

Buformin Suppresses the Expression of Glyceraldehyde 3-Phosphate Dehydrogenase

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The biguanides metformin and buformin, which are clinically used for diabetes mellitus, are known to improve resistance to insulin in patients. Biguanides were reported to cause lactic acidosis as a side effect. Since the mechanism of the side effect still remains obscure, we have examined genes whose expression changes by treating HepG2 cells with buformin in order to elucidate the mechanisms of the side effect. A subtraction cDNA library was constructed by the method of suppressive subtractive hybridization and the screening of the library was performed with cDNA probes prepared from HepG2 cells treated with or without buformin for 12 h. The expression of the gene and the protein obtained by the screening was monitored by real-time RT-PCR with specific primers and Western blotting with specific antibody. The amounts of ATP and NAD⁺ were determined with luciferase and alcohol dehydrogenase, respectively. We found that expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPD) gene was suppressed by treating HepG2 cells with 0.25 mM buformin for 12 h as a result of the library screening. The decrease in the expression depended on the treatment period. The amount of GAPD protein also decreased simultaneously with the suppression of the gene expression by the treatment with buformin. The amount of ATP and NAD⁺ in the HepG2 cells treated with buformin decreased to 10 and 20% of the control, respectively. These observations imply that the biguanide causes deactivation of the glycolytic pathway and subsequently the accumulation of pyruvate and NADH and a decrease in NAD⁺. Therefore, the reaction equilibrium catalyzed by lactate dehydrogenase leans towards lactate production and this may result in lactic acidosis.

Key words biguanide; metformin; buformin; glyceraldehyde 3-phosphate dehydrogenase; side effect

Three biguanides, phenformin, buformin, and metformin, were first used clinically for diabetes mellitus in the 1950s. Since phenformin was found to have a lethal side effect, lactic acidosis, in the late of 1970s,¹⁾ its usage decreased rapidly in many countries and was finally withdrawn. Then use of other biguanides declined gradually because of their own adverse effects. Lactic acidosis, hypoglycemia, and digestive organ dysfunction have been found to be adverse effects of biguanides. Lactic acidosis caused by biguanides results in lethal metabolic acidosis,²⁾ so their use was stopped. Cohen and Woods³⁾ proposed a classification for lactic acidosis that consisted of types A, B₁, B₂, B₃, and B₄, and acidosis caused by biguanides belongs to type B₂ according to this classification. Type B is lactic acidosis occurring when clinical evidence of poor tissue perfusion or oxygenation is absent and type B₂ is due to drugs and toxins.

Metformin was recently reported to decrease the risk of diabetes-related endpoints in overweight diabetic patients⁴⁾ and, therefore, has begun to be widely used again. The following mechanisms of action of biguanides have been proposed⁵⁾: (i) suppression of glucose release from the liver, (ii) increase of glucose intake in peripheral tissues, (iii) decrease of glucose uptake in an alimentary tract, and (iv) suppression of β -oxidation. Metformin was found to improve resistance to insulin in type II diabetes. Zhou *et al.*⁶⁾ postulated that the suppression of SREBP-1 expression through the activation of AMP-activating protein kinase by metformin eliminated the insulin resistance.

The following possible mechanism for the side effect of biguanides has been proposed.^{7,8)} The biguanide binds to the mitochondrial membrane and causes the disruption of oxidative phosphorylation in the mitochondria. This results in an increase in the NADH level, a decrease in pyruvate dehydrogenase activity, and deactivation of the TCA cycle. Further-

more, gluconeogenesis is suppressed and pyruvic acid accumulates in the liver. This leads to an increase in production of lactic acid. However, the molecular mechanisms of the side effects of biguanides are still unclear.

When we researched which gene expressions underwent a change in order to clarify the mechanism of biguanides induced lactic acidosis, we found that the gene expression of glyceraldehyde 3-phosphate dehydrogenase (GAPD) in HepG2 cells decreases significantly by buformin treatment. Since GAPD is involved in the glycolytic pathway, we believe the decrease in GAPD may have caused the lactic acidosis. In this study, we demonstrate that expression of the gene for GAPD in HepG2 cells was suppressed by buformin treatment in a time-dependent manner. We also examined the effect of the biguanide on the protein expression of GAPD and determined the levels of NAD⁺ and ATP in HepG2 cells treated with the biguanide to investigate the mechanism of the lactic acidosis side effect.

