Metformin, an anti-diabetic agent, suppresses the production of tumor necrosis factor and tissue factor by inhibiting early growth response factor-1 expression in human monocytes \textit{in vitro}

Masatoku Arai, Mitsuhiro Uchiba, Hidefumi Komura, Yuichiro Mizuochi, Naoaki Harada, Kenji Okajima

Department of Translational Medical Science Research (M.A., N.H., K.O.) and Anesthesiology and Medical Crisis Management (H.K., Y.M.), Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; and Department of Blood Transfusion and Cell Therapy, Kumamoto University Hospital, Kumamoto, Japan (M.U.)
Running title; Inhibition of Egr-1 expression by metformin

Corresponding author; Kenji Okajima, MD, PhD., Department of Translational Medical Science Research, Nagoya City University Graduate School of Medical Sciences, Kawasumi 1, Mizuho-cho, Mizuho-ku, Nagoya city 467-8601, Japan. Tel: +81-52-853-8191; Fax: +81-52-842-3460; E-mail: whynot@med.nagoya-cu.ac.jp

Number of text pages: 21
Number of tables: 0
Number of figures: 6
Number of references: 33
Number of words in the Abstract: 191
Number of words in the Introduction: 361
Number of words in the Discussion: 1163

Abbreviations: AICAR, 5-Aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; AP-1, activator protein-1; Egr-1, early growth response factor-1; ELISA,
enzyme-linked immunosorbent assay; ERK 1/2, extracellular signal-regulated protein kinase 1/2; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; oxLDL, oxidized LDL; TF, tissue factor; TNF, tumor necrosis factor

Recommended section: Cardiovascular
Abstract

Metformin, an anti-diabetic agent, has been shown to reduce the atherothrombotic disease in diabetic patients independent of anti-hyperglycemic effect. Recent studies have demonstrated that metformin attenuates the proinflammatory responses in human vascular wall cells and macrophages. However, the detailed molecular mechanism(s) underlying these therapeutic effects remains unclear. In the present study, we investigated the effects of metformin on tumor necrosis factor (TNF) production and on tissue factor (TF) expression in isolated human monocytes stimulated with lipopolysaccharide (LPS) or oxidized low density lipoprotein (oxLDL). Metformin significantly inhibited both TNF production and TF expression in isolated human monocytes stimulated with LPS or oxLDL. Metformin also significantly inhibited TNF and TF mRNA in human monocytes stimulated with LPS. Although metformin inhibited neither the activation of nuclear factor-kappa B nor that of activator protein-1, it inhibited the expression of early growth response factor (Egr)-1 and phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2 in monocytes stimulated with LPS or oxLDL. These results suggest that metformin may attenuate the inflammatory responses, at least in part, by suppressing the production of both TNF and TF through the inhibition of ERK1/2-Egr-1 pathway in human monocytes.
Introduction

Metformin is one of the most commonly used anti-diabetic agents. It improves insulin sensitivity in patients with type 2 diabetes (Stumvoll et al., 1995). In addition, metformin reduces the risk of atherothrombotic disease accompanied with diabetes independent of its anti-hyperglycemic effect (UK Prospective Diabetes Study group, 1998).

It is now well-accepted that atherosclerosis is not merely a lipid disorder, but also an inflammatory disease (Ross, 1999; Libby, 2002). Inflammatory cells, such as monocytes/macrophages play a critical role in the initiation, progression and complication of atherosclerotic lesions (Ross, 1999; Libby, 2002).

Various cytokines contribute to pathogenesis of inflammation observed in atherosclerosis (Kleemann et al., 2008). Among these cytokines, tumor necrosis factor (TNF) plays an important role. TNF is shown to present in atherosclerotic lesions but not in normal vessels in humans (Tipping and Hancock, 1993). TNF is associated with the progression of atherosclerotic lesions by regulating the functions of vascular wall cells to promote adhesion, migration, growth and activation of leukocytes (Young et al., 2002), thus leading to the further accumulation of mononuclear phagocytes and amplification of inflammation (Ross, 1999; Libby, 2002). TNF is also associated with complications of atherosclerosis by
increasing the production of matrix metalloproteinases contributing to the vulnerability of the
fibrous cap (Young et al., 2002).

