# Metformin modulates insulin post-receptor signaling transduction in chronically insulin-treated Hep G2 cells

YUAN Li[1], Reinhard ZIEGLER, Andreas HAMANN[2]

[2]*Department of Endocrinology and Metabolism in Hospital of University Heidelberg, Heidelberg 69115, Germany*

## ABSTRACT

**AIM:** To study the effect of chronic insulin treatment on insulin post-receptor signaling transduction and whether the effects of metformin are transmitted throughout the cascade of insulin signaling intermediates in a human hepatoma cell line (Hep G2). **METHODS:** Hep G2 cells were incubated in serum free media containing either insulin 100 nmol/L or insulin 100 nmol/L plus different concentrations (0.01-10 mmol/L) of metformin for 16 h and then were stimulated with insulin 100 nmol/L for 1 min. **RESULTS:** Chronic treatment of insulin 100 nmol/L induced a significant reduction in the phosphorylation and protein expression of IRß, IRS1 and IRS2, which therefore resulted in a downregulation of association of PI3K with IRS. Therapeutic concentrations (0.01-0.1 mmol/L) of metformin prevented the changes induced by chronic insulin treatment in these post-receptor components of insulin signaling pathway. Tyrosine phosphorylation of IRβ, IRS1, and IRS2 was increased by 2.7 fold ($P<0.01$), 6.8 fold ($P<0.01$), and 2.3 fold ($P<0.01$) of chronically insulin-treated cells alone, respectively, after metformin 0.1 mmol/L was added. The association of p85 with IRS1 and IRS2 was also increased from 34 % to 86 % ($P<0.01$) and from 30 % to 92 % ($P<0.01$), respectively. In contrast, metformin in pharmacological concentration (1-10 mmol/L) further inhibited tyrosine phosphorylation of IRβ, IRS1, IRS2 and the interaction of PI3K with IRS. The association of IRS1 with p85 was further decreased by 58 % ($P>0.05$) and of IRS2 by 30 % ($P<0.05$). **CONCLUSION:** Chronic insulin exposure of Hep G2 cells induces the downregulation of insulin signal transduction via PI3K pathway. The effect of metformin on insulin signaling transduction represent a primary mechanism of metformin action in insulin resistant state.

## INTRODUCTION

Metformin is an antihyperglycemic drug that treats insulin resistance and it has become a first-line choice of type 2 diabetes therapy. In recent years, research into the pharmacodynamic properties of metformin and their clinical implications has resulted in a renewal of interesting[1]. The liver is not only a key tissue for glucose metabolism, but also a major site of metformin action. Recently, the importance of hepatic insulin resistance in glucose homeostasis has been emphasized[2]. In hepatocytes, insulin resistance can result from impaired signaling downstream of the insulin receptor[3]. Antihyperglycemic effect of metformin is mainly a consequence of reduced hepatic glucose output.

[1] Correspondence to Dr YUAN Li, now in *Department of Endocrinology in Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China.*
Phn 86-27-8572-6136. Fax 86-27-8577-6343.
E-mail yuanli18cn@yahoo.com.cn

However, the molecular basis of metformin effect on hepatocytes is largely unknown, involving the possible effects of metformin on insulin signaling tranduction, for instance, metformin could directly or indirectly influence protein-protein interactions within the signaling cascade.

Chronic hyperinsulinsm can induce insulin resistance[4]. Therefore, in the present study, we set up an insulin resistant cell model, with the Hep G2 cells chronic exposure to high concentration of insulin. The aim is: (1) to examine the changes in insulin signaling transduction after chronic insulin treatment. (2) to determine whether the antihyperglycemic action of metformin in hepatocytes is associated with effects on signaling protein expression and phosphorylation.

## MATERIALS AND METHODS

**Chemicals** RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco BRL (Karlsruhe, Germany). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad (Hercules, CA). Rabbit polyclonal anti-insulin receptor β-subunit (IRβ), anti-insulin receptor substrate 1 (IRS1), anti-insulin receptor substrate 2 (IRS2), and anti-p85 subunit of phosphatidylinositol 3-kinase (PI3K) antibodies used for Western blotting were purchased from UBI (Lake Placid, NY). Anti-biotin and mouse monoclonal anti-phosphotyrosine (PY) antibody were from New England Biolabs (Frankfurt am Main, Germany). Protein A and protein G sepharose and BCA-assay reagents were purchased from Pierce (Hamburg, Germany). HRPO-conjugated anti-mouse and anti-rabbit antibodies, reagents for ECL were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Human insulin (Actrapid) was obtained from Novo Nordisk (Deisenhofen, Germany). Metformin, aprotinin, pepstatin, leupeptin, and other reagents were from Sigma (Steinheim, Germany).

**Cell culture** Hep G2 human hepatoma cells were grown to confluence in 10-cm plastic culture dishes containing 10 mL RPMI-1640 medium supplemented with 10 % fetal bovine serum, HEPES 25 mmol/L (pH 7.4), 1 % penicillin /streptomycin and glutamine 25 mmol/L. The cells were incubated at 37 °C in a humidified atmosphere of 5 % $CO_2$ and 95 % air.