MATERIALS AND METHODS

Materials Buformin hydrochloride and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-1), and metformin hydrochloride were purchased from Wako Pure Chemicals (Osaka, Japan) and ICN Biomedicals Inc. (Irvine, CA, U.S.A.), respectively. TRIzol and SuperScript III reverse transcriptase, and Oligotex-dT30 (Super) were obtained from Invitrogen Corp. (Carlsbad, CA, U.S.A.) and Takara Bio Inc. (Osaka, Japan), respectively. DNA manipulating enzymes were purchased from Promega Corp. (Madison, WI, U.S.A.). All other chemicals and reagents were of analytical grade.

HepG2 cells were maintained in Dulbecco's modified Eagle MEM supplemented with 10% fetal bovine serum,

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100 $\mu\text{g/ml}$ streptomycin, and 100 units/ml penicillin.

Construction of Subtraction cDNA Library and Library Screening A subtraction cDNA library was constructed by the method of Diatchenko *et al.*⁹⁾ Total RNAs were extracted with TRIzol reagent from HepG2 cells treated with or without 0.25 mM buformin for 24 h and poly(A)⁺ RNAs were prepared using Oligotex-dT30 (Super). First and second strand cDNA was synthesized from poly(A)⁺ RNAs with the Universal RiboClone cDNA Synthesis System (Promega Japan, Tokyo, Japan) according to the manufacturer's instructions. Subtractive suppressive hybridization was performed with a PCR-Select cDNA Subtraction Kit (BD Biosciences Clontech, Tokyo, Japan) according to the manufacturer's instructions. The library was maintained as a plasmid library in *E. coli*.

In order to select the clones whose expression levels changed by treating HepG2 with buformin, the clone in the library was subjected to dot hybridization using labeled probes prepared from the mRNAs of none and 0.25 mM buformin treated HepG2 with a Random Primer Biotin Labeling and Detection Kit (New England Biolabs, Beverly, MA, U.S.A.). The clones were detected with a streptavidin-alkaline phosphatase conjugate in the same kit and CDP-Star detection reagent (Amersham Biosciences, Tokyo, Japan).

DNA Sequencing DNA sequencing was performed using a CEQ 2000 Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter) according to the manufacturer's instruction and with a DNA sequencer model CEQ2000XL. The DNA sequences of clones obtained were searched in the BLAST database and genes were identified from the sequences.

Real Time Reverse Transcription-Polymerase Chain Reaction The template cDNA was prepared from 5 μg of total RNA extracted from HepG2 cells by SuperScript III. Real time RT-PCR was performed with an iQ SYBR Green Supermix and an iCycler iQ System (BIO-RAD Laboratories, Hercules, CA, U.S.A.). The specific primers used for amplification of GAPD, NADH-ubiquinone oxidoreductase, and β -actin were 5'-CAATGACCCCTTCATTGACCC-3' and 5'-GACAAGCTTCCCGTTCTCAG-3', 5'-ATCCAGAGTGGTGAACAGCC-3' and 5'-CTCTGCCCGAGTATAACCGA-3', and 5'-CAAGTACTCCGTGTGGATCG-3' and 5'-AGTCCGCCTAGAAGCATTG-3', respectively.

Western Blotting Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out with 12% polyacrylamide gel according to the method of Laemmli.¹⁰⁾ Western blotting was performed with goat anti-GAPD antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) and anti-goat IgG peroxidase conjugate (Sigma-Aldrich, Saint Louis, MI, U.S.A.) by the method of Towbin *et al.*¹¹⁾ The peroxidase activity was detected with an ECL Western Blotting Detection System according to the manufacturer's instructions (Amersham Biosciences).

Assays The amount of NAD⁺ in HepG2 cells was measured by the method of Klingenberg.¹²⁾ HepG2 cells were sonicated in cold phosphate buffered saline and centrifuged at 105000 $\times g$ for 1 h at 4 $^{\circ}\text{C}$. The supernatant was collected and used for measuring NAD⁺. ATP was extracted from HepG2 cells with a Kinshiro ATP Extraction System LL-100-2 (Toyo Ink Co. Ltd., Tokyo, Japan) and measured with a Kinshiro ATP Luminescence Kit LL-100-1 (Toyo Ink Co. Ltd.)

according to the manufacturer's instructions. Protein was determined by the method of Bradford¹³⁾ using bovine serum albumin as a standard. Proliferation of HepG2 cells was determined with WST-1 according to the method reported by Ishiyama *et al.*¹⁴⁾

RESULT

Effect of Buformin on HepG2 Cell Proliferation To determine the optimum condition for treating HepG2 cells with buformin, the concentration and exposure period of the biguanide were investigated. Since proliferation of the cells was not affected by buformin up to the concentration of 0.25 mM (Fig. 1A), the cells were incubated with this concentration of buformin for various periods. As shown in Fig. 1B, the viability of the cells was almost unaffected by treating with 0.25 mM buformin for 24 h and longer incubation with the biguanide caused lower viability of the cells. Therefore, we decided to treat with 0.25 mM buformin for 12 h, a condition that did not affect the viability of the cells.