The activation of coagulation also contributes to the pathogenesis of atherosclerosis
(Ross, 1999; Libby, 2002). Tissue factor (TF) is an important factor initiating blood
coagulation (Edgington et al., 1991), which is expressed in atherosclerotic lesions in humans
(Tipping et al., 1989; Wilcox et al., 1989). Current evidence suggests that the exposure of TF
positive monocyte-derived macrophages to blood on plaque disruption may trigger both
intravascular thrombosis and a sudden progression of atherosclerotic lesions (Libby, 2002).

Although various causes contributing to the pathogenesis of inflammation observed
in atherosclerosis has been identified, oxidized low density lipoprotein (oxLDL) in
hypercholesterolemia and lipopolysaccharide (LPS) from infectious microorganisms are
recognized as a potentially important source of chronic inflammation in the development of
atherosclerotic lesions (Ross, 1999; Libby, 2002).

In the present study, we examined whether metformin inhibits TNF production and TF
expression in human monocytes stimulated with LPS or oxLDL, and attempted to identify the
underlying molecular mechanism(s).
Methods

Materials.

Metformin (1,1-dimethylbiguanide) was a generous gift from Nippon Shinyaku CO., LTD (Kyoto, Japan). LPS (Escherichia coli, serotype 055:B5) was purchased from Sigma (St. Louis, MO). OxLDL was purchased from Intracel Resources, LLC (Maryland, MD). Polyclonal rabbit antibodies against inhibitor kappa B (IκB) α (#9242), p38 (#9212), phosphorylated p38 (Thr180/Tyr182; #9211), c-Jun N-terminal kinase (JNK; #9252), phosphorylated JNK (Thr183/Tyr185; #9251), extracellular signal-regulated protein kinase (ERK) 1/2 (#9102), phosphorylated ERK1/2 (Thr202/Tyr204; #9101), early growth response factor (Egr)-1 (#4152), and caspase-3 (#9662) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against PU.1 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All reagents used were of analytical grade.

Monocyte preparation and incubation.

Human peripheral blood mononuclear cells were isolated from buffy coats provided by the local Central Institute of Blood Transfusion or obtained from healthy volunteer blood donors by Ficoll-Hypaque density gradient centrifugation on Lymphoprep (Axis-Shield poC
AS, Oslo, Norway) and then were further fractionated as described previously (Uchiba et al., 1997). This study was performed with the approval of the ethics committee of the Nagoya City University Graduate School of Medical Sciences and blood donors enrolled for the study gave informed consent in accordance with the Declaration of Helsinki. To minimize any possible interindividual variation among blood donors, each experiment was conducted using monocytes from 2 to 4 buffy coats and repeated three times using independent mononuclear cell preparations from different donors. The mononuclear cells in plastic dishes with RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 1% calf serum (Hyclone, Logan, UT) were incubated for 16 h at 37°C in a humidified 5% CO₂ incubator. Lymphocytes were then removed from the adherent monocytes by repeated rinsing with serum free RPMI 1640. The resulted cell preparations were > 90% monocytes, as determined by May-Giemsa staining. Cell viability was > 95%, as determined by a trypan blue dye exclusion test. Monocytes thus obtained were adjusted to an appropriate volume and cultured in RPMI 1640 supplemented with 1% calf serum at 37°C in a humidified 5% CO₂ incubator. Various concentrations of metformin were added to cells at 2 h before stimulation with LPS (100 ng/ml) or oxLDL (8 µg/ml). After further incubation under the same culture conditions for required period, the cell suspensions were centrifuged. The resulting supernatant fractions were used to determine the
levels of TNF, whereas the sedimented mononuclear pellets were used for the TF activity measurements.

**Measurement of TNF level.**

Human monocytes (1×10^6 cells/assay) were stimulated with LPS for 6 h or with oxLDL for 12 h in the presence or absence of metformin. The concentrations of TNF in culture media were determined using an enzyme-linked immunosorbent assay (ELISA) kit for human TNF (Biosource International, Camarillo, CA).

