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Metformin reduces endothelial cell expression of both the receptor for advanced glycation end products and lectin-like oxidized receptor 1

Nadjat Ouslimani, Meriem Mahrouf, Jacqueline Peynet, Dominique Bonnefont-Rousselot, Claudine Cosson, Alain Legrand, Jean-Louis Beaudeux*

Département de Biochimie, Faculté de Pharmacie, EA 3617 "Biochimie radicalaire et atteintes vasculaires", F75006 Paris, France

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Abstract

Beyond its antihyperglycemic action, the antidiabetic oral drug metformin possesses antioxidant properties that may contribute to improve the cardiovascular deleterious effects of the diabetic disease. We explored whether metformin could modulate the redox-sensible expression of receptor for advanced glycation end products (RAGE) and lectin-like oxidized receptor 1 (LOX-1), 2 endothelial membrane receptors involved in the arterial endothelial dysfunction observed in diabetes. Bovine aortic endothelial cells, either unstimulated or activated by high levels of glucose (30 mmol/L) or advanced glycation end products, were incubated for 72 hours with metformin at therapeutically relevant concentrations (10^{-5} to 5×10^{-4} mol/L). The expressions of RAGE and LOX-1 were evaluated on cell extracts by Western blot analysis. Metformin was shown to reduce, in dose-dependent manner, such expression of the 2 receptors, both in stimulated (by either glucose or advanced glycation end products) and in unstimulated cells. The effect of metformin was associated with a decrease in intracellular reactive oxygen species as assessed using the 2′,7′-dichlorodihydrofluorescein diacetate fluoroprobe. Taken together, our results suggest that the intracellular antioxidant properties of metformin may result in the inhibition of cell expression of both RAGE and LOX-1, possibly through a modulation of redox-sensible nuclear factors such as nuclear factor κB, that were shown to be involved in such receptor cell expression.
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1. Introduction

Macroangiopathy is a major complication of diabetes mellitus and represents a leading cause of morbidity and mortality. Chronic perturbation of diabetic vasculature leads to the accelerated progression of atherosclerotic plaques. This process is mediated through vascular cell dysfunction and oxidative stress that are key factors in inflammatory events and results in plaque erosion and rupture [1]. Dysfunction of vascular endothelium plays a key role in this process, through the release of proinflammatory mediators and the expression of cell membrane receptors that participate to maintain the chronic vascular inflammatory state.

The hyperglycemic state of diabetes is responsible for glycation of proteins and formation of advanced glycation end products (AGE) accumulating in the vessel wall by interaction with their selective receptor RAGE (receptor for advanced glycation end products) [2]. Advanced glycation end products are also ligands for other cell receptors, including lectin-like oxidized receptor 1 (LOX-1), which is implicated in the deleterious activation of several cell types involved in the atherosclerotic progression. In return, these 2 receptors have a diverse repertoire of ligands (eg, oxidized low-density lipoprotein for LOX-1), some of them being important proinflammatory stimuli for progression of atherosclerosis [3,4]. Activation of RAGE and LOX-1 receptors by these agents contributes to vascular endothelial dysfunction, by inducing proinflammatory redox-sensible transduction and transcription pathways [5].

Metformin (*N*,*N*-dimethylbiguanide) is an antihyperglycemic agent widely used for the management of type 2 diabetes mellitus. Beyond its glucose-lowering effects, metformin exhibits antioxidant properties that contribute to the vasculoprotective effects observed in several epidemiological studies [6]. Metformin possesses a direct scavenging effect against oxygenated free radicals generated

* Corresponding author. Tel.: +33 1 53 73 96 08; fax: +33 1 53 73 97 08.
E-mail address: jean-louis.beaudeux@univ-paris5.fr (J.-L. Beaudeux).

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in vitro [7] and decreases intracellular production of reactive oxygen species (ROS) in aortic endothelial cells through the reduction of both NAD(P)H oxidase and/or the mitochondrial respiratory chain pathways [8]. We and others recently showed that such effect was mediated by the modulation of redox-sensible transduction pathways such as the protein kinase C pathway [9,10], possibly through the activation of adenosine monophosphate kinase that reduces diacylglycerol synthesis [11].

