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Metformin inhibits macrophage cholesterol biosynthesis rate: Possible role for metformin-induced oxidative stress

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ABSTRACT

The aim of the present study was to analyze the metformin (MF) effect on two cellular atherogenic activities: cholesterol biosynthesis and oxidative-stress (OS) as studied in J774A.1 macrophage cell line. MF (2–5 mM) significantly and dose-dependently reduced macrophage cholesterol content and cholesterol biosynthesis rate from acetate, but not from mevalonate, by up to 68% and 71%, respectively. MF inhibitory effect on cholesterol biosynthesis was similar to that of simvastatin. In contrast to the above anti-atherogenic MF effect, MF significantly increased cellular OS as shown by enhancement of reactive oxygen species (ROS) production by up to 70%, and decrement in cellular reduced glutathione (GSH) levels by up to 67%. Macrophage paraoxonase2 (PON2) expression however, increased by MF, by up to 1.5 folds. To overcome the MF oxidation stimulation, macrophages were incubated with MF together with potent dietary antioxidants, i.e. ~5 μg GAE/ml of pomegranate juice (PJ) or 30 μM of vitamin E (VE). Both of these potent antioxidants substantially reduced MF-induced OS, and in parallel, abolished MF inhibitory effect on cholesterol biosynthesis rate. We thus conclude that the inhibition of macrophage cholesterol biosynthesis by MF is related, at least in part, to MF-induced OS.

1. Introduction

Cholesterol accumulation in macrophages, which leads to foam cell formation, is a crucial stage in the development of the atherosclerotic lesion [1–4]. Both cholesterol accumulation and cellular oxidation are known to be enhanced in diabetes [5] and contribute to increased rate of cardiovascular disease (CVD) as one of the major complications associated with type 2 diabetes mellitus (T2DM) [6].

Metformin (MF) has been reported to be associated with reduced rates of CVD [7]. MF decreases intracellular lipid levels and improves insulin sensitivity in pre-adipocytes, hepatocytes and skeletal muscle cells [8–10]. Metformin was shown to activate AMP-activated protein kinase (AMPK) which phosphorylates HMGCoA reductase resulting in its inhibition and thus to the suppression of cholesterol biosynthesis [11,12]. MF was also shown to lower serum VLDL and LDL levels in patients with diabetes [13–15].

The impact of metformin on oxidative stress (OS) appears to be contradictory. While some studies demonstrate reduction in OS [16,17], others indicate the opposite [18,19]. However, none of these studies examined MF effect on OS in macrophages, and the relationship between this effect and MF induced inhibition in macrophages cholesterol biosynthesis.

In the present study we examined the effect of MF on macrophages lipid accumulation and oxidative stress and investigated the linkage between OS and macrophages ability to synthesize cholesterol under MF treatment.

2. Materials and methods

2.1. Reagents

Dihydrocumarin (DHC), 2′, 7′-dichlorofluorescin diacetate CFH-DA), simvastatin, and metformin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Simvastatin was dissolved in DMSO. PBS, DMEM medium, FCS, penicillin, streptomycin, L-glutamine, and sodium pyruvate were purchased from Biological Industries (Beth Haemek, Israel). Pomegranate juice (PJ) was obtained from PomWonderful, Los Angeles, CA, USA. PJ stock solution concentration was 6 mg gallic acid equivalents (GAE)/ml. Analyses of PJ concentrate revealed the presence of some phytosterols such

Abbreviations: T2DM, type 2 diabetes mellitus; MF, metformin; AMPK, AMP-activated protein kinase; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; OS, oxidative stress; ROS, reactive oxygen species; GSH, glutathione; PON2, paraoxonase2; PJ, pomegranate juice; VE, vitamin E.