**Measurement of TF activity.**

Human monocytes (1×10^6 cells/assay) were stimulated with either LPS or oxLDL for 6 h in the presence or absence of metformin. The TF activities on monocytes were measured as described previously (Molor et al., 2005). In brief, human monocytes (1×10^6 cells) were washed twice by a phosphate buffered saline, and then were stored at − 80°C until TF activity was measured. After thawing, the cell pellets were sonicated for 30 sec to scrap the monocyte pellets, and then dissolved in 100 µl clotting buffer (12 mM sodium acetate, 7 mM diethylbarbitrate, and 130 mM sodium chloride). Fifty µl of the resuspended cells were mixed with 50 µl of citrated plasma, and the clotting times were measured after recalcification.
with 50 µl of 20 mM CaCl$_2$ solution at 37°C. The TF equivalents were determined using a
standard curve obtained from rabbit brain thromboplastin (Neoplastin Plus, Boehringer
Mannheim, Mannheim, Germany).

**Detection of specific binding of p65, p50 and c-Fos to DNA by ELISA.**

Human monocytes (1×10$^7$ cells/assay) were stimulated with either LPS or oxLDL
for 1 h in the presence or absence of metformin. Nuclear extracts were prepared as described
previously (Yuksel et al., 2003). The specific binding of p65, p50 and c-Fos to their DNA
consensus oligonucleotides were evaluated in nuclear extracts using ELISA-based assay kits
(Trans AM, Active Motif, Carlsbad, CA) as described previously (Molor et al., 2005).

**RNA isolation and quantitative mRNA analysis.**

RT-PCR assays were used to assess TNF and TF mRNA levels in cultured human
monocytes. Total RNA was extracted from cultured human monocytes using TRIzol
(Invitrogen) reagent according to the manufacturer’s instruction. This procedure yielded 5 to
10 µg of total RNA from 5×10$^5$ cells of cultured human monocytes. RNA samples were
diluted in RNase-free water and stored at -80°C until they were used. Real-time PCR was
performed using the ABI PRISM 7700 Sequence Detection System, TaqMan One-Step RT-PCR Master Mix Reagents Kit and commercially available pre-designed, gene-specific primers and FAM-labeled probe sets for quantitative gene expression (TaqMan Gene Expression Assays; human TNF code Hs00174128_m1, human TF code Hs00175225_ml and human GAPDH code Hs99999905_m1; Applied Biosystems). All probes used in these experiments spanned an exon–intron boundary. TNF, TF and GAPDH mRNA were quantified by parallel estimation. The thermal cycler conditions were 30 min hold at 48°C and 10 min hold at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

**Western blot analysis.**

Human monocytes (2×10⁶ cells/assay) were stimulated with LPS or oxLDL for various times in the presence or absence of metformin (10 µM). Whole cell lysates were collected as described previously (Komura et al., 2008). Samples containing equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and after electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membranes were incubated with appropriate antibodies at 4°C overnight and then subsequently with horseradish peroxidase-conjugated secondary antibody.
for 1 h at room temperature. Specific proteins were visualized using an enhanced chemiluminescence system (Amersham Biosciences Corp, Buckinghamshire, UK). The densitometric quantification of the bands was performed using NIH Image software (Version 1.61).

**Cell Viability.**

Isolated monocytes were stimulated with LPS or oxLDL in the presence or absence of metformin. The number of monocytes was counted at 6 h after stimulation with LPS or 12 h after stimulation with oxLDL. Cell viability was evaluated using a trypan blue dye exclusion test (Uchiba et al., 1997).

**Assessment of Apoptosis.**

Isolated monocytes were stimulated with LPS for 6h or oxLDL for 12h in the presence or absence of metformin (10 μM). Assessing the apoptosis of monocytes was performed by a Western blot analysis probed with anti-caspase-3 antibody.

