An Intracellular Modulation of Free Radical Production Could Contribute to the Beneficial Effects of Metformin Towards Oxidative Stress

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Metformin (dimethylbiguanide) is an antihyperglycemic agent used in type 2 diabetes. Beyond its action on glycemic control, metformin exhibits other intrinsic effects that could play a role in prevention against diabetes complications. Some studies thus reported an improvement in the antioxidant status in patients treated with metformin. This might be in part related to its property to limit formation of advanced glycation end products (AGEs) and to decrease the overproduction of free radicals in diabetic subjects. The aim of this study was to investigate the in vitro ability of metformin to modulate the action of reactive oxygen species (ROS) generated either by water gamma radiolysis or by stimulated human leukocytes. Our results showed that metformin at pharmacologically relevant concentrations was in vitro able to scavenge hydroxyl ($^{\bullet}OH$) but not superoxide ($O_2^{\bullet -}$) free radicals and that hydrogen peroxide did not react with metformin. Nevertheless, when polymorphonuclear cells (PMN) are stimulated by phorbol myristate acetate (PMA), or above all by formyl methionine leucyl phenylalanine (fMLP), a systematic (although nonsignificant) decrease of the ROS-induced chimiluminescence (CL) was observed. These results suggest that metformin could directly scavenge ROS or indirectly act by modulating the intracellular production of superoxide anion, of which NADPH oxidase constitutes the major source. This could contribute to the additional benefits of metformin, especially those related to the improvement in the cardiovascular outcomes in diabetes.

THE BIGUANIDE METFORMIN is an antihyperglycemic agent used for the management of type 2 diabetes.[1] The United Kingdom Prospective Diabetes Study (UKPDS)[2] suggested that this drug, which is an aminoguanidine structurally related compound, likely has other beneficial effects that could be involved in prevention against diabetic complications. Several effects of metformin might thus contribute to the improvement in the predominantly cardiovascular outcomes in diabetes, for example, its ability to reduce the rate of formation of advance glycation end products (AGEs) involved in the pathogenesis of secondary complications of diabetes,[3] to lower systemic methylglyoxal concentrations,[4] and to react with dicarbonyl compounds.[5] This suggests that metformin has aminoguanidine-like activities[6] in relation to its structure. Reactive oxygen species (ROS) play a central role in the formation of AGEs[7] and, conversely, interaction of AGEs with their receptors (RAGE) enhances cellular oxidative stress and leads to an activation of transcription factors.[8] Increased production of superoxide by the mitochondrial electron transport chain is a causal link between formation of AGEs and activation of the pleiotropic trancription factor nuclear factor $\kappa\beta$ (NF$\kappa\beta$) and the other pathways of hyperglycemic damages.[9] In addition, ROS promote enhanced oxidation of low-density lipoproteins (LDLs), which are involved in the pathogenesis of atherosclerosis and induce multiple cell activations and dysfunctions.[10] Until now few studies were focused on the effect of metformin on the oxidative stress present in diabetic subjects and resulting both from an overproduction of free radicals and from a reduction in antioxidant defences.[11] These studies all reported an improvement of the antioxidant status with an increase in antioxidant activities in red blood cells,[12] and hepatic and blood levels[13] in rats, and a decrease in xanthine oxidase activity and lipid peroxidation markers in type 2 diabetic patients.[14-16] Such an improvement of the antioxidant status might result from the above-mentioned effects of metformin on AGE formation but also from a direct or indirect inhibition of free radicals, as reported for aminoguanidine.[17,18] The aim of the present study was therefore to investigate the in vitro ability of metformin to modulate the action of ROS generated either by water gamma radiolysis or by stimulated human leukocytes.

