Metformin, a biguanide, appeared on the European market at the end of the 1950s and was used clinically for diabetes mellitus. Since another biguanide, phenformin, was found to have a severe adverse effect, lactic acidosis, the usage of metformin declined and the drug was eventually withdrawn from the market. However, metformin was recently revaluated in terms of safety and effectiveness. It was reported to reduce the risk of diabetes-related endpoints in overweight diabetic patients in a large clinical study and, therefore, has begun to be widely used again.

Metformin’s mechanisms of action to improve hyperglycemia are proposed to be based on the suppression of glucose’s release from the liver, the limited absorption of glucose in the alimentary tract, the acceleration of glucose’s uptake in peripheral tissues, and the suppression of β-oxidation. It has been recently revealed that AMP-activated protein kinase (AMPK) took part in these pharmacological effects. AMPK is known to play a major role in the control of energy metabolism. This kinase modulates not only lipid metabolism but also glycometabolism as shown by the following findings. Gluconeogenesis was inhibited by suppressing the expression of phosphoenolpyruvate carboxykinase (PEPCK), which catalyzes the conversion of pyruvate to phosphoenolpyruvate in the liver. The absorption of glucose was depressed by the repression of Na⁺/glucose co-transporter 1 (SGLT1), which is abundantly expressed in the intestine and a component of glucose transporter 2 (GLUT2). The utilization of glucose in muscle cells was promoted by inducing the translocation of GLUT1 and 4 to the cell membrane. These observations support that AMPK participates in the improvement of hyperglycemia.

We previously reported that a biguanide, buformin, suppressed the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPD) and suggested that this decrease was one of the causes of lactic acidosis. In this study, we examined the signaling pathway and regulatory factors for the expression of the GAPD gene triggered by metformin in HepG2 cells. The mRNA and protein expression of GAPD, detected by real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, respectively, decreased upon treatment of the cells with 10 mM metformin for 24 h. Under the conditions, metformin induced phosphorylation of AMP-activated protein kinase (AMPK). The expression of GAPD mRNA decreased on treatment with an activator for AMPK, 5-amino-imidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR). Inhibitors for signal transducers, Compound C, H-89, and MDL-12,330A, restored the level of GAPD mRNA. A luciferase reporter plasmid containing bp −1795 to +57 of the 5′-flanking region of the GAPD gene was constructed for a reporter gene assay. The luciferase activity in transfectants decreased on incubation with metformin. A mutant reporter plasmid with an altered cAMP-response element (CRE) counteracted the metformin-mediated repression of GAPD transcription. These results suggest that signal transducers, adenylate cyclase (AC), protein kinase A (PKA), and AMPK, are involved in the signaling pathway triggered by metformin and CRE-binding protein is one of the transcription factors for the GAPD gene down-regulated by metformin.

Key words metformin; glyceraldehyde 3-phosphate dehydrogenase; adenylate cyclase; protein kinase A; AMP-activated protein kinase; cAMP-response element binding protein

Regulation of Glyceraldehyde 3-Phosphate Dehydrogenase Expression by Metformin in HepG2 Cells

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Biguanides are known to have a serious side effect, lactic acidosis. We previously reported that buformin suppressed the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPD) and suggested that this decrease was one of the causes of lactic acidosis. In this study, we examined the signaling pathway and regulatory factors for the expression of the GAPD gene triggered by metformin in HepG2 cells. The mRNA and protein expression of GAPD, detected by real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, respectively, decreased upon treatment of the cells with 10 mM metformin for 24 h. Under the conditions, metformin induced phosphorylation of AMP-activated protein kinase (AMPK). The expression of GAPD mRNA decreased on treatment with an activator for AMPK, 5-amino-imidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR). Inhibitors for signal transducers, Compound C, H-89, and MDL-12,330A, restored the level of GAPD mRNA. A luciferase reporter plasmid containing bp −1795 to +57 of the 5′-flanking region of the GAPD gene was constructed for a reporter gene assay. The luciferase activity in transfectants decreased on incubation with metformin. A mutant reporter plasmid with an altered cAMP-response element (CRE) counteracted the metformin-mediated repression of GAPD transcription. These results suggest that signal transducers, adenylate cyclase (AC), protein kinase A (PKA), and AMPK, are involved in the signaling pathway triggered by metformin and CRE-binding protein is one of the transcription factors for the GAPD gene down-regulated by metformin.