**Statistical analysis.**
The values are distributed in parametric manner, and are expressed as means ± SD of one experiment representative of three separate experiments that gave similar results. Differences in TNF production, TF activity and mRNA levels were assessed using an analysis of variance followed by Scheffé’s post hoc test. In densitometric analysis data of Western blotting, the difference between baseline values (time 0) and subsequent values for each experiment was compared using analysis of variance followed by Scheffé’s post hoc test, and difference between the values with and without metformin treatment at each time point were compared using unpaired t-test. These analyzes were carried out using StatView 5.0 software (SAS Institute, Cary, NC). Statistical significance was defined as a level of p < 0.05.
Results

Effect of metformin on the production of TNF and TF in isolated human monocytes stimulated with LPS.

We examined the effect of metformin on LPS-induced increases in TNF production and TF activities in isolated human monocytes. We previously demonstrated that TNF production and TF activities by monocytes began to increase at 2 h after in monocytes stimulated with LPS (100 ng/ml), peaking at 6 h after the stimulation (Molor et al., 2005; Komura et al., 2008). Metformin significantly inhibited the increases in both TNF production (Fig. 1A; p < 0.05) and TF activities (Fig. 1B; p < 0.05) in isolated monocytes after LPS stimulation. Metformin also inhibited the LPS-induced increases in TNF and TF mRNA levels in isolated human monocytes (Fig. 2, A and B; p < 0.05). The cell viability assessed by trypan blue dye exclusion test and apoptosis analyzed by Western blotting for caspase-3 ruled out the possibility that the observed effects were due to cell death in the experimental condition (data not shown).

Effect of metformin on the activation of nuclear factor-kappa B (NF-κB) and activator protein (AP)-1 pathways in isolated human monocytes stimulated with LPS.
Both NF-κB and AP-1 were demonstrated to be important transcription factors promoting the gene expression of TNF and TF in monocytes stimulated with LPS (Mackman, 1995; Hambleton et al., 1996; Guha et al., 2001). To determine whether metformin inhibits LPS-induced activation of NF-κB in monocytes, we examined the effect of metformin on LPS-induced degradation of IκBα in isolated human monocytes. Metformin did not inhibit LPS-induced degradation of IκBα in monocytes (Fig. 3A). In addition, metformin did not inhibit LPS-induced increases in the DNA-binding activities of p65 and p50 to their consensus oligonucleotides (data not shown). To determine whether metformin inhibits LPS-induced activation of AP-1, we examined the effect of metformin on LPS-induced phosphorylation of p38 and JNK in isolated human monocytes. Metformin did not inhibit LPS-induced phosphorylation of p38 and JNK in monocytes (Fig. 3, B, and C). In addition, metformin did not inhibit LPS-induced increases in DNA-binding activities of c-Fos to their consensus oligonucleotides (data not shown).

Effect of metformin on the expression of Egr-1 and phosphorylation of ERK1/2 in isolated human monocytes stimulated with LPS.
Egr-1 has been demonstrated to be an important transcription factor promoting the gene expression of TNF and TF in monocytes stimulated with LPS (Mackman, 1995; Guha et al., 2001). To determine whether metformin inhibits LPS-induced activation of Egr-1 in monocytes, we examined the effect of metformin on LPS-induced increases in expression of Egr-1 in isolated human monocytes. Intracellular levels of Egr-1 increased after LPS stimulation, peaking at 60 min after stimulation, and decreased thereafter (Fig. 4A). Metformin significantly inhibited the increases of Egr-1 expression at 60 min in monocytes stimulated with LPS (Fig. 4A; p < 0.05). The activation of ERK1/2 was shown to induce transcription of TNF and TF by increasing the expression of Egr-1 (Guha et al., 2001). To determine whether metformin inhibits LPS-induced activation of ERK1/2, thereby suppressing Egr-1 expression, we examined the effect of metformin on LPS-induced phosphorylation of ERK1/2 in isolated human monocytes. Intracellular levels of phosphorylated ERK1/2 increased after LPS stimulation, peaking at 30 min and decreased thereafter (Fig. 4B). Metformin significantly inhibited LPS-induced phosphorylation of ERK1/2 in monocytes at 30 min after stimulation (Fig. 4B; p < 0.05).
Effect of metformin on the production of TNF and TF in isolated human monocytes stimulated with oxLDL.