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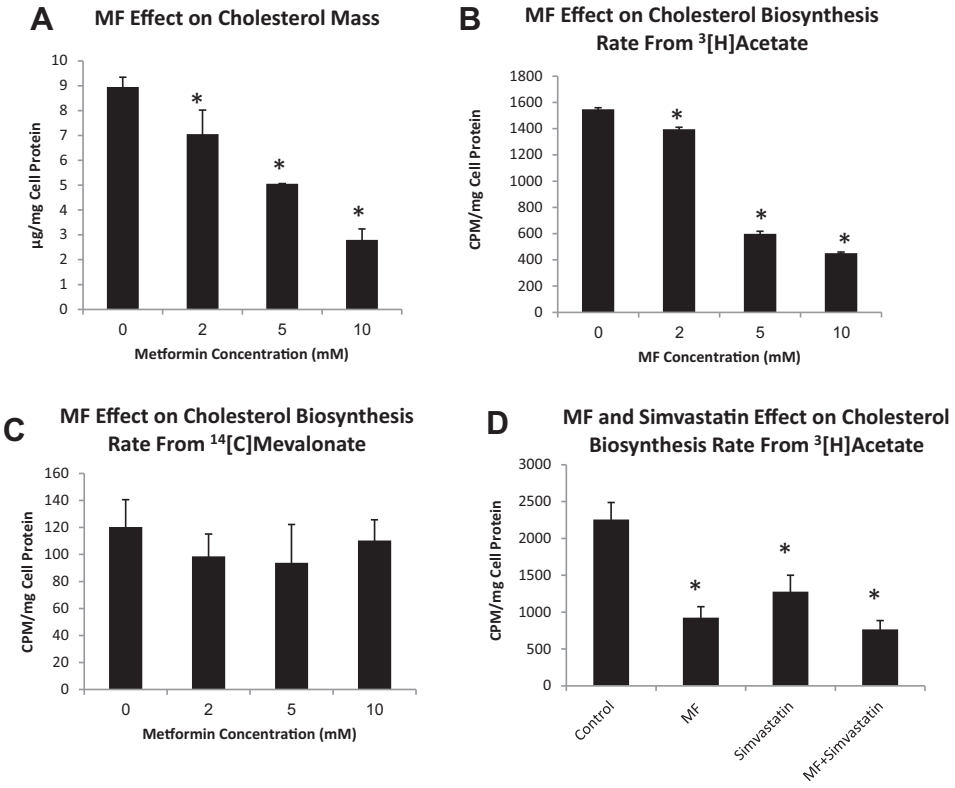

Fig. 1. Effect of MF on macrophage total cholesterol mass and on cholesterol biosynthesis rate. J774.A1 macrophages were incubated without (control) or with increasing concentration of MF as indicated, or with 15 µg/ml simvastatin for 24 h at 37 °C. The cells were lipid extracted with hexane:isopropanol (3:2, v/v) and the amount of total cholesterol was measured in the dried hexane phase (A). The cells were washed and further incubated for 4 h with [3H]acetate (1 mCi/L) (B–C) or with [^{14}C] mevalonate (1 mCi/L) (D). The cellular cholesterol biosynthesis rate was determined as described under methods. Data represent mean ± SEM; $n = 3$; $^{*}P < 0.01$ vs. control.

as: β-sitosterol (115 µg/gr), campesterol (73 µg/gr), and also estriol (36 µg/gr).

2.2. Cells

A J774.A1 macrophage-like cell line was obtained from the American Tissue Culture Collection (Rockville, MD, USA) and maintained in DMEM containing 1000 U/L penicillin, 100 mg/L streptomycin and 5% heat-inactivated (56 °C for 30 min) FCS.

2.3. Pomegranate processing

Pomegranates were washed, chilled at 32 °C and stored in tanks. Then, the fruits were crushed and squeezed to yield pomegranate juice (PJ). The juice was filtered, concentrated and stored at −18 °C.

2.4. Macrophage cholesterol mass

Lipids from J774 A.1 (2×10^6) were extracted with hexane:isopropanol (3:2, v:v) and the hexane phase was evaporated under nitrogen. The amount of cellular cholesterol was determined using a kit (CHOL, Roche Diagnostics GMbH, Mannheim, Germany).

2.5. Macrophage cholesterol biosynthesis

J774 A.1 macrophages were incubated with [^{3}H] acetate, followed by cellular lipid extraction with hexane: isopropanol (3:2, v/v) and separation by thin layer chromatography (TLC) on silica gel plates. The spots of unesterified cholesterol will be visualized by iodine vapor, scraped into scintillation vials, and counted for radioactivity [13].

