Metformin Inhibits Proinflammatory Responses and Nuclear Factor-κB in Human Vascular Wall Cells

Kikuo Isoda, James L. Young, Andreas Zirlik, Lindsey A. MacFarlane, Naotake Tsuboi, Norbert Gerdes, Uwe Schönbeck, Peter Libby

Objective—Metformin may benefit the macrovascular complications of diabetes independently of its conventional hypoglycemic effects. Accumulating evidence suggests that inflammatory processes participate in type 2 diabetes and its atherothrombotic manifestations. Therefore, this study examined the potential action of metformin as an inhibitor of pro-inflammatory responses in human vascular smooth muscle cells (SMCs), macrophages (Mφs), and endothelial cells (ECs).

Methods and Results—Metformin dose-dependently inhibited IL-1β–induced release of the pro-inflammatory cytokines IL-6 and IL-8 in ECs, SMCs, and Mφs. Investigation of potential signaling pathways demonstrated that metformin diminished IL-1β–induced activation and nuclear translocation of nuclear factor-kappa B (NF-κB) in SMCs. Furthermore, metformin suppressed IL-1β–induced activation of the pro-inflammatory phosphokinases Akt, p38, and Erk, but did not affect PI3 kinase (PI3K) activity. To address the significance of the anti-inflammatory effects of a therapeutically relevant plasma concentration of metformin (20 μmol/L), we conducted experiments in ECs treated with high glucose. Pretreatment with metformin also decreased phosphorylation of Akt and protein kinase C (PKC) in ECs under these conditions.

Conclusions—These data suggest that metformin can exert a direct vascular anti-inflammatory effect by inhibiting NF-κB through blockade of the PI3K–Akt pathway. The novel anti-inflammatory actions of metformin may explain in part the apparent clinical reduction by metformin of cardiovascular events not fully attributable to its hypoglycemic action. (Arterioscler Thromb Vasc Biol. 2006;26:611-617.)

Key Words: atherosclerosis • diabetes • inflammation • interleukins • smooth muscle cell

Type 2 diabetes mellitus currently comprises 90% to 95% of all diagnosed diabetes, with an alarming increase in incidence among youth.1 Risk of cardiovascular death and stroke increases 2- to 4-fold in diabetic patients, a prominent comorbidity that accounts for 65% of deaths among the growing diabetic population and underscores the important intersection between this metabolic disease and cardiovascular events.1

The concordance between diabetes and cardiovascular disease highlights a multifactorial relationship, implicating hyperglycemia, insulin resistance, hypertension, and dyslipidemia.2 Although glycemic control represents the classical goal of diabetes therapy, the pathogenesis of the vascular complications of diabetes and the metabolic syndrome extend beyond hyperglycemia and glycated proteins.3 Notably, the United Kingdom Prospective Diabetes Study 34 (UKPDS 34) demonstrated that despite similar glucose-lowering effects, administration of metformin reduced all-cause mortality, myocardial infarction, and stroke more than insulin or sulfonylureas (chlorpropamide, glibenclamide).4,5 UKPDS 34 suggested that glucose-lowering properties alone cannot account for the additional cardiovascular benefit, hence the hypothesis that metformin confers vascular benefits beyond its hypoglycemic action.