OxLDL has been reported to induce TNF production and TF activity in isolated human monocytes in vitro (Jovinge et al., 1996; Petit et al., 1999). The effect of metformin on oxLDL-induced increases in TNF production and TF activities was examined in isolated human monocytes. Metformin (10 µM) significantly inhibited the increases in both TNF production (Fig.5A; p < 0.05) and TF activities (Fig.5B; p < 0.05) in isolated monocytes at 12 h and 6 h after oxLDL (8 µg/ml) stimulation, respectively. The cell viability assessed by trypan blue dye exclusion test and apoptosis analyzed by Western blotting for caspase-3 ruled out the possibility that the observed effects were due to cell death in the experimental conditions (data not shown).

Effect of metformin on the activation of NF-κB, AP-1, and ERK1/2-Egr-1 pathways in isolated human monocytes stimulated with oxLDL.

We examined whether metformin inhibits oxLDL-induced activation of NF-κB and AP-1 in monocytes. Metformin inhibited neither on degradation of IκBα, phosphorylation of
p38 and JNK, nor DNA-binding activities of p65, p50 and c-Fos in isolated human monocytes stimulated with oxLDL (data not shown).

To determine whether metformin inhibits oxLDL-induced activation of Egr-1 in monocytes, we examined the effect of metformin on oxLDL-induced increases in the expression of Egr-1 in isolated human monocytes. Intracellular levels of Egr-1 increased after oxLDL stimulation, peaking at 120 min after stimulation, and decreased thereafter (Fig. 6A). Metformin significantly inhibited the increases of Egr-1 expression at 120 min in monocytes stimulated with oxLDL (Fig. 6A; p <0.05). To determine whether metformin inhibits oxLDL-induced activation of ERK1/2, thereby suppressing Egr-1 expression, we examined the effect of metformin on oxLDL-induced phosphorylation of ERK1/2 in isolated human monocytes. Intracellular levels of phosphorylated ERK1/2 increased after oxLDL stimulation, peaking at 60 min after stimulation, and decreased thereafter (Fig. 6B). Metformin significantly inhibited oxLDL-induced phosphorylation of ERK1/2 in monocytes at 60 min after stimulation (Fig. 6B; p < 0.05).
Discussion

In the present study, we demonstrated that metformin inhibited the production of TNF and TF in isolated human monocytes stimulated with LPS or oxLDL.

The monocytic production of TNF and TF is regulated by various transcriptional factors including NF-κB, AP-1, and Egr-1 (Mackman, 1995; Jovinge et al., 1996; Guha et al., 2001). The activity of NF-κB is primarily regulated via its sequestration in the cytosol by anchoring to inhibitor protein IκBα (Baldwin, 1996). As shown in the present study, pretreatment with metformin affected neither the degradation of IκBα nor the increase in DNA-binding activity of p65 and p50 induced by LPS or oxLDL. These results suggested that the inhibitory effect of metformin on the production of TNF and TF in LPS or oxLDL stimulated monocytes might not be mediated by the inhibition of NF-κB pathway.

AP-1 is another important factor regulating the production of TNF and TF. The activation of p38 and JNK by phosphorylation was shown to enhance the transcriptional activity of AP-1 (Mackman, 1995; Hambleton et al., 1996; Jovinge et al., 1996). In the present study, metformin inhibited neither LPS-induced nor oxLDL-induced phosphorylation of JNK and p38, nor the increase in DNA-binding activity of c-Fos. These results suggested that the inhibitory effect of metformin on the production of TNF and TF in monocytes might not be
due to the inhibition of AP-1 activation.