We studied here whether metformin was able to modulate RAGE and/or LOX-1 receptor expressions in aortic endothelial cells activated by 2 pathophysiologic stimuli of the diabetic state, AGE and high glucose levels. We showed for the first time that the antidiabetic drug significantly reduced endothelial cell expression of the 2 receptors, and we also studied whether it might be related to the inhibitory effect of metformin on the intracellular ROS production.

2. Materials and methods

2.1. Materials, reagents, and culture media

Cell culture materials, media, and reagents were from Sigma-Aldrich (L'isle d'Abeau-Chesnes, France). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) probe was purchased from Interchim (Montluçon, France). Mouse antihuman LOX-1 receptor antibody was provided by Dr Sawamura (Tokyo, Japan); mouse antihuman RAGE and goat peroxidase-conjugated antimouse immunoglobulin G for Western blot revelation were from Chemicon International (Temecula, CA).

2.2. Cell culture

Bovine aortic endothelial cells (BAECs) were provided by Pr P. Duriez (Institut Pasteur, Lille, France). Cells 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. Cells were used between the sixth and tenth passages. Viability was assessed by the neutral red assay; a cell viability of more than 95% was constantly required for performing experiments.

2.3. Preparation of AGE

A methylglyoxal-modified bovine serum albumin (BSA) stock solution was prepared under sterile conditions by incubating 20 mg/mL of BSA with 750 mmol/L methylglyoxal in phosphate-buffered solution at 37°C for 72 hours. A work solution was extemporaneously prepared by a 1/10 000 dilution; when appropriate, BAECs were incubated with methylglyoxal-BSA (AGE-albumin) by a 10-fold dilution of the work solution. The lack of cell viability alteration by AGE in these experimental conditions was controlled by the neutral red assay.

2.4. Experimental procedure and Western blotting

Confluent cells in 75-cm^2 dishes were incubated for 72 hours with stimulating agents (final concentrations of either 30 mmol/L glucose or 2 μg proteins per milliliter of AGE-albumin) with or without metformin at various concentrations (10^{-5} to 5×10^{-4} mol/L), the lowest concentration being at a systemic pharmacologic level. Then cells were harvested by mild trypsination, washed twice in ice-cold PBS (pH 7.5), and lysed by sonication (3×10 seconds at 80 W, Brand Sonicator, Mettler, France); homogenates were centrifuged at 8000g for 5 minutes at 4°C to remove nuclei and unbroken cells. Protein concentration of the supernatant was determined using the Bio-Rad protein assay (Bio-Rad, Marnes la Coquette, France).

Cell homogenates (15 μg total proteins) were analyzed on 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gel then transferred to nitrocellulose membrane (Bio-Rad). Membrane was incubated first with blocking buffer (Tris-HCl 20 mmol/L, NaCl 150 mmol/L, 0.05% Tween 20, nonfat dry milk 5%, pH 7.5) for 1 hour at room temperature and then overnight at 4°C in the presence of primary antibodies (anti-RAGE, 1/1000; anti–LOX-1, 1/2000). After washing, membranes were incubated for 1 hour 30 minutes at room temperature with peroxidase-conjugated antimouse immunoglobulin G (1/5000). Antigen detection was performed with enhanced chemiluminescence detection system (Pierce-Perbio, Brébières, France).

Cells incubated for 72 hours without stimulating agent and metformin served as negative controls. Each experiment was performed at least in triplicate.

2.5. Intracellular ROS detection

The H_2DCF-DA probe 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 prooxidant stimulating agents. Basal values of fluorescence in nonstimulated cells were 3521 $\pm$ 135 relative fluorescence units (microplate fluorescence analyzer, Fluostar, BMG Labtech, Champigny sur Marne, France). Detection characteristics (gain, sensitivity) of the microplate fluorescence reader were maintained identical for all experiment measurements. Results were expressed as a percentage of basal fluorescence value of either nonstimulated or stimulated control cells.