Metformin, a biguanide family member commonly used in treatment for type 2 diabetes, appears to increase liver and peripheral tissue sensitivity to insulin as well as reduce hepatic glucose production; however, its exact mechanism remains unclear.2,6 Previous studies show early evidence of metformin actions beyond its effects on glucose metabolism, including reduction of plasminogen activator inhibitor (PAI)-1, von Willebrand factor (vWF), and smooth muscle cell (SMC) contractility via either agonist-induced increase in intracellular [Ca2+] or a secondary increase in nitric oxide.7–9 Recent clinical studies further suggest that metformin may alter inflammation as determined by decreased inflammatory markers in plasma, including soluble intercellular adhesion molecule, vascular cell adhesion molecule-1, macrophage migration inhibitory factor, and C-reactive protein (CRP) in...
some cases of polycystic ovary syndrome, indicating modula-
tion of inflammation.\textsuperscript{10–12} In the context of the current concept of atherosclerosis as an inflammatory disorder, studies over the past two decades have established that the nuclear transcription factor-kappa B (NF-κB) plays a central role in mediating cytokines, growth factors, receptor signaling proteins, cell adhesion molecules, and other proteins of immunity in cell types resident to the plaque microenvironment, ie, endothelial cells (ECs), SMCs, and macrophages (M\textsubscript{\textdelta}s).\textsuperscript{13–15} Activation of NF-κB transcriptionally activates multiple pro-inflammatory genes, including those that encode the pro-atherogenic cytokines IL-6 and IL-8.\textsuperscript{16} Of interest to the intersection of atherosclerosis and diabetes, recent studies demonstrate enhanced serum IL-6 in obese type 2 diabetes mellitus patients with a concordant elevation of NF-κB and decrease in IkB in blood mononuclear cells.\textsuperscript{17,18} The cytokine IL-8 participates in neutrophil activation and chemotaxis.\textsuperscript{19} Mice with M\textsubscript{\textdelta}s unable to respond to IL-8 show impaired M\textsubscript{\textdelta} recruitment to the atherosclerotic lesion, suggesting a role for IL-8 in monocyte trafficking in vivo.\textsuperscript{19,20}

The present study uses cells that localize in atheroma to test the hypothesis that metformin modulates the inflammatory potential of the atherosclerotic plaque. Metformin reduced elaboration of the pro-inflammatory cytokines IL-6 and IL-8 from activated cells and concomitantly impairs NF-κB nuclear activation in vascular SMCs.

\section*{Methods}

We purchased 1,1-Dimethylbiguanide hydrochloride (metformin), mannitol, EDTA, and trypan blue dye from Sigma (St. Louis, Mo). Recombinant IL-1β, IL-6, and IL-8, as well as antibody for IL-6 and IL-8 with or without biotinylination, were purchased from Pierce Endogen (Rockford, Ill). Anti-human phospho-Akt, Akt, phospho-JNK, phospho-protein kinase C (PKC), phospho-AMP–activated protein kinase (AMPK), and phospho-p65 were obtained from Cell Signaling (Beverly, Mass). Anti-human phospho-Erk, Erk, phospho-p38, p–38, PKC, p65, and IkB were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif), whereas anti-human GAPDH was obtained from Biodigen (Saco, Me).

\subsection*{Cell Isolation and Culture}

Cultured human vascular ECs and SMCs were isolated from saphe-
nous veins as described previously.\textsuperscript{21–23} M\textsubscript{\textdelta}s were isolated from freshly prepared leukocyte concentrates by density gradient centrif-
figation using Lymphocyte Separation Medium (ICN Biomedicals, Aurora, Ohio) and subsequent adherence to plastic culture flasks. M\textsubscript{\textdelta}s were cultured for 10 days in RPMI 1640 containing 2% human serum (Atlanta Biologicals, Lawrenceville, Ga).\textsuperscript{23} All three cell types were cultured in media lacking fetal bovine serum before (12 hours) and during the experiment.\textsuperscript{23} Culture media and fetal bovine serum contained \textlessthan;40 pg endotoxin/mL as determined by the chromogenic Limulus amoebocyte assay (Associates of Cape Cod, Falmouth, Mass).

\subsection*{Enzyme-Linked Immunosorbent Assay}

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described.\textsuperscript{21}

\subsection*{Western Blot Analysis}

Western blot analysis was performed as previously described.\textsuperscript{21} Densitometric analysis of immunoreactive bands used National Institutes of Health Image J (National Institutes of Health) applied to digital images of respective Western blots.