MATERIALS AND METHODS

In Vitro Production of ROS

Aqueous solutions of metformin were prepared by dissolving metformin hydrochloride (Sigma, St Louis, MO; ref. D-5035) in 10 mmol/L sodium phosphate buffer at pH 7, in the absence or presence of 100 mmol/L sodium formate, in order to study the respective effect of $^{\bullet}OH/O_2^{\bullet -}$ or $O_2^{\bullet -}/HO_2^{\bullet}$ free radicals.[19] Pharmacological metformin concentrations were close to 20 μmol/L,[20] so that we studied metformin in the range of concentrations of 10 to 100 μmol/L. Gamma irradiations were performed on 5 mL of metformin solutions with an IBL 637 irradiator (CIS Biointernational, Saclay, France) using a cesium 137 γ-ray source whose activity was approximately 222 TBq (dose rate = 0.17 Gy $\cdot$ s^{-1}). The radiation dose (expressed in Gy) delivered to the LDL solutions directly depended on the time of exposure to the ^{137}Cs source: the longer the exposure time, the higher the radiation dose. No direct interaction of γ radiation with metformin occurred and the radiolytic effect was only due to the radical species produced.[19] Analysis of metformin solutions before and after irradiations was performed by absorption spectrophotometry (Beckman DU 70 spectrophotometer, Gagny, France). Radical-induced metformin consumption was also checked by reverse-phase high-performance liquid chromatography (HPLC), according to an adaptation of the

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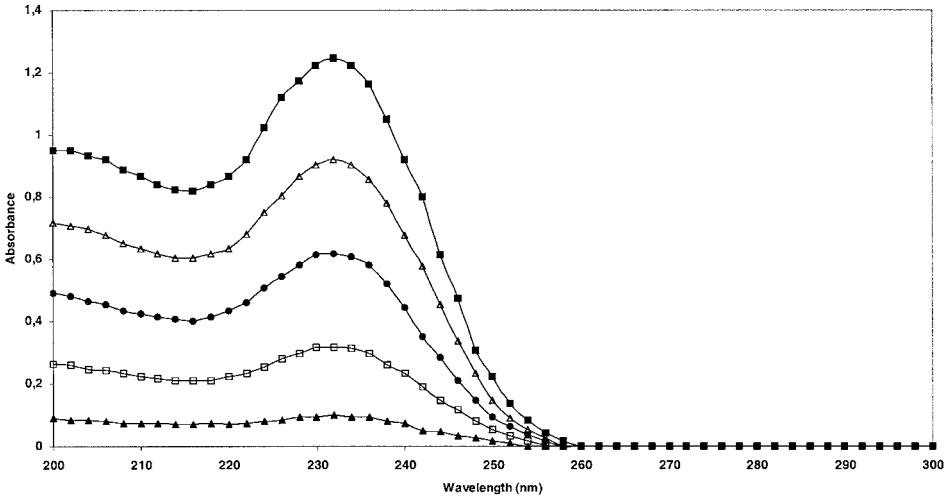

Fig 1. Absolute absorbance spectra of metformin solutions: (▲) 10 μmol/L; (□) 25 μmol/L; (●) 50 μmol/L; (△) 75 μmol/L; (■) 100 μmol/L. Reference: 10 mmol/L sodium phosphate, pH 7. l = 1 cm.

method described by Yuen and Peh,[20] with a Spherisorb CN 5-μm column (250 mm × 4.6 mm) from Ait Chromato (Le Mesnil le Roi, France).

Determination of ROS Production by Phorbol Myristate Acetate–or Formyl Methionine Leucyl Phenylalanine–Stimulated Polymorphonuclear Leukocytes Using Chemiluminescence

Venous peripheral blood (4.5 mL) from 6 healthy volunteers was collected into Vacutainer tubes (Beckton-Dickinson, Le Pont de Clair, France) containing EDTA. Red blood cells were destroyed using ammonium chloride solution (155 μmol/L NH_4Cl, 12 μmol/L $NaHCO_3$, 0.01 μmol/L EDTA). Thereafter, leukocytes were twice washed in RPMI-1640 medium (Sigma-Aldrich, Saint Quentin Fallavier, France). Cells were tested for viability (>95%) using Trypan blue dye exclusion test. The final cell suspension was adjusted in RPMI-1640 medium to the polymorphonuclear leukocyte (PMN) cell density needed (1×10^6 PMN/mL) after counting in a Malassez chamber.

As previously described,[21] ROS production by PMN was determined by luminol-amplified chemiluminescence (CL) assay. PMN suspension (5×10^5) plus luminol (0.2 mmol/L ; Sigma) was introduced in disposable polypropylene vials. The vials were placed in the light-proof chamber of a luminometer (model 1250, LKB Pharmacia, Val de Reuil, France) at 37°C. Metformin (20 μmol/L) or aminoguanidine (AMI, 20 μmol/L) was added to the cell suspension. PMN were then immediately stimulated with phorbol 12-myristate, 13-acetate (PMA, 1 μmol/L; Sigma) or with formyl methionyl leucyl phenylalanine (fMLP, 10 μmol/L; Sigma). The resulting light output was continuously recorded on a chart recorder, simultaneously with a printout set. All results are expressed as mV/10^5 cells using the CL emission peak.

