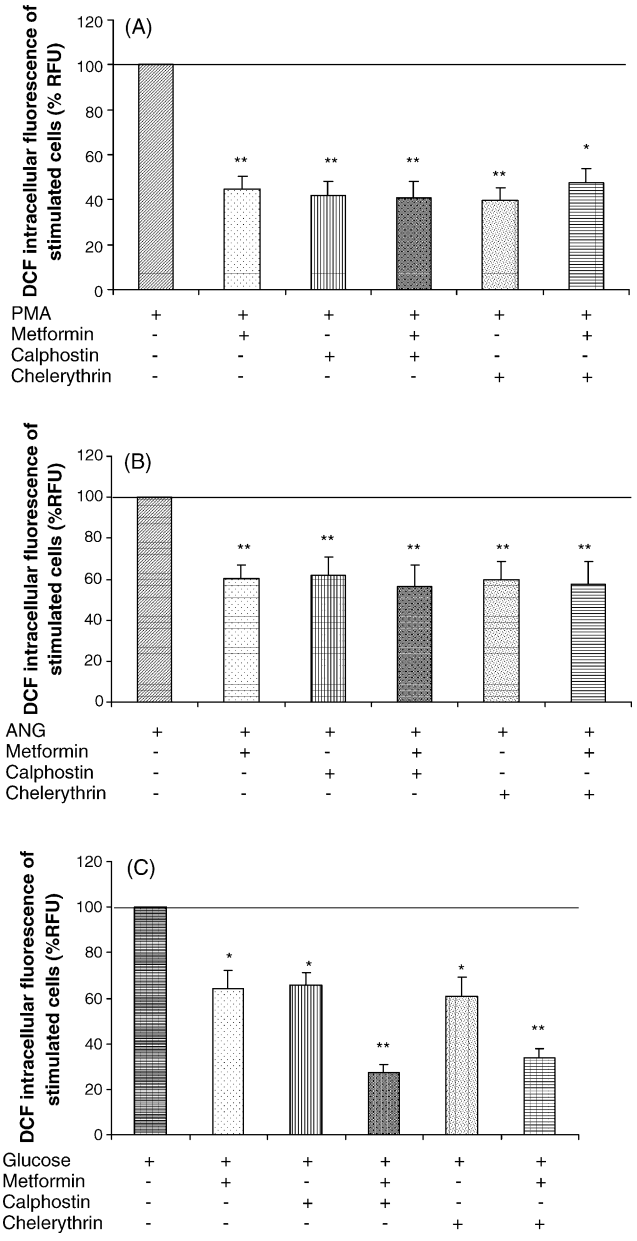

Fig. 1 – Effects of MET and PKC inhibitors caphostin C and chelerythrin on the intracellular ROS production in: (A) PMA-stimulated BAECs (B) ANG-stimulated BAECs and (C) glucose-stimulated BAECs. Confluent cells were incubated with ANG or PMA for (1 h) or with glucose for 72 h and treated with metformin for 1 h and/or PKC inhibitors as described in Section 2, then DCF intracellular fluorescence was measured. Results are expressed taking basal fluorescence of stimulated cells as the 100% reference. $^{*}p < 0.001$; $^{**}p < 0.0001$ vs. stimulated control cells ($n = 6$).

3. Results

3.1. Intracellular ROS production

Incubation of endothelial cells with PMA resulted in a significant increase in ROS production (+42%, $p < 0.0001$). The effects of metformin (10^{-5} M) on PMA-stimulated cells resulted in a significant reduction in intracellular ROS production, which was very close to that obtained with PKC inhibitors, calphostin C 10^{-8} M and chelerythrine 10^{-7} M (−55%, −58% and −60% respectively versus, stimulated cells, $p < 0.0001$). The simultaneous incubation of metformin and either calphostin C or chelerythrine with PMA-stimulated cells led to a similar reduction in ROS production to that observed with metformin or with PKC inhibitors alone without any additive effect (Fig. 1A).

Incubation of endothelial cells with ANG (10^{-6} M) for 1 h significantly increased ROS production (+45%, $p < 0.0001$). The effect of metformin (10^{-5} M) on ANG-stimulated cells resulted in a significant reduction in ROS production which is very close to that obtained with PKC inhibitors, calphostin C and chelerythrine (−40%, −38% and −43% respectively, versus stimulated cells, $p < 0.0001$). The simultaneous incubation of metformin and either calphostin C or chelerythrine with ANG-stimulated cells led to a similar reduction in ROS production as that observed with metformin or with PKC inhibitors alone (Fig. 1B). No additive effect was observed.

