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Metformin reduces the endotoxin-induced down-regulation of apolipoprotein E gene expression in macrophages

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ABSTRACT

The atheroprotective role of macrophage-derived apolipoprotein E (apoE) is well known. Our previous reports demonstrated that inflammatory stress down-regulates apoE expression in macrophages, aggravating atherogenesis. Metformin, extensively used as an anti-diabetic drug, has also anti-inflammatory properties, and thus confers vascular protection. In this study, we questioned whether metformin could have an effect on apoE expression in macrophages in normal conditions or under lipopolysaccharide (LPS)-induced stress. The results showed that metformin slightly increases the apoE expression only at high doses (5–10 mM). Low doses of metformin (1–3 mM) significantly reduce the LPS down-regulatory effect on apoE expression in macrophages. Our experiments demonstrated that LPS-induced NF-κB binds to the macrophage-specific distal regulatory element of apoE gene, namely to the multienhancer 2 (ME.2) and its 5′-deletion fragments. The NF-κB binding on ME.2 and apoE promoter has a down-regulatory effect. In addition, data revealed that metformin impairs NF-κB nuclear translocation, and thus, improves the apoE levels in macrophages under inflammatory stress. The positive effect of metformin in the inflammatory states, its clinical safety and low cost, make this drug a potential adjuvant in the therapeutic strategies for atherosclerosis.

1. Introduction

Metformin (1,1-dimethylbiguanide), a widely prescribed drug for the type 2 diabetes, exhibits beside its anti-hyperglycemic effect [1], anti-hyperlipidemic [2] and anti-inflammatory properties [3]. Cardiovascular protection and survival benefits of metformin in diabetic patients were revealed [4]. Numerous evidence indicated that metformin attenuates pro-inflammatory response in various cell types, including endothelial cells and human vascular smooth muscle cells [3,5,6], and ameliorates macrophage activation [7]. Although its mechanism of action is still not fully understood, there are reports indicating the intracellular signalling pathways modulated by metformin, through AMPK-dependent mechanisms [3,8,9] or AMPK-independent mechanisms, including the inhibition of p70S6K1 kinase [10], protein kinase C and p38 MAPK [11]. In endothelial cells, metformin inhibits NF-κB activation, attenuating the induction of various pro-inflammatory cell adhesion molecules [5].

Apolipoprotein E (apoE) plays a key role in the lipid metabolism [12,13]. ApoE deficiency involves the accumulation of lipoprotein remnants in the plasma, leading to atherosclerosis [14]. ApoE is primarily synthesized in the liver, but is also expressed by various cell types [15], including macrophages, cells playing a significant role in atherogenesis [16]. ApoE secreted by macrophages localized in the atherosclerotic plaque is involved in the cholesterol efflux from this site. In macrophages, the apoE gene regulation implies a highly complex regulatory process requiring the cooperation between the proximal and distal regulatory elements. In the absence of the enhancers, the apoE promoter is not able to direct the gene transcription *in vivo* [17]. Two homologous

Abbreviations: AMPK, adenosine-monophosphate-activated protein kinase; apoE, apolipoprotein E; cAMP, cyclic adenosine monophosphate; CD36, cluster of differentiation 36; Cox2, cyclooxygenase 2; DMEM, Dulbecco's Modified Eagle Medium; DNAP, DNA Pull-down Assays; FBS, foetal calf serum; h, hours; IFN-γ, interferon gamma; iNOS, inducible nitric oxide synthase; IL, interleukin; LPS, lipopolysaccharide; luc, luciferase; Mf, metformin; ME, multienhancer; MPM, murine peritoneal macrophages; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p38 MAPK, p38 mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TFIID, transcription factor II D; TNFα, tumour necrosis factor alpha.

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enhancers involved in macrophage and brain specific apoE expression, multienhancer-1 (ME.1) and multienhancer-2 (ME.2), were identified [18]. Our previous reports demonstrated that the endotoxin stress down-regulates the apoE expression in macrophages [19]. These data indicate that even if apoE could exert a beneficial effect at the atheromatous lesion site, it is diminished by the modulatory impact of the inflammatory factors. We hypothesise that a drug attenuating the negative effects of inflammation could compensate the regulatory balance of apoE expression in macrophages and thus, would restore the cholesterol efflux from the atherosclerotic plaque.