3. Results

3.1. Expression of RAGE

Western blot analysis of cell lysates for RAGE expression confirmed that unstimulated BAECs did not constitutively

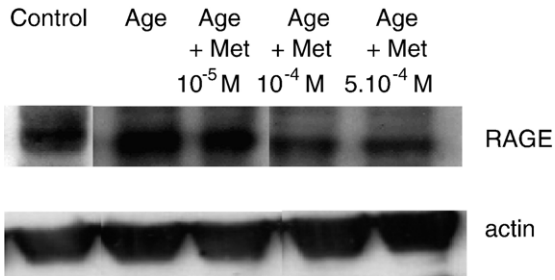

Fig. 1. Receptor for advanced glycation end products Western blots. Effects of metformin (Met) on RAGE expression in AGE-stimulated BAECs. Bovine aortic endothelial cells were incubated for 72 hours with AGE-albumin (final concentration, 20 μg proteins per milliliter) and with or without metformin (10^{-5}, 10^{-4}, or 5×10^{-4} mol/L). The expression of RAGE was analyzed by Western blot analysis on 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gel.

express RAGE. Incubation of unstimulated BAECs with metformin (10^{-5} to 5×10^{-4} mol/L) for 72 hours did not result in the appearance of RAGE expression in any concentration of the drug. On the contrary, stimulation of cells by AGE-albumin, but not by high levels of glucose, led to a sharp increase of RAGE expression. Finally, the simultaneous incubation of cells with AGE-albumin and metformin inhibited such increase in the receptor expression in a concentration-dependent manner (Fig. 1).

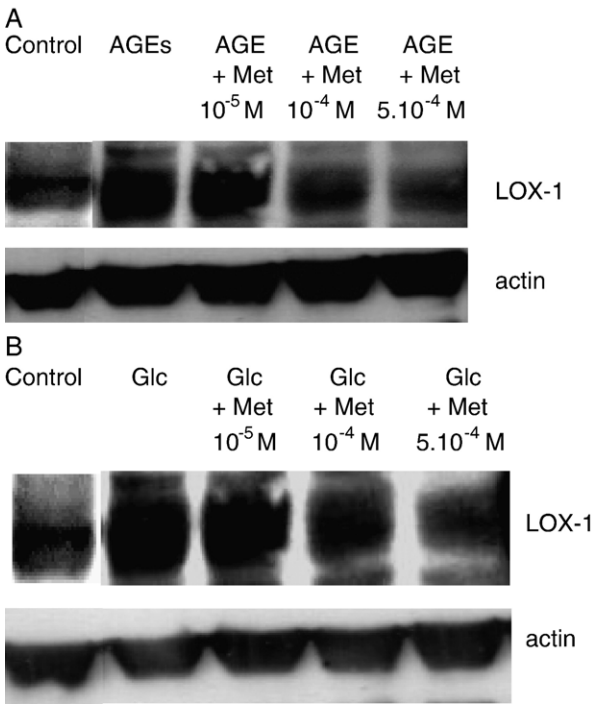

Fig. 2. Lectin-like oxidized receptor 1 Western blots. Effects of metformin (Met) on LOX-1 expression in stimulated BAECs. A, Bovine aortic endothelial cells were incubated for 72 hours with AGE-albumin (final concentration, 20 μg proteins per milliliter) and with or without metformin (10^{-5}, 10^{-4}, or 5×10^{-4} mol/L). Lectin-like oxidized receptor 1 expression was detected by Western blot analysis on 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gel. B, Bovine aortic endothelial cells were incubated for 72 hours with high glucose concentration (30 mmol/L) with or without metformin (10^{-5}, 10^{-4}, or 5×10^{-4} mol/L). Lectin-like oxidized receptor 1 expression was detected by Western blot analysis on 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gel.

3.2. LOX-1 expression

Western blot analysis of cell lysates for LOX-1 expression indicated that unstimulated BAECs constitutively express LOX-1 at a low level. Incubation of unstimulated BAECs with metformin for 72 hours did not modify LOX-1 expression in any concentration of the drug (data not shown). Stimulation of cells with AGE-albumin led to a sharp increase of LOX-1 expression. The simultaneous incubation of cells with AGE-albumin and metformin inhibited such increase in the receptor expression in a concentration-dependent manner (Fig. 2A). In the same way, high levels of glucose for 72 hours resulted in a significant enhancement in LOX-1 cell expression that was at least partly inhibited by the simultaneous incubation of metformin (Fig. 2B). As previously observed, the inhibitory effect of metformin on glucose-induced LOX-1 expression appeared to be concentration-dependent.