Screening of the Subtraction Library Ninety-six clones in the library were screened with the cDNA probes prepared from HepG2 cells treated for 12 h with or without 0.25 mM buformin by the dot hybridization method. The expression of two clones was found to decrease by treatment with 0.25 mM buformin (Fig. 2). The DNA sequences of these clones were analyzed and the clones were identified as

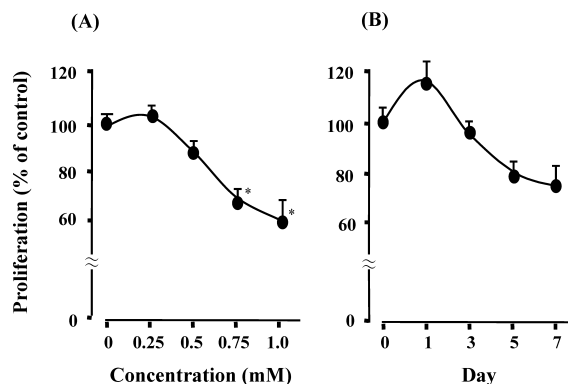


Fig. 1. Proliferation of HepG2 Cells Treated with Buformin

HepG2 cells were cultivated (A) with various concentrations of buformin for 24 h and (B) in the presence of 0.25 mM buformin for the period indicated. Cell proliferation was determined with WST-1. Data represent the mean \pm S.E. of three experiments. * $p < 0.05$ vs. control.

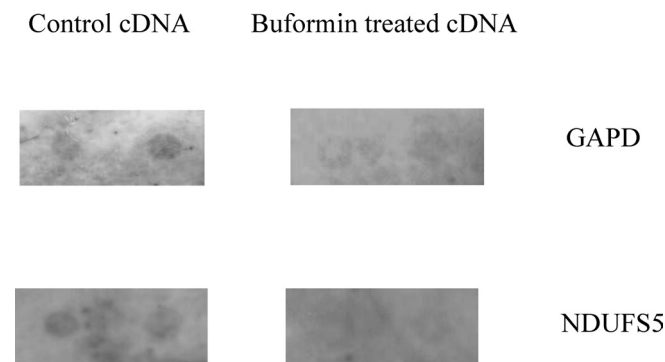


Fig. 2. Dot Blot Analysis of Isolated cDNA Clones

Dot blot analysis was performed with the cDNA probes prepared from HepG2 cells treated with or without buformin for 12 h.

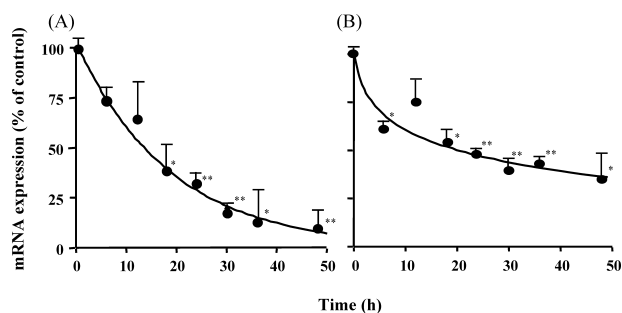


Fig. 3. Effects of Buformin on (A) GAPD and (B) NDUFS5 mRNA Levels in HepG2 Cells

HepG2 cells were incubated with 0.25 mM buformin for the period indicated. The levels of mRNA expression were analyzed with the real-time RT-PCR method and the result was normalized with the β -actin mRNA level. The mRNA level of the control was taken as 100%. Data represent the mean \pm S.E. of three experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

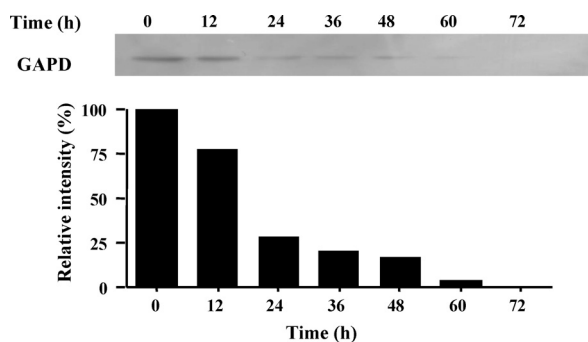


Fig. 4. Western Blot Analysis of GAPD in HepG2 Cells Treated with Buformin

(A) HepG2 cells were cultivated in the presence of 0.25 mM buformin for the period indicated. Cell extracts were separated by electrophoresis with 12% SDS-polyacrylamide gel under reducing conditions. After proteins in the gel were electroblotted on a nitrocellulose membrane, GAPD was probed with the antibody against GAPD and visualized with the second antibody-peroxidase conjugate and ECL system. (B) Band intensities were quantified using the NIH Image program.