Considering all these data, the aim of our current study was to investigate the modulatory potential of metformin on apoE expression in macrophages in normal and inflammatory states and to reveal its mechanism. We report herein that in normal conditions, metformin has a modest upregulatory effect on apoE gene in macrophages, but, more important, this drug significantly diminishes the inflammatory stress-induced down-regulation of apoE, through the inhibition of NF-κB nuclear translocation.

2. Materials and methods

2.1. Materials

Metformin (1,1-Dimethylbiguanide hydrochloride), LPS *E. coli* 0111:B4 and Bay-11-7082 were from Sigma–Aldrich. DMEM, RPMI-1640 and FBS were from EuroClone (MI, Italy). Enhance chemiluminescence kit was from Pierce (Rockford, USA). GoTaq DNA polymerase, PMA and Luciferase assay system were from Promega (Madison, WI). RNA isolation kit was from Analytic Jena (Germany). The oligo(dT), M-MLV reverse transcriptase, and Dynabeads M-280 streptavidin were from Life Technologies. TaqMan probes for apoE (Mm01307193_g1) and actin (Mm00607939_s1) were from Applied Biosystems. Protease Inhibitors cocktail was from Roche. Nuclear Extract kit was from Active Motif (Rixensart, Belgium). Thioglycolate medium was from HiMedia (Mumbai, India). All the antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), excepting anti-human apoE, which was from Immuno-Biological Laboratories Co., LTD. (Japan).

2.2. Cell culture and treatment

Thioglycollate elicited MPM were isolated from C57BL/6J mice. MPM and THP-1 cells were cultured in RPMI with 10% heat-inactivated FBS. THP-1 monocytes differentiation was induced by 7 h exposure to 50 nM PMA; then, PMA was removed and THP-1 macrophages were maintained in culture for 10 days, until further processing. RAW 264.7 cells were cultured in low glucose DMEM and 10% FBS.

2.3. Quantitative real time-PCR

MPM were incubated with different metformin concentrations for 16 h in the presence or absence of 500 ng/ml LPS, and then total RNA was extracted using a kit from Analytic Jena and revers-transcribed into cDNA. ApoE expression was quantified by Real Time-PCR using TaqMan probes for apoE and β-actin (as reference), in a 7900HT Fast Real Time-PCR system (Applied Biosystems). The relative apoE expression was calculated using RQ Manager Software.

2.4. Western blot

The samples obtained from THP-1 differentiated monocytes treated for 16 h with metformin were subjected to SDS-PAGE and then transferred on a nitrocellulose membrane (Bio-Rad). Blocked membranes were incubated with anti-apoE and anti-actin antibodies, followed by HRP-conjugated secondary antibodies. To determine NF-κB p50 subunit distribution between nuclear and cytoplasmic fractions, THP-1 macrophages were 2 h pre-treated with 1 mM metformin and then incubated 4 h with 1 μg/ml LPS and 1 mM metformin. Then, the nuclear and cytoplasmic fractions obtained as described [19] were subjected to SDS-PAGE. After transfer, the blots were incubated with anti-p50, anti-actin or anti-TFIID antibodies, followed by HRP-conjugated secondary antibodies. The proteins were revealed using a chemiluminescence kit and LAS-4000 Chemiluminescent Image Reader (FUJIFILM Europe GmbH, Germany), and quantified using TotalLab Software.

2.5. DNA pull-down assays

This assay was done as we previously described [19], except the following: (i) the primers used for biotinylation were: forward biotinylated RV3 primer (5′-CTAGCAAAATAGGCTGTCCC; Promega) that anneal on pGL3 plasmid and reverse primer 5′-AAGAGCT-CATCCCCGTGCCCCCG for apoE promoter, and 5′-CAAAGCTCT-GAGTGTATGCCCC for ME.2 or its fragments; (ii) the templates used were the plasmids in which the corresponding promoter or ME.2 fragments were previously cloned; (iii) the nuclear extract was prepared from RAW 264.7 cells incubated 18 h with LPS, using a kit from Active Motif. The protein-DNA complexes were subjected to Western blotting using anti-p50 antibodies.

2.6. Transient transfections

RAW 264.7 cells were transfected by calcium phosphate method with following plasmids in which were cloned: (i) apoE proximal promoter ([−500/+73]apoE-luc), (ii) ME.2 (ME-luc), or (iii) apoE promoter and ME.2 (ME[−500/+73]apoE-luc), as described [20]. Eighteen hours from transfection, the medium was changed and the transfected cells were 3 h pre-treated with 1 mM metformin and then incubated for 21 h with 1 μg/ml LPS together with 1 mM metformin. Then, the cells were lysed, and the reporter gene activity was determined as previously reported [20]. All the experiments were done in triplicates and repeated three times.