MATERIALS AND METHODS

Materials 1,1-Dimethylbiguanide hydrochloride (metformin) and FuGene 6 were purchased from ICN Biomedicals Inc. (Irvine, CA, U.S.A.) and Rosch Diagnostics K. K. (Tokyo, Japan), respectively. 5-Amino-imidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was obtained from...
Calbiochem-Merck KGaA (Darmstadt, Germany). TRIzol, Oligo(dT)_12–18 Primer and Super Script III reverse transcriptase were purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.). The GeneEditor in vitro Dual-Luciferase Reporter Assay System, the GeneEditor in vitro Site-Directed Mutagenesis System, and other DNA-manipulating enzymes were acquired from Promega Corp. (Madison, WI, U.S.A.). SYBR Premix Ex Taq, ECL Western Blotting Detection Reagents, and Immobilon Transfer Membranes were purchased from Takara Bio Inc. (Osaka, Japan), GE Healthcare U.K. Ltd. Amersham Place (Buckinghamshire, England), and MILLIPORE Corp. (Billerica, MA, U.S.A.), respectively. Anti-GAPD polyclonal immunoglobulin G (IgG) antibody and anti-β2-microglobulin polyclonal IgG antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Anti-phospho-AMPKalpha (Thr 172) antibody and anti-AMPKalpha antibody were purchased from Cell Signalling Technology, Inc. (Danvers, MA, U.S.A.). All other chemicals and reagents were of analytical grade.

**Cell Culture and Treatment with Compounds** HepG2 cells were maintained in Dulbecco’s modified Eagle’s MEM (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin, and 100 units/ml of penicillin. Cells were plated on 35-mm dishes at a density of 4×10⁵ cells/ml and incubated in a CO₂ incubator for 24 h. They were washed once with phosphate-buffered saline (PBS) and then treated with metformin, AICAR, and inhibitors dissociated from the regulatory regions −1795 to +57 and −714 to +57 upstream of GAPD’s transcription initiation site was prepared by PCR with forward primers containing a KpnI recognition site, 5’-AGTGGTACCTGTAGCAGTGTCGTGAC-3’ and 5’-CGTGGTGACCTGGAACATCGTGAC-3’, respectively, and a reverse primer containing a HindIII recognition site, 5’-CGAAGGCTGGTACGTCG AAGCAGG-3’. After the PCR product was digested with both restriction enzymes, the fragment was ligated into a multiple cloning site of pGL3 basic vector yielding 1795pGL3 and 714pGL3, respectively.

**Western Blotting** HepG2 cells were lysed with extraction buffer. The total amount of protein in the lysate was measured with Bio-Rad’s Bradford reagent. SDS-PAGE was carried out with a 12% polyacrylamide gel according to the method of Laemmli. Western blotting was performed by the method of Towbin et al. After proteins in the gel were electroblotted onto a polyvinylidene difluoride (PVDF) membrane, a desired protein was probed with the specific primary antibody and subsequently the peroxidase-conjugated secondary antibody. The peroxidase activity was detected with an ECL Western Blotting Detection System according to the manufacturer's instructions.

**Construction of Reporter Vector** The genomic DNA in HepG2 cells was extracted with TRIzol reagent according to the manufacturer's instructions. A DNA fragment containing the regulatory regions −1795 to +57 and −714 to +57 upstream of GAPD’s transcription initiation site was prepared by PCR with forward primers containing a KpnI recognition site, 5’-AGTGGTACCTGTAGCAGTGTCGTGAC-3’ and 5’-CGTGGTGACCTGGAACATCGTGAC-3’, respectively, and a reverse primer containing a HindIII recognition site, 5’-CGAAGGCTGGTACGTCG AAGCAGG-3’. After the PCR product was digested with both restriction enzymes, the fragment was ligated into a multiple cloning site of pGL3 basic vector yielding 1795pGL3 and 714pGL3, respectively.