RESULTS

Scavenging Activity of Metformin Towards $^{\bullet}OH/O_2^{\bullet -}$ Free Radicals

Figure 1 shows as an example the absolute absorbance spectra of metformin solutions at 5 concentrations (10, 25, 50, 75, and 100 μmol/L). Metformin solutions exhibited an absorbance maximum at 232 nm. A molar extinction coefficient was determined in the range of concentrations studied: ϵ_{232} = 12,400 ± 400 mol/$L^{-1} \cdot cm^{-1}$.

When metformin solutions were submitted to increasing radiation doses, from 25 to 420 Gy, the absorbance maximum at 232 nm decreased as a function of the radiation dose, whereas 2 absorbance bands appeared that were centered on 208 and 258 nm. Two isobestic points at 223 and 250 nm evidenced the concomitant disappearance of metformin and the formation of a single final product whose nature is by now still unknown. Similar spectral evolutions were observed for all metformin concentrations studied.

Metformin irradiated in the presence of formate (action of $O_2^{\bullet -}/HO_2^{\bullet}$ free radicals) did not exhibit any changes in the differential spectra (data not shown), suggesting that these radicals did not react with metformin under these experimental conditions. Hydrogen peroxide (at concentrations ranging from 50 μmol/L to 1 mmol/L) did not modify the differential spectra of metformin solutions, suggesting no action of H_2O_2 towards metformin.

To quantify the variations of absorbance observed on the differential spectra, we monitored the differential absorbances at 232 nm as a function of the radiation dose. As an example, after a radiation dose of 400 Gy, about 24% and 45% of the initial metformin concentration disappeared for initial metformin concentrations of 50 and 20 μmol/L, respectively.

Determination of ROS Production by PMA- or fMLP-Stimulated PMN Using Chemiluminescence

fMLP produced a shorter and lower CL signal in comparison with PMA activation. In addition, fMLP activation resulted in a transitory PMN activation, contrary to PMA-dependent signal, which reached a plateau (Fig 2). fMLP-induced response was depressed by either AMI or metformin (31% for AMI and 28% for metformin) (Table 1), but in a nonsignificant manner. Activation of PMN by PMA induced a dramatic increase of CL signal in control, as well as in metformin- or AMI-treated wells, producing 368 ± 102, 354 ± 106, and 335 ± 106 mV, respectively (Table 1). A nonsignificant decrease of the CL pick was observed with both metformin and AMI, and the percentages of inhibition were 8% and 15%, respectively.

DISCUSSION

Our radiolysis experiments are in favor of a direct scavenging effect of metformin towards •OH free radicals. This action is similar to that we previously reported for AMI towards those radical species.[22] It is noteworthy that this scavenging effect remained relatively weak, at least for the highest concentrations of metformin (50 and 100 μmol/L). Nevertheless, this could be of interest as regards protection towards LDL peroxidation, which is known to be involved in cardiovascular disease. Indeed, we recently reported that AMI, whose direct scavenging capacity towards •OH free radicals was of the same order of magnitude as that observed with metformin, exhibited a protective effect and acted as an antioxidant upon free radical–induced LDL oxidation.[23]