2.7. Statistical analysis

The results were statistically analysed using One-way ANOVA and graphs show the means ± standard deviation. Statistical significance was considered when p values <0.05.

3. Results

3.1. The effect of metformin on apoE expressed by macrophages in normal and endotoxin-stressed states

The apoE gene expression modulation induced by metformin was evaluated on MPM exposed (16 h) to different metformin concentrations (1–10 mM), by Real Time-PCR, using TaqMan probes. The results showed that the apoE mRNA levels were slightly but significantly increased by 5 mM and 10 mM metformin (~1.5 times as compared with control cells, $p < 0.001$), whereas smaller concentrations (1 mM and 3 mM) did not change the apoE gene expression (Fig. 1A). The apoE protein levels in (16 h) metformin-treated THP-1 macrophages were slightly increased after incubation with 5 mM (~1.57 times) and 10 mM (~1.77 times) metformin, as compared to control (Fig. 1B).

To test the ability of metformin to exert its anti-inflammatory properties and diminish the down-regulatory effect of the

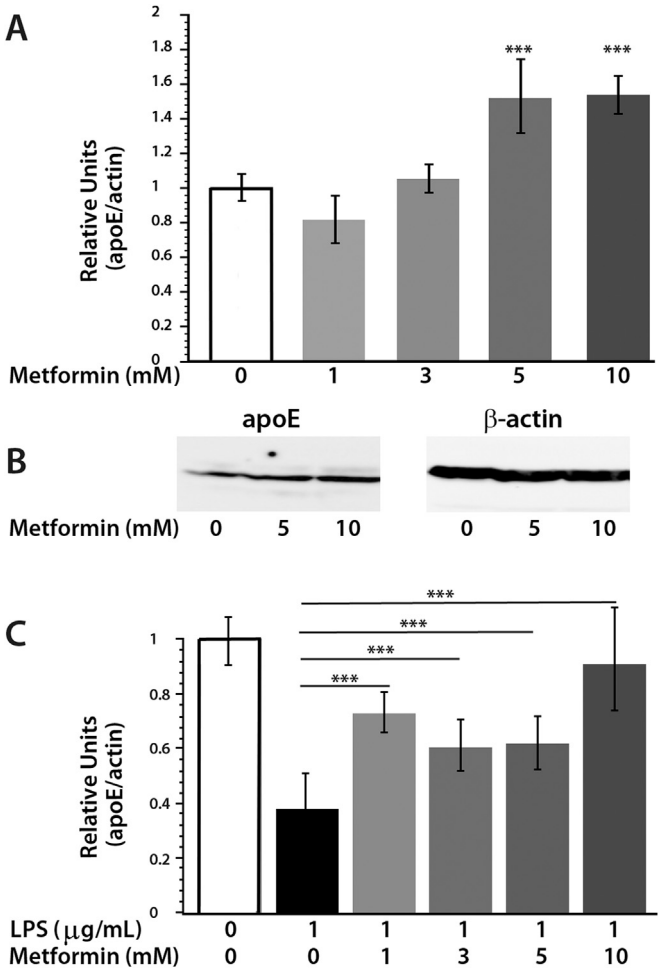

Fig. 1. The effect of metformin on apoE expression in macrophages in normal conditions (A, B) or under endotoxin stress (C). Lower concentrations of metformin (1–3 mM) do not increase the apoE gene expression in macrophages, while higher doses (5–10 mM) increase apoE mRNA levels, as evaluated by RT-PCR (A). ApoE protein levels are increased by high metformin doses (5–10 mM), as determined by Western blotting (B). Metformin significantly reduces the LPS down-regulatory effect on apoE expression in macrophages, as determined by RT-PCR (C).

inflammatory stress on the apoE expression, MPM were treated (16 h) with various concentrations of metformin (1–10 mM) together with LPS and then the apoE expression was analysed by Real Time-PCR. The results presented in Fig. 1C showed that apoE gene expression decreased to 34.9% in LPS-treated cells as compared to control cells ($p < 0.001$), but LPS-induced down-regulation of apoE mRNA was significantly reduced by metformin. Thus, the apoE expression reached ~72.7% of the control values when the cells were simultaneously treated with 1 mM metformin and LPS, ~60.4% and ~61.2% from control for 3 mM and 5 mM metformin in the presence of LPS, respectively. In the case of treatment with 10 mM metformin, the LPS down-regulatory effect on apoE was almost completely reverted.

