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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 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 evidences 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

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