However, our data clearly show no direct scavenging effect of metformin towards $O_2^{\bullet -}$ free radicals and towards hydrogen peroxide. Moreover, with regard to the ROS production by PMA- or fMLP-stimulated leukocytes, the modulating effect of metformin (and of AMI) was dependent on the mode of activation. Indeed, a very weak action was observed after PMA stimulation, whereas an inhibitory effect was shown in fMLP-stimulated cells. The very high CL response recorded after PMA activation might mask the quenching capacity of these 2 compounds. In addition, the binding of fMLP to its specific membrane receptors on phagocytes stimulates the NADPH-oxidase, the enzyme responsible for the production of ROS, mainly by activation via phospholipase C and an increase in Ca^{2+} influx from the medium[24] via protein kinase C and ERK 1/2 pathway cooperation.[25] On the other hand, PMA, a diacyl glycerol-like molecule, behaves as a protein kinase C agonist and activates the NADPH-oxidase via transduction pathways dependent or independent of phosphatidylinositol-3-kinase.[26,27] These 2 distinct mechanisms might explain the different effect of AMI and metformin depending on the activator used. The modulating effect of metformin on fMLP-activated PMN appeared to be similar to that of AMI, which was previously shown to present quenching effects on burst oxidative products.[18] It is the first time that such an effect is reported for metformin. The facts that metformin exhibited no scavenging effect towards superoxide radicals and that the differential effect of metformin on cell ROS production depended on the activator used were in favor of an indirect cellular action of metformin. It could be noted that, in contrast with what has been reported for AMI,[18,28] no conclusive effect for metformin has been found on nitric oxide (•NO), a vasodilator substance produced by NO synthase.[29] Metformin more likely could act as a modulator of the activation of NADPH oxidase. Given that the intracellular metformin concentration has been reported to be very low (eg, <0.1% of metformin was recovered in the cytoplasm after a 60-minute incubation with Xenopus oocytes[30,31]), it could be hypothesized that metformin may recognize some specific membranous sites,[31] allowing metformin to further induce a transduction signal responsible for a modulation of NADPH oxidase activity or of other sources of intracellular ROS. This could be of great interest in the cardiovascular field, since vascular NADPH oxidases share some characteristics of the neutrophil enzyme and produce ROS, which serve as second messengers to activate multiple intracellular signaling pathways.[32] Such an intracellular action of metformin would be in accordance with the aim of developing pharmacological agents that inhibit superoxide overproduction, in order to prevent the progression of diabetic complications.[9]

Thus, in addition to its direct scavenging effect on •OH radical, metformin could act as a modulator of the activity of the NADPH oxidase. These properties could contribute to the additional benefits of metformin, especially those related to the improvement in the cardiovascular outcomes in diabetes.

Table 1. Maximum CL Emission Peaks and Inhibition Percentages Induced by 20 μmol/L Metformin or AMI in PMA- or fMLP-Stimulated PMN

	PMA		fMLP	
	Maximum (mV)	% Inhibition	Maximum (mV)	% Inhibition
Control (n = 6)	368 ± 102	—	66 ± 34	—
Metformine (n = 6)	354 ± 106	8 ± 4	50 ± 27	28 ± 8
AMI (n = 6)	335 ± 106	15 ± 6	47 ± 24	31 ± 10

NOTE. Values are means ± SE.

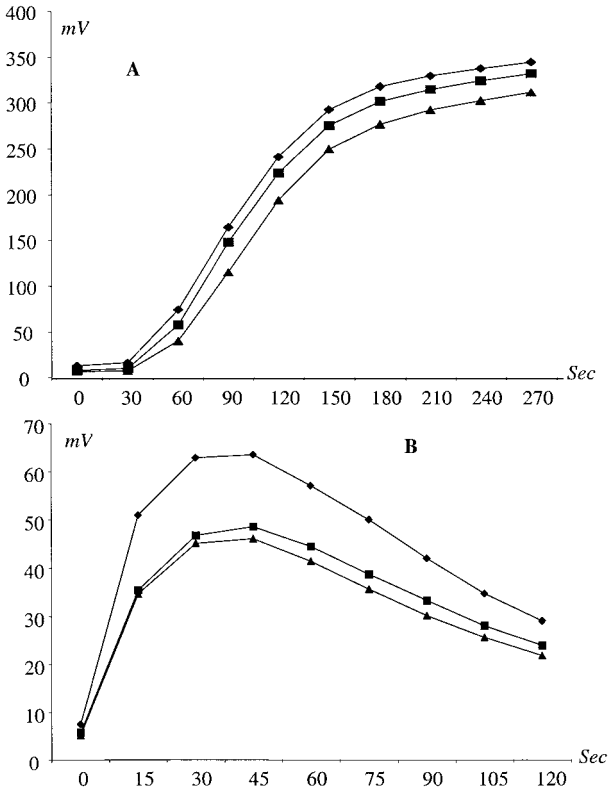

Fig 2. ROS production by PMA- or fMLP-stimulated PMN (5×10^5) measured using luminol-amplified CL assay. PMN were preincubated either with metformin (2×10^{-5} mol/L, ■) or AMI (2×10^{-5} mol/L, ▲) and, thereafter, stimulated by PMA (A) or fMLP (B). A control well, without metformin or AMI treatment, was also studied (◆).

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