\section*{Evaluation of Cell Viability}

Cell viability was determined by trypan blue dye exclusion after 12 hours of metformin treatment. Additional experiments monitored cytoplasmic oligonucleosome formation (Roche Applied Science, Indianapolis, Ind).

\subsection*{Immunostaining of NF-κB in Cultures of Human SMCs}

After fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS), cells were permeabilized by methanol treatment (5 minutes) and blocked with 10% goat serum in PBS. Cells were incubated with primary antibody anti-p65 NF-κB before application of Alexa-fluor 594 anti-mouse IgG1 antibody (Molecular Probes, Eugene, Ore). Cells were washed 3 times with PBS between each step. Immunostained cells were visualized with a Nikon IX 710 fluorescence microscope.

\subsection*{PI3-Kinase Activity Assay}

The PI3-kinase activity was assayed as previously described.\textsuperscript{24}

\subsection*{Statistical Analysis}

Results are shown as mean±SEM. Differences between groups were determined using ANOVA with Bonferroni post hoc test. Two groups were compared using the Student \(t\) test. A value of \(P<0.05\) was regarded as a significant difference.

\section*{Results}

\subsection*{Metformin Inhibits IL-1β–Induced IL-6 and IL-8 Production in SMCs, ECs, and M\textsubscript{\textdelta}s}

To investigate whether metformin modulates inflammatory functions in cells found in atherosclerotic plaques, cultured cells (SMCs, ECs, M\textsubscript{\textdelta}s) were stimulated with IL-1β (1 ng/mL; 12 hours) under serum-free conditions and then incubated with fresh media in the absence or presence of metformin (1 mmol/L to 1 mmol/L) for 6 hours. Cell culture supernatants were assayed for IL-6 and IL-8 protein levels by ELISA. Unstimulated cultured cells released no IL-6 and IL-8 and, as expected, IL-1β stimulation markedly induced release of these cytokines. Metformin inhibited IL-1β–induced IL-6 (Figure 1A) and IL-8 (Figure 1B) release in SMCs, ECs, and M\textsubscript{\textdelta}s in a concentration-dependent manner. Inhibition of IL-6 and IL-8 occurred at metformin concentrations in the nanomolar to micromolar range consistent with those achieved clinically.

\subsection*{Metformin Does Not Impair Cell Viability}

Metformin did not induce death of SMCs, ECs, or M\textsubscript{\textdelta}s at concentrations up to 10 mmol/L, as determined by trypan blue exclusion and phase contrast micrographic inspection, indicating that toxic effects of metformin do not explain the attenuated inflammatory response (data not shown). Additional studies measuring oligonucleosome formation, an index of apoptosis, in SMCs and ECs support this conclusion (data not shown).

\subsection*{Metformin Limits IL-1β–Induced NF-κB Activation in SMCs}

Further experiments explored the molecular mechanisms by which metformin diminishes the inflammatory response in vascular SMCs by analyzing activation of the central pro-inflammatory transcription factor NF-κB. Human vascular SMCs pre-incubated with metformin (1 mmol/L; 30 minutes) had markedly attenuated IL-1β–induced phosphorylation of
Metformin Inhibits the PI3-Kinase/Akt Pathway

To obtain further evidence that metformin could regulate inflammatory pathways, we analyzed the effect of metformin on IL-1β-induced phosphorylation of Akt (Figure 4A), Erk (Figure 4B), p38 (Figure 4C), and c-Jun N-terminal kinase (JNK) (Figure 4D) in SMCs by Western blotting. Resting SMCs did not contain phosphorylated forms of Akt, Erk, p38, or JNK (data not shown). However, on stimulation with IL-1β, SMCs showed rapid phosphorylation of Akt, peaking at 10 minutes and beginning to decline by 30 minutes after stimulation (Figure 4A). Pretreatment with metformin decreased phosphorylation with a maximum inhibitory effect of 68%, as observed at 10 minutes after stimulation.