The LPS-induced inflammatory stress involves NF-κB activation that represents, at least in part, the mechanism of apoE down-regulation in macrophages [19]. Based on this data, we examined whether metformin has the capacity to revert the negative effect of LPS on the apoE regulatory elements. For this, we performed transient transfections on RAW 264.7 cells, using plasmids containing ME.2 along with the proximal region of apoE promoter, cloned in pGL3 basic vector (ME.2[−500/+73]apoE-luc). Transiently transfected cells were: (i) incubated with 1 μg/ml LPS for 24 h; (ii) pre-treated for 3 h with 1 mM metformin and then incubated for 21 h with 1 μg/ml LPS together with 1 mM metformin; (iii) treated with 1 mM metformin for 24 h. As illustrated in Fig. 2, LPS reduced the activity of the construct containing the apoE promoter and ME.2 to a value of ~10% of the control (Fig. 2, LPS column). When transiently transfected macrophages were incubated simultaneously with LPS and metformin, the inhibitory effect of LPS on apoE promoter/ME.2 activity was significantly decreased, the value obtained representing ~70% ($p = 0.01$) of the control (Fig. 2, LPS + Mf column). The treatment of the transfected macrophages with 1 mM metformin had no significant effect on apoE promoter activity (Fig. 2, Mf column).

3.2. Metformin interferes with NF-κB nuclear translocation

To study the ability of metformin to inhibit the NF-κB translocation in the nucleus, THP-1 differentiated macrophages were incubated with metformin or simultaneously with metformin and LPS (4 h). Then, nuclear and cytoplasmic fractions were prepared using the treated cells and analysed by Western blotting for NF-κB p50 distribution. The results showed that THP-1 macrophages exposed to LPS exhibited an accumulation of NF-κB p50 subunit in the nuclear fraction (Fig. 3, lane 'LPS') as compared to control (Fig. 3, lane 'ctr'). In contrast, 2 h pre-incubation of THP-1 differentiated macrophages with 1 and 10 mM metformin attenuated by ~96%, and 89% respectively the nuclear accumulation of the NF-κB p50 subunit (Fig. 3, lane 'LPS+1 mM Mf', and 'LPS+10 mM Mf'), an effect similar to that of the known NF-κB inhibitor, Bay-11-7082 (Fig. 3, lane 'LPS + Bay'). In cells treated only with metformin (1 or 10 mM) as well as with Bay-11-7082, p50 remains in the cytoplasmic fraction (Fig. 3, lanes '1 mM Mf', '10 mM Mf' and 'Bay'). As control for the protein loading, TFIID was analysed (Fig. 3, TFIID). The intensity graph of p50 nuclear localization is illustrated in the Fig. 3. In the cytoplasmic fraction, the precursor of p50 and actin were found in each sample analysed (Fig. 3, cytoplasmic fraction).

3.3. NF-κB p50 subunit binds on apoE promoter and multienhancer

Considering that LPS-induces p50 nuclear translocation, we further tested the ability of NF-κB p65/p50 heterodimer to bind on the apoE regulatory elements. First, we performed DNAP assays to test the p50 binding to ME.2, using the biotinylated [1-619 ME.2] DNA and nuclear extracts prepared from RAW 264.7 cells treated with 1 μg/ml LPS for 24 h. The results showed that p50 proteins

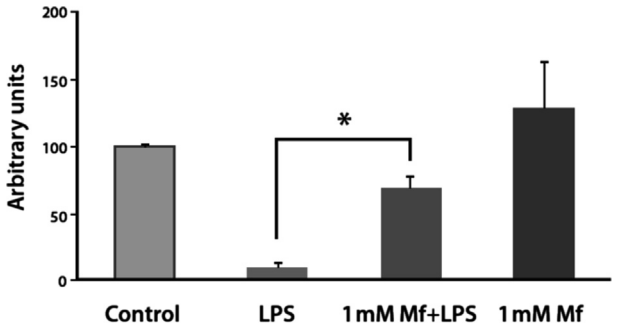

Fig. 2. Metformin diminishes the negative effect of LPS on the activity of apoE promoter and multienhancer in RAW 264.7 cells. RAW 264.7 macrophages were transiently transfected with ME.2[−500/+73]apoE-luc construct, and treated with 1 μg/ml LPS or 1 mM metformin in the presence or absence of LPS. The treatment with 1 mM metformin significantly reduced the inhibitory effect of LPS (LPS column) on apoE promoter/ME.2 activity (LPS + Mf column) as compared with the control, while 1 mM metformin has no significant effect on apoE regulatory elements (Mf column).