3.3. Intracellular ROS production

Incubation of unstimulated cells with metformin resulted in a slight decrease in intracellular ROS production, from the lowest concentration tested (−13%, 10^{-5} mol/L). Higher metformin concentrations resulted in statistically significant decrease in ROS production (−39% and −46% for metformin concentrations of 10^{-4} and 5×10^{-4} mol/L, respectively).

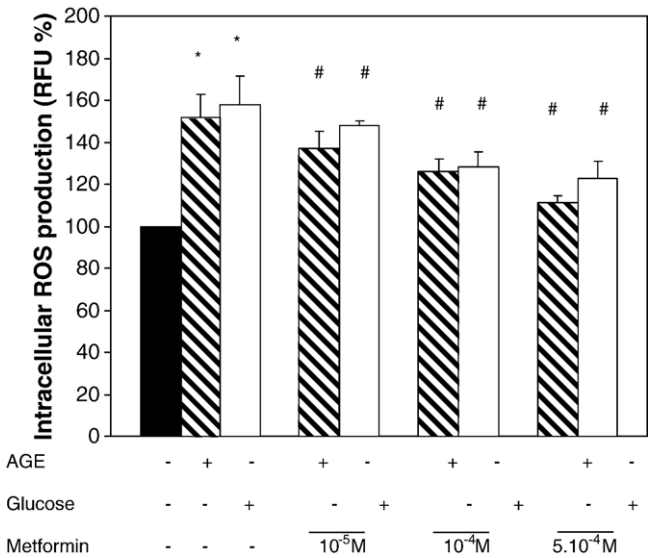

Fig. 3. Effects of metformin on the intracellular ROS production in BAECs. Confluent cells were incubated with metformin (10^{-5}, 10^{-4}, or 5×10^{-4} mol/ L) for 72 hours with and without high concentrations of glucose (30 mmol/L) or AGE-albumin (final concentrations, 20 μg proteins per milliliter). DCF intracellular fluorescence was measured as described in Materials and methods. Results are expressed taking basal fluorescence of unstimulated cells as the 100% reference. *$P < .05$ vs unstimulated control cells; #$P < .01$ vs the corresponding stimulated cells. RFU = relative fluorescence units.

Experiments of BAEC stimulation with AGE or high glucose levels for 72 hours led to a significant increase in intracellular ROS production, reaching +52% and +58% ($P < .05$), respectively (Fig. 3). As observed with unstimulated cells, incubation of AGE- or glucose-stimulated cells with metformin resulted in a statistically significant dose-dependant inhibition of the ROS production.

4. Discussion

Vascular endothelial dysfunction is a determinant for atherogenesis and plaque progression, particularly in the context of diabetic macroangiopathy. Cell-cell interactions and endothelium activation participate to this process, and are mediated by ligand-receptor interactions. We showed here that pathophysiologic stimuli of the diabetic disease, especially AGE, were able to increase endothelial cell expression of RAGE and LOX-1 receptor, and we demonstrated for the first time that the overexpression of these 2 receptors was reversed by pharmacological concentrations of the antidiabetic drug metformin in our experimental conditions.

The action of AGE and high levels of glucose on endothelial cell have been reported to result in several molecular events such as the generation of ROS (essentially by activation of 2 major sources of ROS, NAD(P)H oxidase and mitochondrial respiratory chain [12-16]), and the activation of p44/p42 mitogen-activated protein kinase pathway [17,18], thus leading to the activation of nuclear factor κB (NF-κB) [19,20]. As a consequence, stimulation of endothelial cells by AGE and glucose was shown to enhance the release of endothelial proinflammatory mediators such as monocyte chemoattractant protein 1, vascular cell adhesion molecule 1, and tumor necrosis factor α [21,22].

In addition, AGE was reported to enhance the endothelial cell expression of LOX-1 [23] as well as the expression of its own specific receptor RAGE [24]. Finally, high levels of glucose were shown to enhance LOX-1 [3,25], although Chen et al [26] did not observe such an effect, but were unable to modify endothelial RAGE expression. By return, activation of these 2 receptors results in the intracellular enhancement in ROS formation [27-29], and to the activation of the above-mentioned redox transduction/transcription pathways [25,17], thus contributing to the vicious circle of endothelial dysfunction in the diabetic state.