**Site-Directed Mutagenesis** Site-directed mutagenesis in the cAMP response element (CRE) and NF-κB-binding element was performed with the GeneEditor in vitro Site-Directed Mutagenesis System according to the manufacturer's instructions. Five micrograms of 1795pGL3 as a template DNA and oligonucleotides for the mutagenesis, 5’-CACACACTAATGAGTATGCCTCC-3’ and 5’-CAAAAGGGCCTGCGCCCTCCCATGCA-3’ for CRE and NF-κB-binding element, respectively, were used. These mutated plasmids were named CREmutpGL3 and NF-κBmutpGL3, respectively.

**Transcriptional Analysis** HepG2 cells were plated onto a 24-well culture plate at a density of 4×10⁴ cells/well and cultured for 24 h in a CO₂ incubator. After attaching to the plate, the cells were co-transfected with 0.5 μg of pGL3, 10 μg of the reporter vectors prepared and 5 ng of phRL-TK as a reference plasmid with 1.2 μl of FuGene6 according to the manufacturer's instructions. Transfected cells were maintained in the regular culture medium for 24 h and treated with 10 μM metformin for an additional 48 h. The activities of luciferases from the firefly and Renilla were detected with the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions.

Table 1. Sequences of Oligonucleotide Primers and Conditions for Real-Time RT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product size (bp)</th>
<th>PCR condition</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPD</td>
<td>5’-CCA GCA AGA GCA CAA GAG GA-3’</td>
<td>5’-GCA ACT GTG AGG AGG GGA-3’</td>
<td>98</td>
<td>94 °C, 30 s</td>
<td>19</td>
</tr>
<tr>
<td>β2-Microglobulin</td>
<td>5’-CGG CAG GCA TAC TCA TCT TT-3’</td>
<td>5’-GTT TTC ATC CAT CCG ACA-3’</td>
<td>157</td>
<td>94 °C, 30 s</td>
<td>20</td>
</tr>
<tr>
<td>CREB</td>
<td>5’-ACG CCA GGA GTC ATG AAG AG-3’</td>
<td>5’-TGT CCC TAA GGC AAT CAA GG-3’</td>
<td>160</td>
<td>94 °C, 30 s</td>
<td>20</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5’-TTG AAG CAC GAA TGA CAG AG-3’</td>
<td>5’-TGA GGT CCA TCT CCT TGG TC-3’</td>
<td>176</td>
<td>94 °C, 30 s</td>
<td>30</td>
</tr>
</tbody>
</table>
Statistical Analysis  The significance of differences between two groups was calculated with Student’s t-test, and the significance of differences between multiple groups was assessed by one-way analysis of variance followed by Dunnet’s test.

RESULTS

Effect of Metformin on the mRNA and Protein Expression of Glyceraldehyde 3-Phosphate Dehydrogenase (GAPD) in HepG2 Cells

We first examined whether metformin affected the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPD) mRNA in HepG2 cells since the expression was previously reported to decrease upon treatment of the cells with buformin.9) The expression of GAPD mRNA was observed to decrease with an increase in the concentration of metformin for 24 h (Fig. 1A). HepG2 cells were incubated with 10 mM metformin for the period indicated. Although the expression decreased significantly for 24 h, it was restored 36 and 48 h later (Fig. 1B). The protein expression of GAPD continued to decrease 72 h after treatment with 10 mM metformin (Fig. 1C). The reason for this discrepancy in the expression of the mRNA and protein remains unclear. However, it may be that metformin inhibits the translation of GAPD mRNA as Dowling et al.16) reported that metformin inhibited a mammalian target of rapamycin-dependent translational initiation in MCF-7 cells. Further study is necessary to elucidate this interesting observation.