2.6. Macrophage peroxides content (DCFH assay)

Cellular peroxide levels were determined by the flow-cytometric assay with Dichlorofluorescin-diacetate (DCFH-DA). DCFH-DA is a nonpolar dye that diffuses into the cells. In the cells it is hydrolyzed into the nonfluorescent derivate 2′, 7′–dichlorofluorescin (DCFH), which is polar and trapped within the cells. Under oxidative stress, DCFH is oxidized to DCF, which is a fluorescent compound [8]. J774 A.1 (2×10^6) were preincubated with the by-products for 1 h at 37 °C in DMEM medium +5% FCS. After cell wash, the cells were further incubated with 2.5×10^{-5} mol/L DCFH-DA for 30 min at 37 °C. Reaction was stopped by washes with PBS at 4 °C. Cellular fluorescence was determined with a flow cytometric apparatus (FACS-SCAN, Becton Dickinson, San Jose, CA, USA). Measurements were done at 510–540 nm after excitation of cells at 488 nm with an argon ion laser.

2.7. Macrophage reduced glutathione (GSH) content

All the preparation steps were carried out on ice. MPM derived from the mice from triplicate dishes (2×10^6 per dish were washed, scraped from the dish and sonicated in an ultrasonic processor (3×20 s at 80 W). The protein content was measured by the Lowry method [9] and the GSH level by the DTNB-GSSG reductase recycling assay [6].

2.8. Real Time RT-PCR for macrophage PON2

Total RNA was extracted with Epicentre commercial kit (Tamar, Israel). cDNA was generated from 1 µg of total RNA using Thermo Scientific commercial kit (Tamar, Israel). Products of the RT were subjected to Quantitative PCR using TaqMan Gene Expression

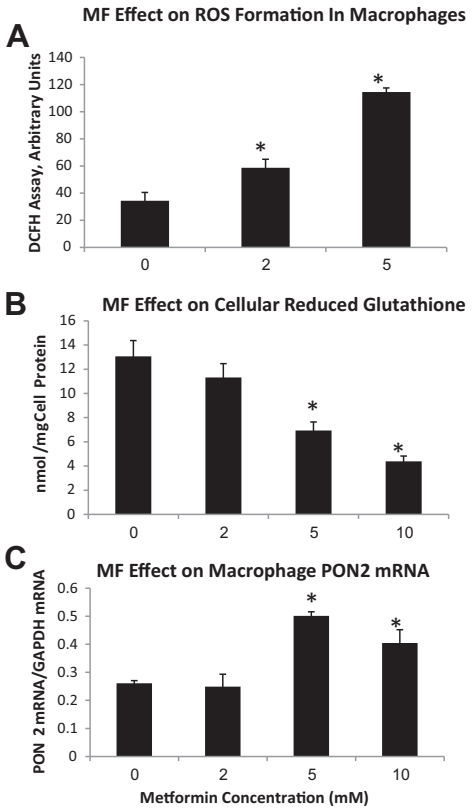

Fig. 2. Effect of MF on macrophage oxidative status. J774.A1 macrophages were incubated without (control) or with increasing concentrations of MF as indicated for 24 h at 37 °C. Then, the level of cellular peroxides was determined by the DCFH assay. (A). Reduced glutathione (GSH) content was measured in cell sonicate (1.5×10^6 cells/ml PBS) by the DTNB-GSSG recycling assay. Cellular protein content was measured by the Lowry assay (B). Paraoxonase2 (PON2) mRNA levels in J774.1A were determined as described under the Methods section (C). Data represent mean ± SEM; ($n = 5$); *P<0.01 vs. control.

Assays. Quantitative PCR was performed on the Rotor-Gene 6000 system (Corbett Life science, Australia). To normalize the data obtained for each gene expression, the amount of GAPDH mRNA was measured by quantitative PCR as internal standard in all treatments. The primers and probes for all analyzed genes were design by PrimerDesign (South Hampton, UK).

2.9. Statistical analysis

Student's *t* test was performed for all statistical analyses.