Our results indicated that a 72-hour incubation of AGE- or glucose-stimulated endothelial cells with metformin resulted in a significant reduction in intracellular ROS production. Such in vitro intracellular antioxidant properties of metformin were already described by our group using shorter incubation times of metformin with endothelial cells. We showed that metformin reduced intracellular ROS production by modulating both NAD(P)H oxidase activity and the mitochondrial respiratory chain [8]. We and others also recently reported that metformin was able to negatively modulate endothelial cell PKC activity, and this effect appeared closely linked to intracellular antioxidant properties of the drug [9,10]. Finally, the recent study of Isoda et al [30] and Li et al [31] confirmed that metformin, at therapeutically relevant concentrations, was able to inhibit the activation of NF-κB in vascular cells, although only reported in smooth muscle cells. Actually, antioxidant activity of the antidiabetic drug is now demonstrated by both experimental [32,33] and clinical studies [34], and probably participates to the vasculoprotective effects of metformin observed in epidemiological studies [6]. Because the expressions of RAGE and LOX-1 receptor appeared to be mediated through redox-dependent pathways, the decrease in ROS production induced by metformin certainly contributes to the negative modulation of both RAGE and LOX-1 expressions we originally described in this report. As a unifying model of the relation of the consequences of AGE or glucose cell activation and the modulation of RAGE/LOX-1 receptor by metformin, we can postulate that AGE or high levels of glucose are able to bind to constitutively expressed (although at a low level) endothelial cell LOX-1 receptor, thereby increasing intracellular ROS production (Fig.4). Besides, such increase may be directly induced by glucose through activation of mitochondrial respiratory chain and NAD(P)H oxidase, as we previously observed [8]. The resulting activation of redox-sensible transcription factors such as NF-κB may induce synthesis and release of proinflammatory mediators as well as the overexpression of both LOX-1 and RAGE that in return will be able to increase intracellular redox imbalance, thus amplifying the effects of initial stimulus.

In any way, the effects of metformin on RAGE end LOX-1 expressions may improve several adverse effects of endothelial dysfunction in the diabetic state. Inhibition of NF-κB lowers proinflammatory response by vascular endothelium [30]. On the other hand, inhibition of LOX-1 cell expression, as induced by different pharmacological agents, results in decreased CD40/CD40L [35] and adhesion molecule [36,37] expressions, as well as decreased apoptosis. Such effects, which can be mediated by the inhibition of redox transcription factors such as NF-κB, could be

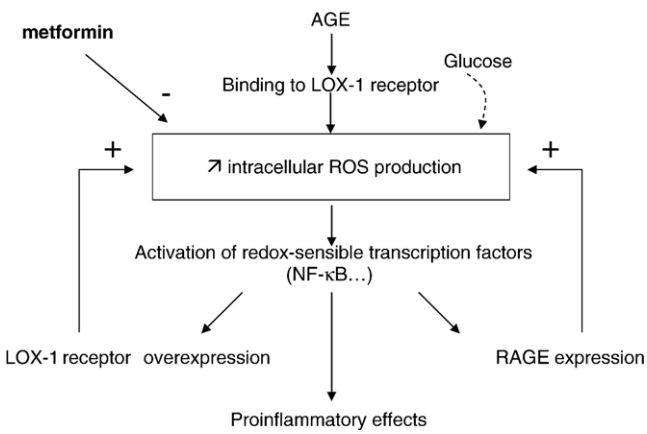

Fig. 4. Possible mechanism for the inhibitory action of metformin on RAGE and LOX-1 expressions.

reproduced by metformin, and therefore could account for the pharmacological vasculoprotective actions of this drug beyond its antihyperglycemic properties. The concentrations used in this work included pharmacological systemic levels (10^{-5} mol/L) and higher levels; however, several reports indicated that peripheral blood levels may underestimate tissue levels because tissues and subcellular organites were shown to accumulate metformin [38,39]. Thus, inhibition of RAGE/LOX-1 expressions by metformin we described here could be relevant in the atheromatous lesion environment and could contribute to the beneficial properties of the drug on macrovascular complications of diabetes.

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