the genes of GAPD (entry No. 2597) and NADH dehydrogenase (ubiquinone) Fe-S protein 5 (NDUFS5, entry No. 4725) in the GenomeNet database.

Effects of Buformin on the Gene Expression of GAPD and NDUFS5 The expression of the genes for GAPD and NDUFS5 in HepG2 cells treated with 0.25 mM buformin for various incubation periods was analyzed by the real time RT-PCR method. As shown in Fig. 3, the gene expression of GAPD and NDUFS5 in HepG2 cells was suppressed by the biguanide. The expression amounts of GAPD and NDUFS5 genes decreased to about 10 and 50%, respectively, compared with the control after exposure for 50 h.

Effect of Buformin on Protein Expression Since the mRNA expression of GAPD in HepG2 cells decreased depending on the period of exposure to 0.25 mM buformin, the protein amount of GAPD was determined by Western blotting with anti-GAPD antibody. As shown in Fig. 4, GAPD protein decreased over time by treatment with the biguanide. The decrease in GAPD mRNA and protein seemed to simultaneously occur.

Effect of Buformin on the Amounts of Cellular NAD^+ and ATP in HepG2 Cells The amounts of NAD^+ and ATP in the cytosol fraction of HepG2 cells were measured when the cells were treated with 0.25 mM buformin for various periods (Fig. 5). The NAD^+ amount was observed to decrease

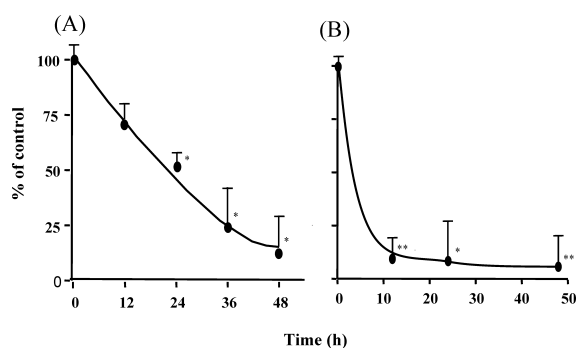


Fig. 5. Effects of Buformin on (A) NAD^+ and (B) ATP Levels in HepG2 Cells

HepG2 cells were cultivated in the presence of 0.25 mM buformin for the period indicated. The amount of NAD^+ in the cytosol fraction of the cells was measured with alcohol dehydrogenase and recorded as a change in the fluorescence intensity at 340 and 460 nm for the excitation and emission wavelengths, respectively. The amount of ATP in the cell-free extract was measured utilizing the luciferin-luciferase reaction. Data represent the mean \pm S.E. of three experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

gradually with time by treatment with buformin, while the ATP amount was shown to decrease rapidly within 12 h.

DISCUSSION

Biguanides are well known to cause lactic acidosis as a side effect, although they are frequently used for improving the resistance to insulin in the clinical therapy of diabetes mellitus.¹⁵⁾ To clarify the mechanism of this side effect, we have attempted to find genes whose expression changed by treatment with buformin through the screening of the subtraction cDNA library constructed by the suppression subtractive hybridization method.⁹⁾ We found that buformin suppressed expression of the GAPD gene in HepG2 cells and the suppression was dependent on the period of exposure. It was observed that not only the GAPD gene but also the GAPD protein decreased with time by treatment with 0.25 mM buformin. The amounts of NAD^+ and ATP in HepG2 cells were also found to decrease with time by this treatment.

The gene expressions of glucokinase, glucose 6-phosphatase, L-pyruvate kinase, and phosphoenolpyruvate carboxykinase were reported to change by treatment with metformin.¹⁶⁾ Glucokinase (GK) and L-pyruvate kinase (L-PK), and glucose 6-phosphatase (G6P) and phosphoenolpyruvate kinase (PEPK) are involved in the glycolytic and gluconeogenic pathways, respectively, and all of these enzymes catalyze irreversible reactions which were rate-limiting steps in these pathways. The gene expressions of GK and L-PK, and G6P and PEPK were demonstrated to increase and decrease, respectively. GAPD is well known to be a component enzyme in the glycolysis pathway and catalyzes the reversible reaction between glyceraldehyde 3-phosphate and 1,3-bisphosphoglyceric acid. Although GAPD is involved in both glycolytic and gluconeogenic reactions, the gene and protein expressions of GAPD were suppressed by biguanides. The decrease in GAPD activity is thought to cause a lowering of both glycolysis and gluconeogenesis reactions and suppression of the whole pathway.