Lastly, incubation of BAEC with high glucose level (25 mmol/L) for 72 h significantly increased intracellular ROS production (+39%, $p < 0.0001$) as compared to cells incubated in a 5.5 mM-glucose medium. The effects of metformin (10^{-5} M) on glucose-stimulated cells resulted in a significant reduction in ROS production, which is very close to that obtained with PKC inhibitors (−35%, −34% and −39%, respectively versus stimulated cells, $p < 0.001$) (Fig. 1C). The simultaneous incubation of metformin and either calphostin C or chelerythrine led to the addition, at least partly, of the inhibitory effects of each molecule.

3.2. PKC translocation

Western blot analyses indicated that treatment with metformin alone did not modify cellular PKC distribution. Incubation of cells with PMA for 1 h induced a complete translocation of PKC from cytosol to membrane extracts. The pretreatment with metformin did not result in a visible reduction in PMA-induced PKC translocation (Fig. 2A).

Incubation of cells with ANG induced a noticeable PKC translocation from the cytosol to the cell membrane, which was partially restored by metformin pretreatment (Fig. 2B).

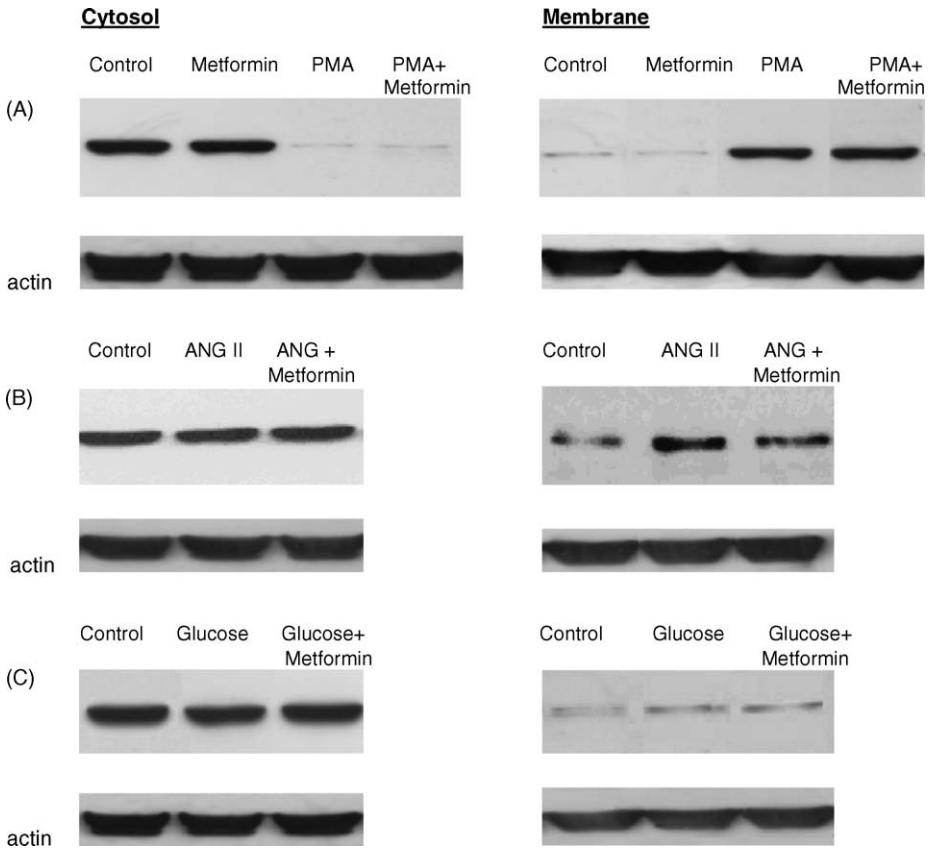

Fig. 2 – Effects of MET on PKC translocation analyzed by Western blotting using the specific antibody against the cPKC. Confluent cells were pretreated with MET for 1 h then stimulated with (A) PMA for 1 h; (B) with ANG for 20 min and (C) with high glucose for 72 h. Cytosolic and membrane fractions were prepared as described in Section 2, then the extracts were analyzed on 10% SDS-polyacrylamide electrophoresis gel. Immunoblots shown are representative of four independent experiments.