Metformin, on the other hand, inhibited LPS-induced or oxLDL-induced increases in intracellular levels of Egr-1 as shown in the present study. Egr-1 is shown to rapidly and transiently expressed in monocytes in response to LPS or oxLDL (Guha et al., 2001; Harja et al., 2004). Since Egr-1 is critically involved in the production of TNF and TF by monocytes (Mackman, 1995; Guha et al., 2001), metformin might inhibit the production of TNF and TF by inhibiting the activation of Egr-1 in monocytes stimulated with LPS or oxLDL. Egr-1 gene expression is regulated by various transcriptional factors, including Elk-1 and Sap-1a. Guha et al. reported that Elk-1, activated by phosphorylated-ERK1/2, plays an important role in the Egr-1 expression induced by LPS (Guha et al., 2001). They also reported that an inhibitor of ERK1/2 pathway reduces LPS-induced production of TNF and TF via inhibition of Egr-1 expression in monocytes. Since metformin inhibited LPS-induced or oxLDL-induced phosphorylation of ERK1/2 in the present study, it is probable that metformin may inhibit the expression of Egr-1 by inhibiting ERK1/2 activation, and thereby suppressing the production of TNF and TF in monocytes stimulated with LPS or oxLDL.

The precise mechanism(s) by which metformin inhibits phosphorylation of ERK1/2 in monocytes stimulated with LPS or oxLDL remains unclear at present. Metformin is known
as a pharmacological activator of AMP-activated protein kinase (AMPK) in various cell types (Zou et al., 2004; Hattori et al., 2006). However, it still remains controversial whether metformin exhibits an anti-inflammatory effect through the activation of AMPK. Hattori et al. demonstrated that high concentrations of metformin (>1 mmol/L) reduce TNF-α-induced NF-κB activity through the activation of AMPK in human umbilical vein endothelial cells (Hattori et al., 2006). Isoda et al. reported that metformin reduces high-glucose induced proinflammatory signaling independent of AMPK activation in human saphenous vein endothelial cells (Isoda et al., 2006). Another AMPK activator, 5-Aminoimidazole-4-carboxamide riboside (AICAR), was shown to exhibit anti-inflammatory effect independent of the activation of AMPK in RAW264.7 cells stimulated with LPS (Jhun et al., 2004; Kuo et al., 2008). These observations implicate that the inhibitory effects of metformin on the production of TNF and TF in LPS or oxLDL stimulated monocytes might be independent of its activation of AMPK.

TNF plays a critical role in proinflammatory responses in the development of inflammation observed in the atherosclerotic lesions of the vasculature (Tipping and Hancock, 1993; Libby, 2002). TNF can regulate the various functions of vascular wall cells. TNF induces expression of adhesion molecules such as vascular cell adhesion molecule-1,
intercellular adhesion molecule-1, E-selectin, and P-selectin (Collins T et al., 1995; Marui et al., 1993; Takahashi et al., 1996; Young et al., 2002), crucial to the recruitment of mononuclear leukocytes to endothelial cells. TNF also increases the production of monocyte chemoattractant protein-1 and interleukin-8, which are capable of inducing the migration of mononuclear leukocytes into the intima (Rollins et al., 1990; Young et al., 2002). TNF augments the production of macrophage colony stimulating factor by vascular wall cells, which stimulates the transition of monocytes to lipid-laden macrophages (foam cells). Consequently, it promotes the survival and growth of foam cells, and increases production of cytokines and growth factors by foam cells (Libby, 2002; Young et al, 2002). Therefore, TNF is involved in accumulation of mononuclear phagocytes and amplification of inflammation of the lesions leading to the development of atherosclerosis. The degradation of extracellular matrix by matrix metalloproteinases is thought to be important in plaque rupture (Young et al, 2002). TNF is shown to stimulate the expression of various types of matrix metalloproteinases in endothelial cells and smooth muscle cells as well as macrophages (Young et al, 2002). Since the present study has evidenced that metformin inhibits TNF production in LPS or oxLDL stimulated human monocytes, it is possible that the inhibition of TNF production by metformin in monocytes may at least partly contribute to prevent the progression and
complications of atherosclerosis.