Furthermore, stimulation of SMCs with IL-1β (1 ng/mL) maximally induced the phosphorylation of Erk and p38 at 30 minutes after stimulation (Figure 4B, 4C). Interestingly, pretreatment of SMCs with metformin before addition of IL-1β inhibited Erk and p38 phosphorylation by 30% and 52%, respectively, at 30 minutes after stimulation as compared with SMCs treated with IL-1β alone.

Stimulation of SMCs with IL-1β (1 ng/mL) induced JNKp54 phosphorylation, with maximum phosphorylation at 60 minutes after stimulation (Figure 4D). However, inhibition caused by treatment with metformin occurred at 30 minutes after stimulation when JNKp54 phosphorylation was 42% less than in SMCs treated with IL-1β alone. Exposure to
metformin did not significantly suppress activation of PI3-kinase at 10 minutes after stimulation, suggesting no functional deficit of PI3-kinase (Figure 5). Taken together, these results point to a mode of metformin action downstream of PI3-kinase yet upstream of Akt.

Therapeutically Relevant Concentration of Metformin Inhibits Glucose-Induced Inflammation

A recent report demonstrated that treatment of ECs with high glucose (HG) (30 mmol/L) in ECs leads to activation of PI3K and Akt, as well as subsequent NF-κB activation.25 To address the importance of the anti-inflammatory effects of a therapeutically relevant plasma concentration of metformin (20 μmol/L), we investigated the effect of metformin on pro-inflammatory signaling in ECs exposed to HG (30 mmol/L). Western blots evaluated the effect of metformin on HG-induced phosphorylation of Akt (Figure 6A) in ECs pretreated with 20 μmol/L metformin for 1 hour and exposed to HG. On stimulation with HG, ECs showed phosphorylation of Akt, but the peak (30 minutes) was delayed compared with IL-1β stimulation (10 minutes). Pretreatment with metformin decreased Akt phosphorylation at 30 and 60 minutes.

Gallo et al showed that metformin prevents glucose-induced PKC activation in ECs.26 We also examined the effect of metformin on phosphorylation of PKC (Figure 6B). Stimulation of ECs with HG induced PKC phosphorylation, with maximum phosphorylation at 30 minutes. Of note, metformin inhibited PKC phosphorylation at 30 and 60 minutes. Although a recent report showed that metformin activates AMPK in ECs,27 we found no AMPK phosphorylation at any time point using a pharmacologically relevant concentration of metformin (data not shown). We further treated ECs with a range of metformin concentrations (2 to 2000 μmol/L) for 1 hour. Western blot analysis revealed that metformin ≥200 μmol/L could induce AMPK phosphorylation (Figure 6C).

Discussion

Metformin enjoys wide use in the management of type 2 diabetes. Clinical trials with metformin have indicated improvement in cardiovascular events in diabetic patients apparently beyond its glucose-lowering properties.4 However, metformin’s possible mechanisms of action beyond glycemic control remain poorly understood. The present study shows a novel anti-inflammatory function of metformin, disclosed by inhibited release of IL-6 and IL-8 from human vascular
SMCs, ECs, and MØs. These anti-inflammatory functions occurred at concentrations on the order of plasma levels achieved during conventional clinical regimens in either type 2 diabetics or healthy nondiabetic adults. The current study reveals insight into the potential underlying anti-inflammatory mechanisms of metformin, which may contribute to the observed clinical benefits in cardiovascular outcomes.

Inflammation undoubtedly participates in coronary heart disease. White blood cell count and levels of CRP predict the incidence of type 2 diabetes. Furthermore, obesity elevates levels of pro-inflammatory cytokines, considered mediators of increased cardiovascular morbidity in these patients. Cytokines such as IL-8 likely contribute to monocytic recruitment and adhesion to ECs in atherosclerosis, whereas IL-6 drives the acute phase response. This study demonstrates that metformin inhibits IL-1β-induced IL-6 and IL-8 expression, showing mechanisms by which treatment with metformin may attenuate inflammation.

