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Metformin decreases intracellular production of reactive oxygen species in aortic endothelial cells

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Abstract

Beyond its antidiabetic activity justifying its use in the treatment of the type 2 diabetes, metformin (MET [dimethylguanidine, Glucophage]) has been shown to exhibit antioxidant properties in vitro, which could contribute to limit the deleterious vascular complications of diabetes. We investigated whether MET, at the pharmacological level of 10^{-5} mol/L, was able to modulate intracellular production of reactive oxygen species (ROS) both in quiescent bovine aortic endothelial cells (BAECs) and in BAECs stimulated by a short incubation with high levels of glucose (30 mmol/L, 2 hours) or angiotensin II (10^{-7} mol/L, 1 hour). Intracellular ROS production was measured by fluorescence of the DCF (2,7′-dichlorodihydrofluorescein) probe. Our results showed that MET was able to reduce the intracellular production of ROS in both nonstimulated BAECs (−20%, $P < .05$) and BAEC stimulated by high levels of glucose or angiotensin II (−28% and −72%, respectively, $P < .01$). Experiments performed in the presence of the nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase inhibitor apocynin or the respiratory mitochondrial chain inhibitor rotenone indicated that MET exerted its effect partly through an inhibition of the formation of ROS produced mainly by NAD(P)H oxidase and also, to a lesser extent, by the respiratory mitochondrial chain.

1. Introduction

Amplification and acceleration of atherosclerosis account for more than 75% of all death in diabetes mellitus. Prolonged exposure to hyperglycemia is recognized as a major factor in the pathogenesis of atherosclerosis in diabetic patients, by inducing a large number of alterations at the cellular level of vascular tissue [1]. The most important mechanisms involved in this process are glycation of proteins, formation of advanced glycation end products accumulating in vessel wall by interaction with their specific receptor RAGE (receptor for advanced glycation end products) [2], and oxidative stress by impairment of antioxidant defensive capacity as well as by an overproduction of reactive oxygen species (ROS) [3-5]. Excess of ROS is known to exert multiple deleterious cardiovascular actions by promoting oxidation of low-density lipoproteins, widely involved in atherosclerosis [6]. At the cellular level, ROS promote lipid peroxidation in membranes, decrease of nitric oxide bioavailability, and activation of redox-sensible signaling pathways with resultant changes in gene expression [7].

The biguanide metformin (MET [dimethylguanidine, Glucophage]) is an antihyperglycemic agent used for the management of type 2 diabetes [8-10]. Its glucose-lowering effects are mainly the 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 [11]. Several studies have shown reduced cardiovascular-related mortality rates in MET users compared with sulfonylurea monotherapy users [12-14]. These observations suggest that MET might have some additional cardiovascular protective effects beyond its antihyperglycemic properties. Until now, few works focused on the potential antioxidant properties of MET. These studies reported an improvement of antioxidant status, consisting in an increase of antioxidant enzymatic activities in red cells [15] and hepatic and blood glutathione levels in rats [16], and a decrease of xanthine oxidase activity and lipid peroxidation markers in type 2 diabetic patients [17-19]. Chelating properties of MET toward metals such as

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copper or iron [20] might participate to its antioxidant action because metal ions are involved in the radical-generating Fenton system. On the other hand, a recent study of our group demonstrated that MET exhibited direct antioxidant properties by scavenging oxygenated free radicals generated in vitro and was able to decrease the oxidative burst induced in stimulated human leukocytes [21].

Because vascular endothelial dysfunction contributes to atherosclerosis development, we investigated here whether MET could decrease oxidative stress in quiescent and activated endothelial cells. We tested the action of MET on ROS production in bovine aortic endothelial cells (BAECs), either nonstimulated or stimulated by a high glucose level, or by angiotensin II, a potent activator of vascular nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase system involved in cardiovascular complications [22]. We showed that MET was able to significantly decrease intracellular ROS level in nonstimulated cells and in cells stimulated by glucose or angiotensin, by reducing both NAD(P)H oxidase and/or mitochondrial respiratory chain–induced activities, 2 main sources of ROS production in endothelial cells.