TF is a cell membrane associated protein initiating blood coagulation. TF expressed by macrophage-derived foam cells and in the necrotic core of the plaque could contact with the coagulation factors and initiate clotting after plaque disruption (Ross, 1999; Libby, 2002). TF expression has been shown to be higher in atheroma from patients with unstable angina in comparison to those with stable angina (Annex et al., 1995). Higher levels of TF expression are observed in symptomatic than in asymptomatic patients with high-grade stenosis of the internal carotid artery (Jander et al., 2001). These observations suggest that high levels of TF exposed on plaque rupture can trigger acute thromboembolic complications, such as myocardial infarction or stroke. Since metformin inhibited TF expression in LPS or oxLDL stimulated human monocytes as presented in this study, it is possible that the inhibition of TF expression by metformin in monocytes may at least, in part, contribute to a reduction in the risk of atherothrombosis.

The present study demonstrated that metformin inhibited LPS-induced or oxLDL-induced TNF production and TF expression at a concentration of 10 µM in isolated human monocytes in vitro. Since the maximum plasma concentrations are 8.7 and 13 µM after taking metformin at doses of 1000 and 2000 mg, respectively, in the single dose test of
healthy volunteers (Cullen et al., 2004), it is likely that metformin might inhibit the production of TNF and TF in the actual clinical setting.

In conclusion, the present study suggests that the therapeutic effects of metformin against atherosclerotic thrombosis in patients with type 2 diabetes might at least partly be explained by the inhibition of the production of TNF and TF through the inhibition of Egr-1 expression in monocytes/macrophages.
Acknowledgments

We thank Ms Natsuki Dohi and Yumi Sakamoto for the technical assistance.
References


Guha M, O’Connel MA, Pawlinski R, Hollis A, McGovern P, Yan SF, Stern D, and


Jander S, Sitzer M, Wendt A, Schroeter M, Buchkremer M, Siebler M, Müller W,


Legends for figures

Figure 1. Effects of metformin on increases in TNF production and TF activities in isolated human monocytes stimulated with LPS. (A) Human monocytes were preincubated with various concentrations of metformin for 2 h and then stimulated with LPS (100 ng/ml). Six hours after the stimulation, the supernatants were collected and TNF levels were measured by ELISA as described in Methods. Data presented are means ± SD (n = 4) and are representative of three separate experiments that gave similar results. *, p<0.01 versus LPS (-) without metformin; †, p<0.05 versus LPS (+) without metformin. (B) Human monocytes were preincubated with various concentrations of metformin for 2 h and then stimulated with LPS (100 ng/ml). Six hours after the stimulation, mononuclear cell pellets were collected and TF activities were measured using the clotting assay method as described in Methods. Data presented are means ± SD (n = 4) and are representative of three separate experiments that gave similar results. *, p<0.01 versus LPS (-) without metformin; †, p<0.05 versus LPS (+) without metformin.

Figure 2. Effects of metformin on increases in TNF and TF mRNA in isolated human monocytes stimulated with LPS. (A) TNF mRNA levels in isolated human monocytes preincubated with or without metformin (10 µM) for 2 h were determined at 1 h after stimulation...
with LPS (100 ng/ml). TNF mRNA levels in human monocytes were detected by quantitative RT-PCR as described in Methods. Data presented are means ± SD (n = 3) and are representative of three separate experiments that gave similar results. *, p<0.01 versus LPS (-) without metformin; †, p<0.05 versus LPS (+) without metformin. (B) TF mRNA levels in isolated human monocytes preincubated with or without metformin (10 µM) for 2 h were determined at 1 h after stimulation with LPS (100 ng/ml). TF mRNA levels in human monocytes were detected by quantitative RT-PCR as described in Methods. Data presented are means ± SD (n = 3) and are representative of three separate experiments that gave similar results. *, p<0.01 versus LPS (-) without metformin; †, p<0.05 versus LPS (+) without metformin.

Figure 3. Effects of metformin on the degradation of IκBα and increases in intracellular levels of phosphorylated p38 and phosphorylated JNK in isolated human monocytes stimulated with LPS. Isolated human monocytes pretreated with or without metformin (10 µM) for 2 h were stimulated with LPS (100 ng/ml) for the indicated times. Intracellular levels of IκBα (A), phosphorylated p38 (p-p38) (B), and phosphorylated JNK (p-p54 and p-p46) (C) in human monocytes were determined by a Western blot analysis. β-actin, non-phosphorylated p38 (p38) and non-phosphorylated JNK (p54 and p46) were detected as the loading control. The
results of a densitometric analysis were also shown. Data presented are means ± SD of three samples in one experiment representative of three separate experiments that gave similar results. Open circles: LPS without metformin; closed circles: LPS with metformin. *, p < 0.01 versus time 0.