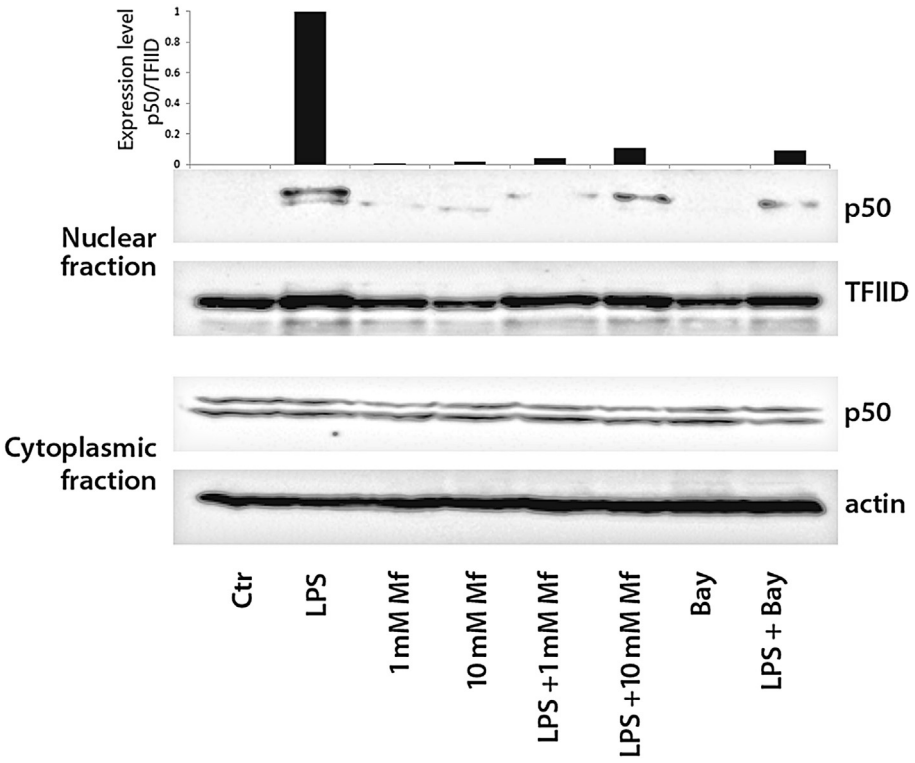

Fig. 3. Metformin inhibits NF-κB nuclear translocation. The nuclear and cytoplasmic distribution of NF-κB p50 subunit in response to LPS in the presence or absence of metformin, in THP-1 derived macrophages, was determined by Western Blotting using anti-p50 antibodies. Cells treated 4 h with LPS present an accumulation of p50 in the nuclear fraction (lane 'LPS') as compared with the control (lane 'ctr'), while metformin treatment attenuates p50 nuclear accumulation (lane 'LPS+1 mM Mf', and lane 'LPS+10 mM Mf'), an effect similar with that of Bay-11-7082 (lane 'LPS + Bay'). In the absence of LPS, Bay-11-7082 has no significant effect on p50 distribution (lane 'Bay'). As control for the protein loading, TFIID (for the nuclear fraction) and β-actin (for the cytoplasmic fraction) were assessed. The normalised p50 expression level in the nuclear fraction was also graphically represented. In the cytoplasmic fraction, the precursor of p50 is observed in each sample analysed.

bind to the whole ME.2 sequence in LPS-treated macrophages (Fig. 4A, lane '1-619 ME'). Then to check for the localization of NF-κB p50 subunit binding site on ME.2, DNAP assays using two 5′ deletion fragments (249-619 ME and 407-619 ME) were performed. The results showed that both deletion fragments were able to bind NF-κB p50 subunit (Fig. 4A, lane '249-619 ME', and '407-619 ME'). As positive control, we used the apoE promoter (Fig. 4A, lane 'apoE promoter') previously shown to bind NF-κB [19], and a validated sequence for NF-κB binding (Fig. 4A, lane 'NF-κB'). As negative control, a DNA sequence containing the glucocorticoid receptor binding site was employed and no band was detected (Fig. 4A, lane 'Random DNA').