3. Results

3.1. Effect of MF on cholesterol biosynthesis rate in macrophages

Incubation of murine macrophage cell line J774A.1 with increasing concentrations of MF for 20 h resulted in a significant and dose-dependent decrement in macrophage cholesterol content, by up to 69%, as compared to control cells incubated without MF (Fig. 1A). This phenomenon could be attributed, at least in part, to inhibition in macrophage cholesterol biosynthesis rate. Indeed, [^{3}H] acetate incorporation into newly synthesized cholesterol was inhibited by MF in a dose-dependent manner, reaching a maximum inhibition of up to 71% as compared to control cells (Fig 1B). In contrast, macrophage cholesterol biosynthesis rate from [^{14}C] mevalonate was not affected by MF (Fig. 1C), possibly indicating that the inhibitory effect of MF involves the cholesterol biosynthesis rate limiting enzyme HMGCoA reductase. We next compared the inhibitory effect of MF to Simvastatin, a most potent inhibitor of HMGCoA reductase, on macrophage cholesterol synthesis. A substantial decrease in cholesterol biosynthesis rate was noted in cells administered with MF or with simvastatin, by 59% or 44%, respectively. The combination of Simvastatin and MF resulted in an additional decrease in cholesterol biosynthesis rate, by 66%, as compared to cells incubated with MF alone (Fig. 1D). These data strongly suggest that MF regulate macrophage cholesterol synthesis by inhibiting cellular HMGCoA reductase activity.

3.2. Effect of MF on macrophages oxidative status

Incubation murine macrophage cell line J774A.1 cells with increasing concentrations of MF for 20 h resulted in a significant and dose-dependent increment in macrophage total peroxides level, by up to 3.3 folds, as compared to control cells incubated without MF (Fig. 2A). This effect was associated with a dose-dependent significant lowering of macrophage reduced glutathione (GSH), the major cellular anti-oxidant agent, by up to 67%, compared to control cells (Fig. 2B). Since oxidative stress was shown to affect macrophage PON2 expression [17,26], we have next analyzed the effect of MF on macrophage PON2 expression (mRNA). Incubation of the cells with MF resulted in a significant and does-dependent increased PON2 mRNA expression, by up to twofold, as compared to control cells incubated without metformin (Fig. 2C). These results indicate that macrophages exposure to MF is associated with increased oxidative stress.

3.3. Association of cholesterol biosynthesis with oxidative stress in MF-treated macrophages

We next examined whether the effects of MF on cholesterol biosynthesis is indeed inversely associated with oxidative stress in macrophages. In order to study this relationship we used pomegranate juice (PJ) which was previously shown to be a most potent anti-oxidant [20]. Whereas ROS formation in the presence of MF alone was enhanced by 3.3 folds, the addition of PJ to MF-treated macrophages not only inhibited MF-induced increased OS, but it also abolished OS to levels 85% below control cells incubated without MF (Fig. 3A). Concomitantly, the addition of PJ to macrophages treated with MF, not only completely abolished the decrease in cholesterol synthesis rate by MF alone, but rather resulted in some elevation of cholesterol biosynthesis as compared to control cells (Fig. 3B). Similar results were obtained in an experiment where vitamin E, another known anti-oxidant agent was employed. ROS formation was reduced by vitamin E in MF treated macrophages to levels 63% below control (Fig. 3C). The inhibition of cholesterol biosynthesis rate by MF was counteracted by vitamin E and was not significantly different from control cells (Fig. 3D).

4. Discussion

The present study demonstrates that MF suppresses macrophage cholesterol biosynthesis rate and this effect is inversely associated, at least in part, with the effect of metformin on cellular oxidative state, and involves MF inhibition of the cholesterol biosynthesis rate limiting enzyme, HMGCoA reductase.

The impact of metformin on cellular oxidative stress appears to be inconsistent. While some studies indicate that MF is an anti-oxidant agent [16,17], others show the opposite [18,19]. However, none of the above studies examined MF effect on OS in macrophages.

In the present study we demonstrated, for the first time, a significant increment in macrophages cellular OS upon MF treatment.