Phosphorylation of AMP-Activated Protein Kinase by Metformin and 5-Amino-imidazole-4-carboxamide-1-β-D-ribofuranoside

Metformin was recently reported to induce the activation of AMP-activated protein kinase (AMPK) via its phosphorylation.5) Therefore, it was examined whether AMPK was phosphorylated by metformin under our experimental conditions. 5-Amino-imidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is an AMPK activator and was used as a positive control for the experiment. As shown in Fig. 2, phosphorylation of AMPK was observed to increase with time on treatment with 100 μM AICAR and on incubation with 10 mM metformin for 30 and 60 min.

Expression of GAPD mRNA Mediated by AMPK

Having confirmed the phosphorylation of AMPK evoked by metformin, we investigated whether the activation of AMPK participated in the down-regulation of GAPD expression caused by metformin. When HepG2 cells were incubated with various concentrations of AICAR for 24 h, the expression of GAPD mRNA decreased significantly on treatment with 100 μM AICAR (Fig. 3A). To examine further whether AMPK was involved in the pathway of metformin signaling, HepG2 cells were incubated with an AMPK inhibitor, Compound C, for 1 h and subsequently treated with 10 mM metformin for 24 h. The decrease in the expression of GAPD mRNA induced by metformin disappeared on pretreatment with 1 μM of Compound C (Fig. 3B). These results suggest that AMPK modulates the expression of GAPD mRNA and the signaling is mediated by the phosphorylation of AMPK. Protein kinase A (PKA) was present upstream of AMPK and glucagon repressed signaling through the mammalian target of rapamycin in rat liver by activating AMPK.17) Therefore, it was investigated whether PKA and a related upper signal transducer, adenylyl cyclase (AC), took part in the signal transduction evoked by metformin in HepG2 cells using MDL-12,330A and H89, inhibitors for AC and PKA, respectively. The decrease in expression of GAPD mRNA caused
by 10 mM metformin was diminished by pre-treatment of the cells with 100 nM H89 and 1 mM MDL-12,330A (Fig. 3C), suggesting that AC and PKA are involved in the signaling pathway triggered by metformin.

Transcriptional Regulation of the GAPD Gene Evoked by Metformin

The binding sites of two transcription factors, cAMP response element binding protein (CREB) and nuclear factor-kappa B (NF-κB), which were reported to be responsive to the signaling mediated by AMPK, were found in the promoter region of the GAPD gene from bp −1795 to +57 5′-upstream of the transcription initiation site as a result of searching the MOTIF Search database (Fig. 4A). Whether metformin affected the mRNA expression of these factors was examined by the real-time RT-PCR method with specific primers. As shown in Figs. 4B and C, the expression of CREB mRNA was significantly suppressed by the incubation of HepG2 cells with 10 mM metformin for 12 h but that of NF-κB mRNA significantly increased after 24 h of incubation.

Whether the promoter activity of the GAPD gene was affected by metformin was examined using a luciferase reporter plasmid with GAPD’s promoter region ligated into a reporter vector, pGL3, and by introducing this construct, 1795pGL3, into HepG2 cells. Luciferase activity significantly decreased to 50% of the control level on treatment with 10 mM metformin for 24 h (Fig. 4D). Since the expression of CREB and NF-κB mRNAs was observed to decrease and increase, respectively, mutant constructs with altered sequences for the binding sites of these transcriptional regulatory factors in the reporter plasmid were prepared by site-directed mutagenesis and transfected into HepG2 cells. The mutation of the binding sites for CREB and NF-κB resulted in a significant loss of luciferase activity, suggesting that these factors are responsible for the transcription of the GAPD gene. Although the luciferase activity in the mutant with the altered NF-κB-binding sequence decreased on incu-
Metformin was reported to activate AMPK by increasing the ratio of AMP/ATP caused by the inhibition of complex I in the mitochondrial respiratory chain after it bound to mitochondria producing ATP in the cells. AMPK was phosphorylated by LKB1 serine/threonine kinase which was activated through this pathway, stimulates the pathway, or both. However, it is unclear from our results whether AMPK is activated through this pathway, stimulates the pathway, or both.