The functionality of the NF-κB binding sites found on apoE regulatory elements was assessed by transient transfection experiments. RAW 264.7 macrophages transfected with the following plasmids: [−500/+73]apoE-luc, ME.2/[−500/+73]apoE-luc and ME.2-luc were incubated (24 h) in medium containing or not 1 μg/ml LPS. The activity of apoE promoter in the presence or absence of ME.2 (Fig. 4B, columns ME.2/[−500/+73]apoE, and −500/+73apoE, respectively) as well as that of ME.2 (Fig. 4B, columns ME.2) was significantly decreased by LPS (black columns) to ~5–7% ($p < 0.001$) from the control values (white columns).

4. Discussion

Metformin is a drug discovered in the 1920s, intensively used for treating patients with type 2 diabetes mellitus, being currently prescribed to over 100 million patients worldwide. Several clinical studies indicate its beneficial effects in improving the survival rate for type 2 diabetes patients [21–23]. The main effect of metformin is the inhibition of the glucose production in the liver [24]. Metformin was shown to be efficient in treating diseases with an inflammatory component, such us uveitis [25], and polycystic ovary syndrome [26]. Moreover, Forouzandeh and co-workers, using non-diabetic mice models, reported that metformin has vasculoprotective effects in attenuating atherosclerosis [27].

Apolipoprotein E has an atheroprotective role being implicated in lipoprotein clearance and cholesterol efflux. ApoE deficiency leads to atherosclerosis [14]. Its beneficial properties in cholesterol efflux can be achieved only when apoE reaches the core of the atherosclerotic plaque. At this site, apoE is secreted by the macrophages differentiated from the infiltrated monocytes. Our previous data revealed that inflammatory factors, such as LPS, repress apoE gene expression in macrophages [19]. One can safely assume that restoring apoE levels expressed by macrophages could contribute to slow down the progression of atherosclerosis and may diminish the atherosclerotic plaque.

Therefore, in this study we investigated a possible intervention in the inflammatory stress signalling pathways leading to apoE gene down-regulation in macrophages, in order to attenuate its effect. Our data showed that only high doses (5–10 mM) of metformin slightly increase apoE gene expression in macrophages (Fig. 1A and B). However, particularly interesting results showed that although lower concentrations of metformin (1–3 mM) did not increase the apoE mRNA level, these concentrations were enough to reverse the down-regulatory effect of LPS on apoE gene expression (Fig. 1C). In good agreement with these results, we demonstrated that the activity of apoE regulatory elements that was decreased by LPS was significantly restored by 1 mM metformin treatment (Fig. 2).

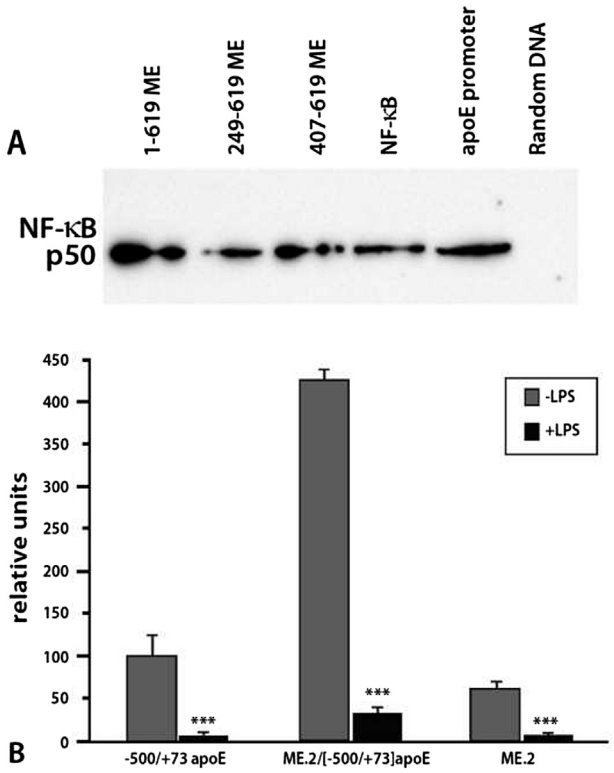

Fig. 4. Interaction of NF-κB p50 subunit with apoE promoter and multienhancer. Panel A. DNAP experiments for NF-κB p50 subunit. The whole ME.2, [249-619 ME] or [407-619 ME] fragments and apoE promoter bind p50 (lanes '1-619 ME', '249-619 ME', '407-619 ME, and 'apoE promoter', respectively). No bands appear in the negative control (lane 'Random DNA'); as positive control, a consensus sequence for NF-κB was used (lane 'NF-κB'). Panel B. Down-regulation of apoE promoter and multienhancer in RAW 264.7 macrophages by LPS, as revealed by transient transfections.