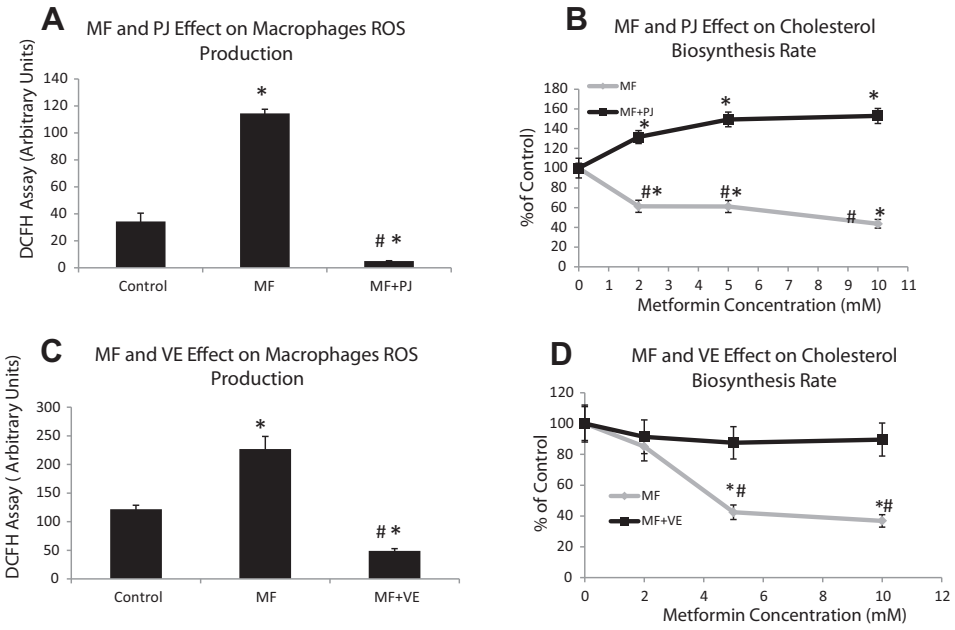

Fig. 3. Effects of antioxidants on macrophage oxidative stress and on cellular cholesterol biosynthesis rate. Macrophages were incubated without or with increasing concentration of MF for 24 h alone or together with Pomegranate juice (75 M of PJ total polyphenols) or together with 30 μM vitamin E (VE). The level of cellular peroxides was determined by the DCFH assay (A, C). The cellular cholesterol biosynthesis rate was determined as described in Fig. 1 (B, D). Data represent mean ± SEM; (n = 5). $^{*}P$<0.01 (vs. control), $^{\#}P$ < 0.005 (vs. MF).

The increase in cellular reactive oxygen species production by MF, as measured by DCFH assay, was associated with changes in two other parameters which affect free radicals scavenging, depletion of cellular GSH levels, and enhanced PON2 expression (a compensatory effect), which were previously shown to be affected by increased OS [21,22]. Consistent with our results, MF was shown to inhibit mitochondrial complex 1 of the respiratory chain [18,23,24], resulting in increased OS stress in white adipocyte [19].

The results of the current study demonstrate a significant inhibitory effect of MF on cholesterol biosynthesis rate from [^{3}H]acetate, but not from ^{14}[C]mevalonate, suggesting that MF inhibits macrophage HMGCoA reductase. The observation that the effect of MF is enhanced by statin treatment, further suggest the involvement of HMGCoA reductase in the inhibitory effect of MF on cholesterol biosynthesis in macrophages. These results are in agreement with a previously study showing that MF inhibits the expression of HMGCoA reductase in a rat liver by a post-transcriptional mechanism [11].

Although metformin was shown to activate AMPK which further phosphorylates HMGCoA reductase, resulting in its inhibition and to suppression of cholesterol biosynthesis rate [11,12], our data clearly indicate for the first time, that the reduction of macrophage cholesterol biosynthesis rate is a direct consequence of metformin-induced oxidative stress. Oxidative stress has been shown to considerably attack lipids in LDL as well as in arterial macrophages [25,26]. Oxidized lipids (i.e. oxysterols) have been shown to down regulate HMGCoA reductase and to block endogenous cholesterol synthesis more than native cholesterol in suppressing cholesterol biosynthesis rate [27]. Abolishing the inhibitory effect of MF on cholesterol biosynthesis by the two potent antioxidants and by hydrophobic vitamin E, suggests that the inhibitory effect of MF on cholesterol biosynthesis rate could be secondary to its effect on OS.

In summary, we may suggest that in macrophages, MF increases OS, which in turn, inhibits HMGCoA reductase, thus resulting in the reduction of cellular cholesterol synthesis, which may contribute to anti-atherogenicity of MF.

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