Benfluorex (1-(3-trifluoromethylphenyl)-2-[N-(benzyloxyethyl)amino]propane) was reported to exhibit hypoglycemic and hypolipidemic effects in model animals of diabetes mellitus¹⁶⁾ and to reduce $\text{HbA}_{1\text{C}}$ and fasting plasma glucose

levels in hyperglycemic patients.¹⁷⁾ A proposed action mechanism for the hypoglycemic effect by benfluorex was that this drug lowered gluconeogenesis by suppressing GAPD and pyruvate carboxylase activities and stimulating the availability of glucose. The expression of genes encoding enzymes of G6P and phosphoenolpyruvate carboxykinase was decreased by benfluorex, whereas mRNAs encoding GK and PK were increased.¹⁷⁾ These observations about the expression of these genes seemed to be very similar to those reported with metformin¹⁶⁾ although some effects of benfluorex and metformin on glycolytic enzymes appeared to be different to each other and it is unclear whether benfluorex causes the suppression of the gene expression of GAPD.

Several researchers^{19–21)} have reported that metformin suppresses the respiratory chain complex I in mitochondria. Since the gene expression of NDUFS5 involved in complex I was observed to decrease with time in our study, the inhibitory effect of metformin on complex I appears to be based on the suppression of this gene expression. This observation implies that the correct cDNA clones of GAPD and NDUFS5 are selected as genes changing their expressions through the processes of the construction and screening of the subtraction library by the suppression subtractive hybridization method in this study. Since the amount of ATP produced in mitochondria was reported to decrease by metformin treatment,^{22–26)} a part of the reason for this decrease in ATP amount seems to be based on the loss of complex I activity and suppression of the gene expression in complex I by the biguanide. Buformin was also demonstrated to cause a rapid decrease in ATP within 12 h in the present study, even though the expression of NDUFS5 mRNA was observed to decrease gradually. This suggests the possibility that buformin directly or indirectly suppresses the function of respiratory chain complex I in mitochondria. Furthermore, failure to supply electrons to the respiratory chain may result from suppression of the glycolytic pathway based on the decreases in the expressions of GAPD, pyruvate kinase, and glucokinase by biguanides. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea, an inhibitor of the respiratory chain, has been reported to suppress gluconeogenesis in the liver through the loss of pyruvate carboxylase and pyruvate kinase activities.^{27,28)} Suppression of the respiratory chain appeared to be correlated to suppression of the glycolytic pathway.

Metformin was previously demonstrated to raise the intracellular ratio of NADH to NAD⁺.¹⁶⁾ Our observation that the amount of NAD⁺ in the cytosol fraction decreased by buformin treatment is thought to result from suppression of the glycolytic pathway and was dependent on the loss of GAPD and the above glycolytic enzyme activities by biguanides. The increase in the ratio of NADH to NAD⁺ seems to be based on the decrease in NAD⁺ in the cytosol fraction. The ratio of NADH to NAD⁺ in the cytosol and mitochondrial fractions has been proposed to correlate to that of lactate to pyruvate and β -hydroxybutyrate to acetoacetate, respectively.²¹⁾ The decrease in NAD⁺ and the accumulation of pyruvate may lead the reaction equilibrium catalyzed by lactate dehydrogenase to shift towards lactate production.

In this study, we showed that buformin suppressed the gene expression of GAPD and NDUFS5. Since the results

were obtained by the exposure of HepG2 cells to a high dose of buformin for a short period, 12 h, they appear to reflect acute toxicity against buformin. However, this observation implies the possibility, at least, that the biguanide causes deactivation of the glycolytic pathway and complex I in the respiratory chain, resulting in the accumulation of pyruvate and NADH and a decrease in NAD⁺. These phenomena are thought to induce the side effect of biguanides, lactic acidosis, although further experiments on the chronic exposure of HepG2 cells to a low dose of buformin for a long period are necessary. Thus, we propose that one of the mechanisms of the side effect is based on the suppression of GAPD expression by biguanides. Further study is needed to determine how biguanides suppress the gene expression of GAPD.

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