Figure 4. Effect of metformin on increases in intracellular levels of Egr-1 and phosphorylated ERK1/2 in isolated human monocytes stimulated with LPS. (A) Isolated human monocytes pretreated with or without metformin (10 µM) for 2 h were stimulated with LPS (100 ng/ml) for the indicated times. Intracellular levels of Egr-1 in human monocytes were determined by a Western blot analysis. PU.1 was detected as the loading control. The results of a densitometric analysis were also shown. Data presented are means ± SD of three samples in one experiment representative of three separate experiments that gave similar results. Open circles: LPS without metformin; closed circles: LPS with metformin. *, p < 0.01 versus time 0; †, p<0.05 versus LPS without metformin. (B) Isolated human monocytes pretreated with or without metformin (10 µM) for 2 h were stimulated with LPS (100 ng/ml) for the indicated times. Intracellular levels of phosphorylated ERK1/2 (p-ERK1/2) in human monocytes were determined by a Western blot analysis. Non-phosphorylated ERK1/2 (ERK1/2) was detected as the loading
control. The results of a densitometric analysis were also shown. Data presented are means ± SD of three samples in one experiment representative of three separate experiments that gave similar results. Open circles: LPS without metformin; closed circles: LPS with metformin. *, p < 0.01 versus time 0; †, p<0.05 versus LPS without metformin.

Figure 5. Effects of metformin on increases in TNF production and TF activities in isolated human monocytes stimulated with oxLDL. (A) Human monocytes were preincubated with metformin (10 µM) for 2 h and then stimulated with oxLDL (8 µg/ml). Twelve hours after the stimulation, the supernatants were collected and TNF levels were measured by ELISA as described in Methods. Data presented are means ± SD (n = 4) and are representative of three separate experiments that gave similar results. *, p<0.01 versus oxLDL (-) without metformin; †, p<0.05 versus oxLDL (+) without metformin. (B) Human monocytes were preincubated with metformin (10 µM) for 2 h and then stimulated with oxLDL (8 µg/ml). Six hours after the stimulation, mononuclear cell pellets were collected and TF activities were measured using the clotting assay method as described in Methods. Data presented are means ± SD (n = 4) and are representative of three separate experiments that gave similar results. *, p<0.01 versus oxLDL (-) without metformin; †, p<0.05 versus oxLDL (+) without metformin.
Figure 6. Effect of metformin on increases in intracellular levels of Egr-1 and phosphorylated ERK1/2 in isolated human monocytes stimulated with oxLDL. (A) Isolated human monocytes pretreated with or without metformin (10 µM) for 2 h were stimulated with oxLDL (8 µg/ml) for the indicated times. Intracellular levels of Egr-1 in human monocytes were determined by a Western blot analysis. PU.1 was detected as the loading control. The results of a densitometric analysis were also shown. Data presented are means ± SD of three samples in one experiment representative of three separate experiments that gave similar results. Open circles: oxLDL without metformin; closed circles: oxLDL with metformin. *, p < 0.01 versus time 0; †, p < 0.05 versus oxLDL without metformin. (B) Isolated human monocytes pretreated with or without metformin (10 µM) for 2 h were stimulated with oxLDL (8 µg/ml) for the indicated times. Intracellular levels of phosphorylated ERK1/2 (p-ERK1/2) in human monocytes were determined by a Western blot analysis. Non-phosphorylated ERK1/2 (ERK1/2) was detected as the loading control. The results of a densitometric analysis were also shown. Data presented are means ± SD of three samples in one experiment representative of three separate experiments that gave similar results. Open circles: oxLDL without metformin; closed circles: oxLDL with metformin. *, p < 0.01 versus time 0; †, p < 0.05 versus oxLDL without metformin.
Figure 2

(A) TNF/GapDH ratio

(B) IFN/GapDH ratio