These data are well integrated in the context of anti-inflammatory properties of metformin. The anti-inflammatory effects of metformin in cells involved in atherosclerotic plaque formation, such as vascular smooth muscle cells, endothelial cells and macrophages were reported by different research groups. It was demonstrated that metformin attenuated TNFα-induced gene expression of pro-inflammatory and cell adhesion molecules in endothelial cells [5], inhibited IL-1β-induced IL-6 and IL-8 expression [3,28], and drastically reduced the expression of pro-inflammatory cytokines (TNFα, IL-6, and IFN-γ), iNOS and Cox2 in LPS-treated macrophages [29]. Similar effects of metformin were reported by Hyun et al., for IL-4, IL-10, iNOS, Cox2, IL-1β, IL-6, TNFα; in addition, metformin reduced the level of CD36 and scavenger receptor A in LPS-treated RAW 264.7 cells and MPM [28]. Metformin inhibited high-mobility group protein B1 release, and thus improved the survival in a murine model of lethal endotoxemia [30]. A recent study has shown that metformin suppresses liver inflammation in obesity-associated non-alcoholic fatty liver disease by decreasing the LPS-induced pro-inflammatory cytokines, c-Jun N-terminal protein kinase 1 phosphorylation and NF-κB activation in hepatocytes and macrophages [31].

According to Buldak et al., metformin can direct macrophages toward alternative activation, this phenotype being involved in the healing processes and repression of inflammation [32]. Moreover, Sag et al. suggested that the macrophage polarization and the switch between the pro-to anti-inflammatory state, may be induced by AMPK through distinct regulation of some transcription factors such as: inhibition of NF-κB and activation of Protein kinase B and cAMP response element binding protein [33]. AMPK-activation was described as the first signalling pathway of metformin action which drives a multitude of alternative downstream molecules leading to various effects, as recently reviewed [34]. Our current study indicates that, in macrophages, metformin prevents NF-κB activation, blocking the NF-κB nuclear translocation (Fig. 4). Similar mechanisms were reported for the inhibition of various pro-inflammatory cytokines (TNFα, IL-6, IL-1β and IFN-γ) production and LPS-induced iNOS and Cox2 expression in macrophages treated with metformin [28,29]. Moreover, this mechanism was also demonstrated for other cell types, such as smooth muscle cells in which metformin treatment inhibited IL-1β-induced IL-6 and IL-8 expression via a mechanism involving reduction of the nuclear translocation of NF-κB [3].

We investigated whether NF-κB has binding sites on apoE regulatory elements in macrophages. The DNA Pull-down assays indicated that NF-κB p50 subunit could bind on ME.2, that drives the expression in macrophages (Fig. 4A), as well as on the apoE promoter, as previously shown [19]. Moreover, 5'-deletion fragments of ME.2 have also binding capacity for p50. The p65/p50 binding on the apoE regulatory elements decreases their activity (Fig. 4B), leading to the down-regulation of the apoE gene expression in macrophages. Despite this fact, computer analysis of NF-κB binding site on ME.2 and apoE promoter showed no consensus sequences for p50 binding. Thus, an indirect binding of p50/p65 on the apoE regulatory elements is suggested. A similar mechanism was described by Wang et al. for NF-κB-mediated repression of troponin-I2 gene, also lacking NF-κB binding sites; in addition, YinYang1, a transcriptional repressor, was shown to mediate this regulatory process in myoblasts [35].

In conclusion, the novel data of this study are: (i) high doses of metformin slightly increase apoE expression in macrophages; (ii) low doses of metformin up-regulate apoE gene in endotoxin-stressed macrophages; (iii) the mechanism by which metformin counteracts LPS effect involves the inhibition of NF-κB, a transcriptional factor playing an important role in atherosclerosis. The valuable effect of metformin on the cells implicated in atherogenesis, the clinical safety and the low cost, make this drug a potential prospective adjuvant in the therapeutic strategies for atherosclerosis.

Conflict of interest

None

Acknowledgments

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