Compelling evidence suggests that in SMCs, cytokines mediate a range of their pro-inflammatory effects by activating NF-κB. SMCs in human atherosclerotic plaques display activation of this multipotent pro-inflammatory transcriptional regulator. Therefore, limiting NF-κB activation in plaques could represent a molecular mechanism by which metformin modulates inflammatory responses to IL-1β. Metformin suppressed cytokine-induced NF-κB–dependent gene transcription in the present study, caused by modulation of cytokine-induced IκB degradation and NF-κB nuclear translocation. These findings suggest a novel anti-inflammatory mechanism for metformin, i.e., inhibition of NF-κB activation.

To elucidate the molecular mechanisms leading to inhibition of NF-κB activation by metformin, we explored IL-1 signaling cascades including 3 distinct types of mitogen-activated protein (MAP) kinases (p38, JNK, and Erk). IL-1–stimulated SMCs showed activation of all three MAP kinase pathways studied. The MAP 3-kinase family MEKK activates the protein kinase MEK, which subsequently activates p38, JNK, or Erk. Once activated, MAP kinases translocate to the nucleus and activate transcription factors. The p38 kinase regulates various transcription factors, including NF-κB, considered a central hub of pro-inflammatory gene regulation in atherosclerosis. Moreover, the transcriptional activity of NF-κB p65 links with versatile coactivator proteins such as p300, CBP, TFIIB, and TBP. Thus, p38 kinase may regulate these coactivators to facilitate NF-κB–dependent gene expression. We observed that metformin inhibited IL-1–induced p38 phosphorylation in SMCs.

Most cells express 2 JNK isoforms, JNK1 and JNK2, which share similar modes of regulation. Activation of JNK greatly enhances c-Jun transcriptional activity and expression of the AP-1 target gene. Moreover, recent data demonstrated that AP-1 participates importantly in the regulation of the IL-6 gene in IL-1–stimulated cells. In our study, metformin also inhibited IL-1–induced phosphorylation of JNK, suggesting that blocking JNK activation contributed to the inhibition of cytokine expression, potentially via c-Jun or AP-1.

Previous studies showed that Akt and Erk1/2 act upstream of NF-κB. Akt also associates directly with IKK, and activates IKK-α via phosphorylation at Thr-2. Mutation of this amino acid blocks Akt-induced IκBα phosphorylation and NF-κB activation. However, Erk1/2 activation induces IκB degradation. These reports support our findings that metformin suppressed the phosphorylation of Akt and Erk1/2 with consequent inhibition of NF-κB translocation and of IκB degradation.

This study demonstrates that metformin may suppress the phosphorylation of all three MAP kinases (p38, JNK, and Erk) and Akt. Therefore, we investigated PI3-kinase phosphorylation because of its established role as an upstream activator of Akt. Metformin did not significantly suppress phosphorylation of PI3-kinase targets, inconsistent with a functional deficit of PI3-kinase. Taken together, these results indicate that metformin blocks pro-inflammatory signal transduction in SMCs via NF-κB downstream of PI3-kinase by suppressing Akt, Erk1/2, and, finally, NF-κB translocation.

Our signaling studies used metformin (1 mmol/L) at a level that exceeds its therapeutic plasma concentration (Cmax 20 μmol/L). Further experiments therefore investigated the effect of a therapeutically relevant plasma concentration of metformin (20 μmol/L) on Akt phosphorylation in ECs exposed to HG. Pretreatment with metformin (20 μmol/L) decreased Akt phosphorylation at 30 and 60 minutes, estab-
lishing that inhibition of pro-inflammatory signaling occurs at therapeutic plasma concentrations of metformin and in support of the potential clinical relevance of these “pleiotropic” effects of the agent. Interestingly, metformin also inhibited PKC activation in response to HG, concordant with a recent report. PKC activation in EC leads to extracellular matrix accumulation, increased endothelial permeability, and base-


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