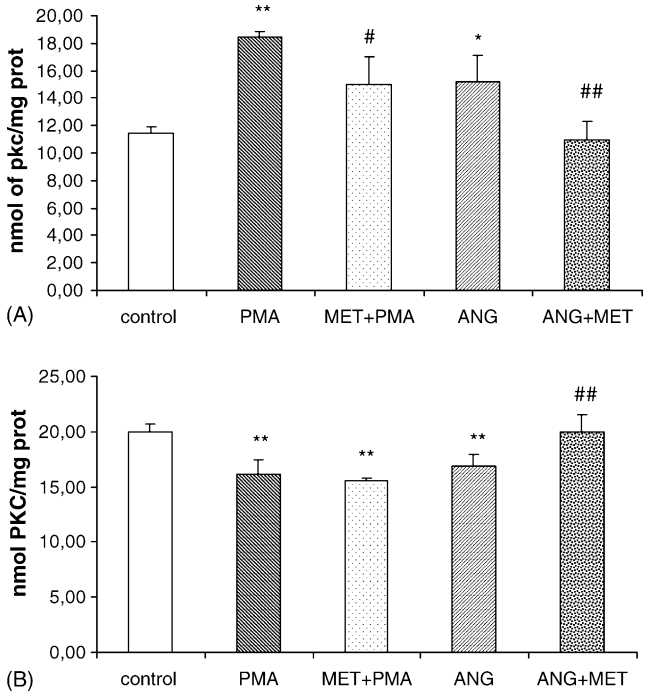

Fig. 3 – Effect of metformin on PKC membrane and cytosolic activity: (A) membrane PKC activity in cells stimulated by PMA and ANG, (B) cytosolic PKC activity in cells stimulated by PMA and ANG. Confluent BAEC were pre-treated with metformin then stimulated with PMA or ANG, cytosolic and membrane fractions were prepared as described in Section 2, then the fractions were subjected to the PepTag® non-radioactive PKC assay. The phosphorylated peptide bands were excised than PKC activity was measured spectrophotometrically at 570 nm. $^{*}p < 0.05$; $^{**}p < 0.005$ vs. control non-stimulated cells, $^{\#}p < 0.05$; $^{\#\#}p < 0.005$ vs. stimulated cells. These results are from four independent experiments.

However, we did not observe a noticeable reduction in cytosolic PKC due to the high initial intensity of the band.

Finally, we failed to observe any modification in PKC intracellular distribution when cells were incubated with high levels of glucose for 72 h (Fig. 2C).

3.3. 3.3. Protein kinase C (PKC) activity

Treatment of cells with PMA significantly increased membrane PKC activity (+61%, $p < 0.005\%$) and decreased PKC cytosolic activity (−20%, $p < 0.005$). Pretreatment of cells with metformin (10^{-5} M) for 1 h before PMA activation resulted in a decrease in membrane PKC activity (−19%, $p < 0.05$ versus PMA-stimulated cells), while the cytosolic PKC activity remained unchanged (Fig. 3). Treatment of cells with ANG (10^{-6} M) for 20 min resulted in an increase in membrane PKC activity (+33%, $p < 0.05$), that was associated with a decrease in PKC cytosolic activity (−16%, $p < 0.005$). Pretreatment of cells with metformin for 1 h before incubation with ANG resulted in a normalization of both membrane and cytosolic activities, leading to the restoration of a basal values of PKC activity (Fig. 3).

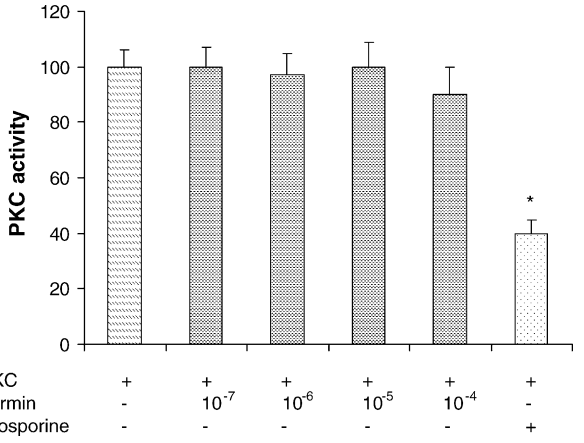

Fig. 4 – Direct effect of metformin on PKC activity. Active recombinant PKC standard (recPKC) was incubated with or without metformin (10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M) or with 10^{-5} M of staurosporine, then PKC activity was measured using the Stressgen's StressXpress® non-radioactive PKC kinase activity assay as described in Section 2. $^{*}p < 0.01$ vs. active recombinant PKC activity ($n = 3$).

3.4. Direct effect of metformin on PKC activity

The direct incubation of metformin (10^{-7} to 10^{-4} M) with recombinant PKC failed to alter the measured activity of the enzyme, whatever the concentration of metformin (Fig. 4). In opposite, the positive control staurosporin, a kinase inhibitor, which binds to the site of attachment of the ATP, significantly inhibited PKC activity.

4. Discussion

Results of the present study showed that: (1) metformin was able to decrease intracellular ROS production in PMA-, ANG- and in glucose-activated cells in a similar manner to PKC specific inhibitors calphostin C and chelerythrine; (2) except for glucose-activated cells, no additive effect was observed after the simultaneous incubation of metformin and either calphostin C or chelerythrine on PMA- and ANG-stimulated cells; (3) metformin inhibited both PKC membrane translocation and activity induced by ANG; (4) metformin had no effect on PMA-induced PKC translocation but decreased its membrane activity; (5) metformin was unable to alter in vitro PKC activity in a cell-free system.