2. Materials and methods

2.1. Materials, reagents, and culture media

All cell culture materials, media, and reagents were from Sigma-Aldrich (L'isle d'Abeau-Chesnes, France), except 2,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Interchim-Molecular Probe, France), apocynin (Fluka), and dark microfluor 96-well plates (ATGC, France).

2.2. Cell culture

BAECs were cultured in Dulbecco modified Eagle medium 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. Glucose concentration in this medium was 5.5 mmol/L. The cells used in this study were between the fifth and tenth passages. Cells were grown at confluence in 96-well plates. Viability was assessed by the neutral red assay; a cell viability >95% was constantly required for performing experiments.

2.3. ROS detection

The probe H_2DCF-DA was used to detect intracellular production of ROS. H_2DCF-DA freely penetrates into cells, and the acetate moiety is cleaved off by intracellular esterases leaving the nonfluorescent H_2DCF, whose oxidation (mainly by endogenous H_2O_2) results in the formation of the fluorescent compound DCF. The fluorescence measured at 530 nm by microspectrofluorimetry is proportional to the H_2O_2 formed into cells at a basal level and under the action of pro-oxidant–stimulating agents [23-25]. Basal values of fluorescence in nonstimulated cells were 5220 ± 308 relative fluorescence units (RFU; microplate fluorescence analyzer, Fluostar-BMG). Detection characteristics of the microplate fluorescence reader were maintained identical for all experiment measurements. Results were expressed as a percent of basal fluorescence value of either nonstimulated or stimulated control cells.

2.4. Experimental procedure

Experiments were performed as previously described [26-28]. Confluent cells in 96-well microplates were incubated for 20 hours with or without the NAD(P)H oxidase inhibitor apocynin (1 mmol/L) or with rotenone (10^{-5} mol/L), an inhibitor of the mitochondrial respiratory chain complex 1. H_2DCF-DA (10 μmol/L) was then added in the culture medium for 45 minutes at 37°C. Cells were washed twice with Hank's balanced salt solution (HBSS) to remove extracellular H_2DCF-DA, and MET (10^{-5} mol/L) was added for 1 hour. After washing with HBSS, fluorescence in cells was measured. In experiments with stimulated cells, cells were treated before the fluorescence measurement (ie, following the incubation time with MET) by either glucose (Glc, 30 mmol/L) or tert-butylhydroperoxide (tBHP, 1 mmol/L) for 2 hours or by angiotensin II (Ang, 10^{-7} mol/L) for 1 hour [29-34].

In preliminary experiments, incubation times of cells with MET (final concentrations 10^{-4} and 10^{-5} mol/L) from 1 to 3 hours were tested (data not shown); the pharmacologically relevant MET concentration of 10^{-5} mol/L and the incubation time of 1 hour, for which the maximal effect on intracellular ROS formation was observed, were chosen for this study.

Control cells were treated in the same conditions as assays except the addition of MET, stimulating agents (Glc and Ang), and/or inhibitors of ROS formation (apocynin and rotenone). When using apocynin or rotenone, the 2 inhibitors were present in culture medium for all steps of experiments. We verified that neither apocynin nor rotenone nor the stimulating agents Glc and Ang induced endothelial cell cytotoxicity under our experimental conditions, as evaluated by the neutral red assay.

2.5. Statistical analysis

Results are expressed as mean ± SEM of at least 6 separate and independent experiments. Statistical significance was determined by the nonparametric Mann-Whitney U test. P values of <.05 were considered to be statistically significant.

3. Results

3.1. Effect of MET on ROS production in nonstimulated BAEC

Incubation of confluent quiescent BAEC with MET resulted in a significant decrease of intracellular ROS production (−19%, $P < .05$, Fig. 1). The treatment of confluent BAEC with the NAD(P)H oxidase inhibitor