The expression of a transcription factor, CREB, was suppressed on treatment with metformin and CREB was suggested to be one of the regulators responsible for transcription of the GAPD gene in HepG2 cells. GAPD mRNA decreased after 24 h incubation with metformin (Fig. 1B) but the expression of CREB mRNA was suppressed after just 12 h (Fig. 4B). The time-lag may be explained as follows. The decrease in CREB mRNA on incubation with metformin for 12 h caused a decrease in the amount of CREB 12 h later, because it generally takes at least 6 to 12 h until proteins function after transcription of the gene. The decrease in CREB probably results in functional depression of the transcriptional activity by CREB. Therefore, the decrease in GAPD mRNA modulated by CREB was observed 24 h after treatment with metformin. Although the activation of AMPK resulted in the inhibition of gluconeogenesis through the suppression of PEPCK expression, the decrease in the expression of GAPD caused by metformin decreased the expression of PEPCK and glucose-6-phosphatase, concurrent with reduced expression of the gene encoding peroxisome proliferator-activated receptor γ coactivator-α. Thus, inhibition of NF-κB activity seems to play important roles in the regulation of gluconeogenesis. We found the binding motif for NF-κB in the promoter region of the GAPD gene.

Fig. 4. Transcriptional Regulation of the GAPD Gene Evoked by Metformin

(A) The binding motifs of cAMP response element-binding protein (CREB) and nuclear factor-kappa B (NF-κB) in the promoter region of GAPD HepG2 cells were incubated with 10 mM metformin for the period indicated. Cells not treated with metformin were used as a control. Total RNA was isolated and subjected to a real-time RT-PCR analysis using specific primers for (B) CREB and (C) NF-κB. The results were normalized with the β2-microglobulin mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean±S.D. from three experiments. * p<0.05, ** p<0.01 vs. control. (D) Effect of metformin on the promoter activity of GAPD in HepG2 cells. HepG2 cells were co-transfected with the reporter vector containing the promoter region of the GAPD gene, GAPD genes having a mutated CREB and NF-κB binding motif, and a reference plasmid, phRL-TK. Transfected cells were maintained in regular culture medium for 24 h and further cultured in the presence or absence of 10 mM metformin for 24 h. Luciferase activities were measured with a Dual-Luciferase Reporter Assay System. Firefly luciferase activity was normalized with Renilla luciferase activity and is expressed relative to the control treated without metformin. Data represent the mean±S.D. from three experiments. * p<0.05 vs. 1795pGL3 control, ** p<0.01 vs. 1795pGL3 with an altered NF-κB-binding sequence, † p<0.05 vs. 1795pGL3 control.
remained suppressed on incubation with metformin (Fig. 4D). Metformin promoted the transcription of NF-κB mRNA after 24 h of incubation (Fig. 3C). These results suggest that NF-κB was almost unaffected by the down-regulation of GAPD expression by metformin.

Biguanides are known to have a severe adverse effect, lactic acidosis, caused by a high level of lactate in the blood. Metformin was reported to raise the intracellular ratio of NADH to NAD⁺.9,31) This increase was proposed to result in less lactate being converted to pyruvate by lactate dehydrogenase causing the lactate to accumulate.32) Since pyruvate is produced not only by glycolysis but also by gluconeogenesis via an intermediate in the citric acid cycle, the reaction equilibrium catalyzed by lactate dehydrogenase appears to shift towards lactate production. The decrease in GAPD caused by metformin seems to induce reduction in both glycolysis and gluconeogenesis and partially suppress the conversion between NADH and NAD⁺ because GAPD catalyzes the reversible reaction between glyceraldehyde 3-phosphate and 1,3-bisphosphoglycerate. The depression of glycometabolism by metformin implies an increase in the accumulation of lactate. Although the hypoglycemic effect of metformin is now believed to be modulated via AMPK,24) the activation of AMPK by metformin seems to cause the onset of lactic acidosis. Attention needs to be paid to the abnormal activation of this kinase in order to prevent the side effect of biguanides.

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REFERENCES