Experimental studies revealed that diabetes mellitus is associated with an enhancement of cell PKC activity, likely through an increase in the formation of DAG, a physiologically required molecule for the activation of PKC [39]. PKC is a family of serine–threonine kinases consisting in classic/conventional PKC (cPKC—α, βI, βII and γ) that require DAG, Ca^{2+} and phosphatidylserine (PS) for activation, novel PKC (nPKC—δ, ε, θ and η) that does not respond to Ca^{2+} but are activated by DAG and PS; and atypical PKC (aPKC—ζ and ι/λ) that require only PS for its activation [40,41]. Classic/conventional PKC isoforms are the main subgroup present in endothelial cells. Both in vivo and in vitro studies demonstrated the role of glucose-induced

activation of PKC in the endothelial dysfunction in diabetes. Indeed, the administration of specific PKC inhibitors ameliorated vascular complications in diabetic rats [39], probably through an improvement of the endothelial cell dysfunction [23]. Both the enhancement of PKC activity and the increase in intracellular ROS generation are closely dependent, since the inhibition of PKC activity was reported to suppress high glucose-induced ROS production in different vascular cell types [26] and to improve in vivo the vascular complications of diabetes [30]. Activation of PKC by phorbol esters such as PMA, a structural analogue of DAG, is able to induce NAD(P)H oxidase activation through phosphorylation of several subunits of the enzyme, including p47phox, p67phox and p22phox [42]. Thus, PKC-dependent activation of NAD(P)H oxidase may be a critical mechanism responsible for the enhanced ROS production in diabetic vascular tissues [26].

Our experiments clearly indicated that metformin, at pharmacological concentrations, was able to inhibit increased ROS production in ANG-, PMA- or glucose-stimulated endothelial cells as the PKC inhibitors calphostin C and chelerythrine did. Such inhibitory effect of metformin was shown to be partially additive to that of PKC inhibitors in glucose-activated cells; this result are consistent with our previous report, indicating that metformin exerts its effect partly through an inhibition of ROS produced by NAD(P)H oxidase but also by the respiratory mitochondrial chain [17]. On the contrary, the effects of metformin and PKC inhibitors appeared not additive when cells were activated by PMA or ANG, that are two stimuli of PKC activity, either directly (PMA) or via the interaction of ANG with its specific membrane AT1 receptor. Cellular effects of ANG are mediated by the activation of AT1 receptor, leading to phospholipase C (PLC) activation and the subsequent formation of inositol trisphosphate (IP3) and DAG, which then respectively increase intracellular calcium and activate PKC [43,44].

The increase in angiotensin activity due to an alteration of renin–angiotensin system in the diabetic disease lead to an activation of endothelial NAD(P)H oxidase which is the major source of ROS in endothelial cells. Inhibition of ANG production induced by an angiotensin-converting enzyme inhibitor (ACEi) was shown to reduce ROS production in both myocardium and aorta in diabetic rats [45] and in vascular endothelial cells [46]. In addition, a recent study reported that treatment with the PKC inhibitor chelerythrine decreased ANG-induced superoxide production in endothelial cells [47]. We originally showed here that metformin was able to inhibit both translocation and activity of PKC induced by ANG. On the contrary, metformin did not affect PKC translocation induced by PMA that agrees with the recent report of Gallo [48], but diminished PMA-induced PKC membrane activity. Such dissociation between PKC translocation and activity was already proposed [40,49] and might be explained by the decrease of membrane-translocated PKC activation. Lastly, we failed to observe any PKC translocation and/or activation when cells were stimulated by high glucose levels; a similar result was previously described by Gallo et al., who reported that the translocation of PKC only occurred when endothelial cells were previously cultured in a medium containing 10 mM glucose [48].

In conclusion, we report here evidence that short incubations of pharmacologically relevant concentrations of metformin exert antioxidant properties at the cellular level, by inhibition of intracellular ROS production in stimulated endothelial aortic cells, through the reduction of PKC membrane translocation and/or activity. Whether such inhibition of the PKC pathway by metformin might be associated to a modulation of the AMPK pathway, the proposed redox-dependent mechanism for the pharmacological effect of the antidiabetic drug [18], remains to be clarified. The inhibitory effect of metformin on PKC translocation may be due to the inhibition of PKC activation by DAG, since metformin could not affect PKC translocation induced by PMA. Inhibition of ANG-enhanced PKC activation pathway certainly participates to the improvement of vascular endothelial cell functions, and thus explain at least partly, the additional cardiovascular benefits of metformin beyond its antihyperglycaemic action in diabetes mellitus.

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