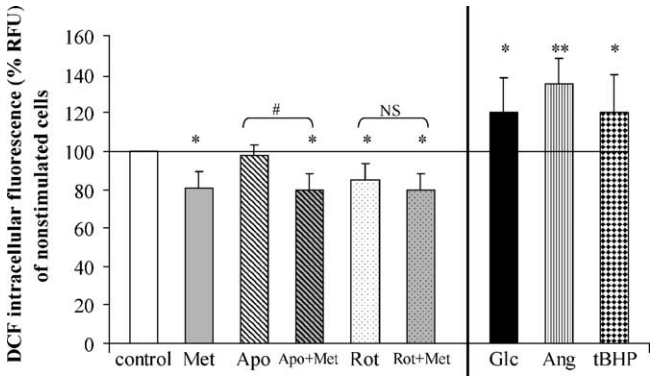

Fig. 1. Effects of MET on the intracellular ROS production in non-stimulated BAECs. Confluent cells were incubated with MET (1 hour) with and without the NAD(P)H oxidase inhibitor apocynin (Apo) or the mitochondrial respiratory chain complex I inhibitor rotenone (Rot) as described in the Materials and methods, then DCF intracellular fluorescence was measured. Results are expressed taking basal fluorescence of non-stimulated cells as the 100% reference. Bars on the right of the graph represent the increases in intracellular ROS production in cells induced by high levels of glucose (Glc, 2 hours), Ang, or tBHP (1 hour) as described in the Materials and methods. *$P < .05$, **$P < .01$ vs nonstimulated control cells; #$P < .05$ vs cells treated in the same conditions except MET.

apocynin alone did not significantly modify basal values of intracellular ROS production (−2%, not significant). The effect of MET in the presence of apocynin resulted in a significant decrease in ROS production (−20%, $P < .05$). Such decrease was similar to that observed with MET alone and statistically differed from the negligible effect of apocynin alone ($P < .05$). On the other hand, treatment of cells by the mitochondrial respiratory chain complex I inhibitor rotenone alone led to a significant, although moderate, decrease in ROS production (−15%, $P < .05$). Treatment of cells with MET in the presence of rotenone resulted in a significant decrease in ROS production (−20%, $P < .05$), which did not significantly differ neither from those observed with MET alone nor from that of rotenone alone.

3.2. Effect of stimulating agents on ROS production in BAEC

As positive controls, stimulation experiments of BAEC by high levels of Glc or by Ang (for 2 hours and 1 hour, respectively) led to a significant increase in intracellular ROS production, reaching +20% ($P < .05$) and +35% ($P < .01$) for Glc and Ang, respectively. Such increases did not differ from that of the pro-oxidant agent tBHP (stimulating time 2 hours), which was used as a positive control of cell-induced oxidative stress (Fig. 1).

3.3. Effect of MET on ROS production in stimulated BAEC

Incubation of MET with stimulated cells resulted in a significant inhibition of the ROS production. When compared with the intracellular ROS production in untreated cells, the decrease in ROS production in the presence of MET reached −28% ($P < .05$) and −72% ($P < .01$) for Glc (Fig. 2A) and Ang (Fig. 2B), respectively.

Treatment of cells by either apocynin or rotenone before incubation with MET, then with high levels of Glc, induced a significant inhibition of ROS production (−32% and −39%, respectively, vs ROS production in stimulated cells without both inhibitor and MET, $P < .01$). These inhibitory effects appeared more marked than those observed in cells treated with MET alone, that is, without any inhibitor. Apocynin alone and rotenone alone reduced ROS production in these cells by 17% and 23%, respectively ($P < .05$ vs basal value of stimulated cells) (Fig. 2A).

Treatment of cells by either apocynin or rotenone before incubation with MET, then with Ang, also induced a significant inhibition of ROS production (−84% and −55% vs ROS production in stimulated cells without both inhibitors and MET, $P < .01$). The decreases appeared significantly greater than in cells treated with MET alone, that is, without any inhibitor. Apocynin alone and rotenone alone reduced ROS production in these cells, respectively, by 66% ($P < .01$ vs basal value of stimulated cells) and 8% (not significant) (Fig. 2B).

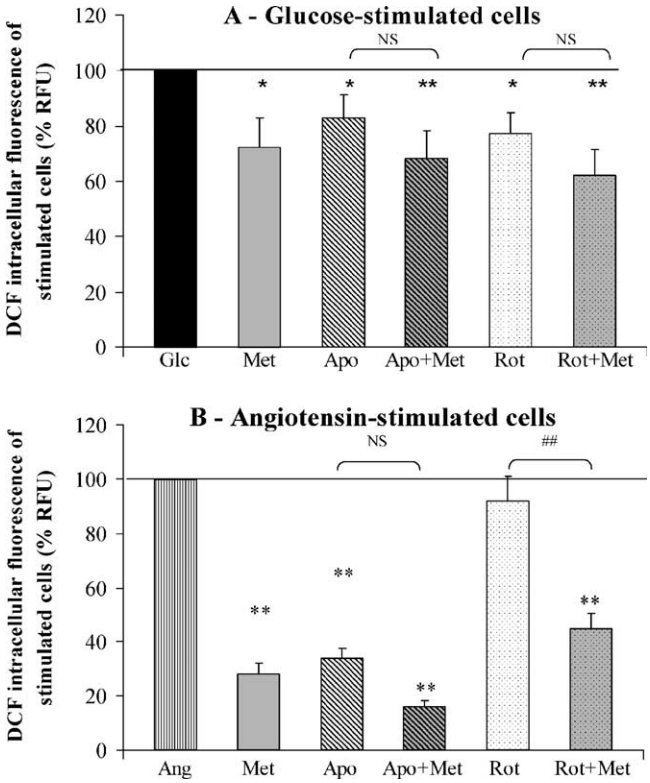

Fig. 2. Effect of MET on the intracellular ROS production in stimulated BAECs. Confluent cells were incubated with MET (1 hour) with and without the NAD(P)H oxidase inhibitor apocynin (Apo) or the mitochondrial respiratory chain complex I inhibitor rotenone (Rot) as described in the Materials and methods, then cells were stimulated by high levels of glucose (Glc 30 mmol/L, A) or angiotensin II (Ang 10^{-7} mol/L, B). DCF intracellular fluorescence was measured as described in the Materials and methods. Results are expressed taking basal fluorescence of stimulated cells as the 100% reference. $P < .05$, **$P < .01$ vs stimulated control cells; #$P < .05$, ##$P < .01$ vs stimulated cells treated in the same conditions except MET.

4. Discussion

The present data showed that MET, at the pharmacologically relevant concentration of 10^{-5} mol/L, significantly reduced the intracellular production of ROS in both nonstimulated and stimulated BAEC. They suggest that the antidiabetic drug was able to limit the activation of both NAD(P)H oxidase and mitochondrial respiratory chain complex 1, although differently according to the nature of cell stimulation.

As a first result, we observed that MET significantly reduced intracellular ROS production in nonstimulated endothelial cells. The effect of MET certainly could not be mediated through an inhibition of the membrane NAD(P)H oxidase activity, because apocynin alone did not modify endothelial intracellular ROS production. This result also indicates that basal NAD(P)H oxidase activity was very low in nonstimulating conditions. On the other hand, our results suggest that the effect of MET might be mediated through an inhibition of ROS issued from mitochondrial respiratory chain, because (i) rotenone alone led to a similar decrease in ROS production, (ii) the simultaneous action of rotenone and MET resulted in a similar decrease in ROS production as the one obtained by each alone, and (iii) the inhibitory effects of MET were similar with and without rotenone.

As a second main result of our study, MET was able to completely blunt the increases of ROS production in both Glc- and Ang-stimulated cells. High blood glucose levels, which occur in vivo during the postprandial state and in nonequilibrated diabetic subjects, stimulate endothelial ROS production through protein kinase C (PKC)-dependent activation of NAD(P)H oxidase in cultured endothelial cells [35], and also by increasing mitochondrial superoxide production [36]. Recent studies reported that short incubation times of cells (one to several hours) with high glucose levels led to an enhancement of intracellular ROS production [37]. Such short effects of glucose at high concentrations were chosen in our work to mimic the in vivo pathophysiological state of postprandial hyperglycemia occurring in diabetes. On the other hand, increased angiotensin activity caused by alteration of renin-angiotensin system in the diabetic disease may favor stimulation of endothelial NAD(P)H oxidase activity, through the activation of angiotensin II type-specific receptors and phosphorylation of the p47phox subunit [38-40]. Although the incubation time of cells with the stimuli was limited to 1 or 2 hours, intracellular endothelial ROS production was significantly enhanced by Ang as well as by high levels of Glc, and in a similar manner than that of the well-known pro-oxidant agent tBHP [41]. Because these stimulating agents induce intracellular ROS production in a different manner (both quantitatively and qualitatively), we assumed that the potential cellular targets of MET would be either mitochondrial respiratory chain or NAD(P)H oxidase, or both. ROS formation in Ang-stimulated cells was essentially caused by the activation of NAD(P)H oxidase and, as expected, was quite completely blunted via apocynin inhibition of NAD(P)H oxidase. The combined action of apocynin and MET led to a slightly and not significantly higher inhibition than the one of MET alone or apocynin alone. Thus, we can conclude that the target for MET in Ang-stimulated BAEC would be NAD(P)H oxidase. On the other hand, ROS formation in Glc-stimulated cells resulted from both NAD(P)H oxidase activation and the mitochondrial respiratory chain pathway, because apocynin and rotenone were able to reduce, each partially, endothelial ROS production. Under these conditions, the action of MET was partially maintained in the presence of apocynin or rotenone, suggesting that MET interacted with each of these 2 pathways. Further studies are required to specify the action of MET on these 2 pathways and whether other cellular sources of ROS (eg, xanthine oxidase activity) might be a target for MET way of action.

In a recent paper, Zou et al [42] reported that MET was able to increase intracellular reactive nitrogen and oxygen species, thus contributing to the activation of adenosine monophosphate (AMP)-activated protein kinase, a potential target for the pharmacological action of MET. Using quite different experimental conditions and higher MET concentrations than in our work (10^{-4} mol/L), these authors observed an increase in intracellular ROS formation in nonstimulated BAEC submitted to a 24-hour exposure to MET. As an explanation, a biphasic cellular effect of MET in ROS production, depending of either MET concentration or incubation time of cells with the drug (or both), may be proposed to explain these apparently discrepant results. Such a biphasic effect was reported by Detaille et al [43] concerning the metabolic action of MET.

Chelating properties of MET toward metal irons certainly are not involved in the decrease of ROS detection by the DCF-DA probe, which requires iron to be oxidized [44], for 2 main reasons: (1) chelation of intracellular metal ions by MET was unlikely, because penetration of MET into cells was reported to be quite weak (<1% [45]), and (2) the experimental procedure we used allowed a contact of cells with culture medium free of MET for 1 hour or 2 hours (according to the stimulating agent) before the measurement of DCF fluorescence. By this way, metal ions such as iron were present in the medium, and their penetration into cells remained possible [44]. Taken together, the cellular effects we observed more likely resulted from the alteration of a signal transduction pathway, probably mediated by a modulation of intracellular ROS production systems. To exert its antihyperglycemic effect, MET was shown to enhance cellular insulin signaling, in part by enhancing the decrease in cyclic (c)AMP levels induced by insulin [46], by increasing tyrosine kinase activity [47,48], as well as by decreasing tyrosine phosphatase activity [49], in relation to the modulation of cellular AMP kinase (AMPK) activity [50,51]. Such interaction of MET with intracellular signaling pathways could be the central point for both the antihyperglycemic effect and the intracellular antioxidant

properties of guanidic drugs. This could be of great interest in the cardiovascular field, in which involvement of ROS and of intracellular redox-signaling pathways (including the PKC pathway), especially the role of vascular NAD(P)H oxidase, is now well established [52,53].

In conclusion, our results showed that MET significantly decreased intracellular ROS production in nonstimulated endothelial cells as well as in stimulated cells (Glc and Ang). Depending on the nature of cell stimulation, such effects likely resulted from the reduction of NAD(P)H oxidase endothelial activity and/or mitochondrial respiratory chain–induced ROS production. The action of MET in endothelial oxidant/antioxidant equilibrium, through diminished ROS generation, therefore might participate to its additional benefits, such as those related to the improvement in the cardiovascular complications of diabetes.

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