Confluent Hep G2 cells were incubated in serum free media including either insulin 100 nmol/L or insulin 100 nmol/L plus metformin 0.01-10 mmol/L for 16 h and then were stimulated with insulin 100 nmol/L for 1 min, as specified in the results section. Cells under basal conditions were used as controls.

**Immunoprecipitation and Western blotting** Phosphorylated proteins of IRβ, IRS1, IRS2 and interaction of PI3K with IRS were determined by immunoprecipitation assay. protein lysates 1000 μg were subjected to immunoprecipitstion using corresponding specific antibodies (anti-IRβ, anti-IRS1, anti-IRS2) and incubation at 4 °C for 2 h. Protein-antibody complexes were conjugated with protein A and Protein G for another 1 h. Precipitates were take up in 30 μL sample buffer containing Tris 62.5 mmol/L, dithiothreitol (DTT) 100 mmol/L, 10 % glycerol, 2 % SDS, 0.01 % bromphenolblue and denatured at 95 °C for 10 min. Protein samples were subjected to 7.5 % SDS-PAGE. After SDS-PAGE, electrotransfer of protein from the gel to nitrocellulose membranes was performed by Western blotting. Subsequently, nitrocellulose filters were incubates at 4 °C overnight with one of the following antibodies (1st antibody) at a concentration of 1 mg/L: anti-PY, anti-IRβ, anti-IRS1, anti-IRS2 or anti-p85, each of those suspended in 2.5 % non-fat dried milk. Blots were then incubated with 1:3000 HRPO conjugate (2nd antibody) at room temperature (28 °C) for 1 h. Immunoreactive bands were determined with the ECL detected reagents.

**Immunoblotting** Relative protein levels of IRβ, IRS-1, IRS-2, and p85 were determined by Western-immunoblotting in total cell lysates. Similar size aliquots of sample (150 μg) were processed for 7.5 % SDS-PAGE and proteins were separated by electrophoresis. Immunoblotting was performed as described above.

**Statistical analysis** Relative amounts of immunoreactive proteins were quantitated by Image Quant scanning densitometry. Experimental results were expressed as mean±SD. Statistical analysis was performed by Student's *t*-test. Statistical significance was assessed at *P*<0.05.

## RESULTS

**Effect of metformin on protein levels and phosphorylation of signaling proteins following chronic insulin treatment** In the basal state, no or little tyrosine phosphorylation of IRβ, IRS1, and IRS2 was detectable. Acute stimulation with insulin for 1 min resulted in an extensive phosphorylation on tyrosine

residues of signaling proteins. After cells were treated with insulin 100 nmol/L for 16 h, insulin-induced autophosphorylations of IRβ, IRS1, and IRS2 were significantly decreased. By comparison with control cells, tyrosine phosphorylation of IRβ was decreased to 22 % (*P*<0.01) of control level. For IRS1 an even more significant reduction in insulin-induced phosphorylation was observed, as anti-PY detectable protein was reduced to 15 % (*P*<0.01). Similar to the changes in IRS1, tyrosine phosphorylation of IRS2 was decreased to 22 % (*P*<0.01) of control levels (Fig 1).

Therapeutic concentrations of metformin 0.01 mmol/L and 0.1 mmol/L reversed the reduction of tyrosine phosphorylation of insulin signaling protein induced by chronic insulin treatment. Tyrosine phosphorylation of IRβ was increased by 2.3 fold (*P*<0.01) and 2.7 fold (*P*<0.01) of chronically insulin treated cells alone. Tyrosine phosphorylation of IRS1 was increased by 6.4 fold (*P*<0.01) and 6.8 fold (*P*<0.01), and phosphorylation of IRS2 was increased 2.8 fold (*P*<0.01) and 2.3 fold (*P*<0.05) after cells were preincubated with insulin 100 nmol/L for 16 h in the simultaneous presence of metformin 0.01 mmol/L and 0.1 mmol/L, respectively.

When the concentration of metformin was further increased to pharmacological doses (1-10 mmol/L), the effect of metformin on insulin signal transduction became inhibitory. Tyrosine phosphorylation of IRβ was further decreased to 3 % (*P*<0.01) of control levels. Quite similar observations were made for IRS1 and IRS2. When metformin 10 mmol/L was added, IRS1 tyrosine phosphorylation was further decreased to only 4 % (*P*<0.01) and IRS2 phosphorylation to 11 % (*P*<0.01) of control levels (Fig 1).

There were also significant changes in protein expression levels of IRβ, IRS1, and IRS2 following insulin 100 nmol/L chronic treatment. Protein level of IRβ was decreased to 42 % (*P*<0.01) of control levels, IRS1 was decreased to 63 % (*P*<0.01) and IRS2 was decreased to 47 % (*P*<0.05) compared to controls. However, this chronic hyperinsulinism induced reduction of protein levels was reversed after cells were preincubated with different concentrations of metformin. Level of IRβ was increased by 1.1 fold (*P*<0.01) in the presence of metformin 0.01 mmol/L and 1.6 fold (*P*<0.01) in the presence of metformin 10 mmol/L. Levels of IRS1 and IRS2 did not significantly changed following physiological concentration of metformin treatment and increased by 43 % (*P*<0.01) and 112 %
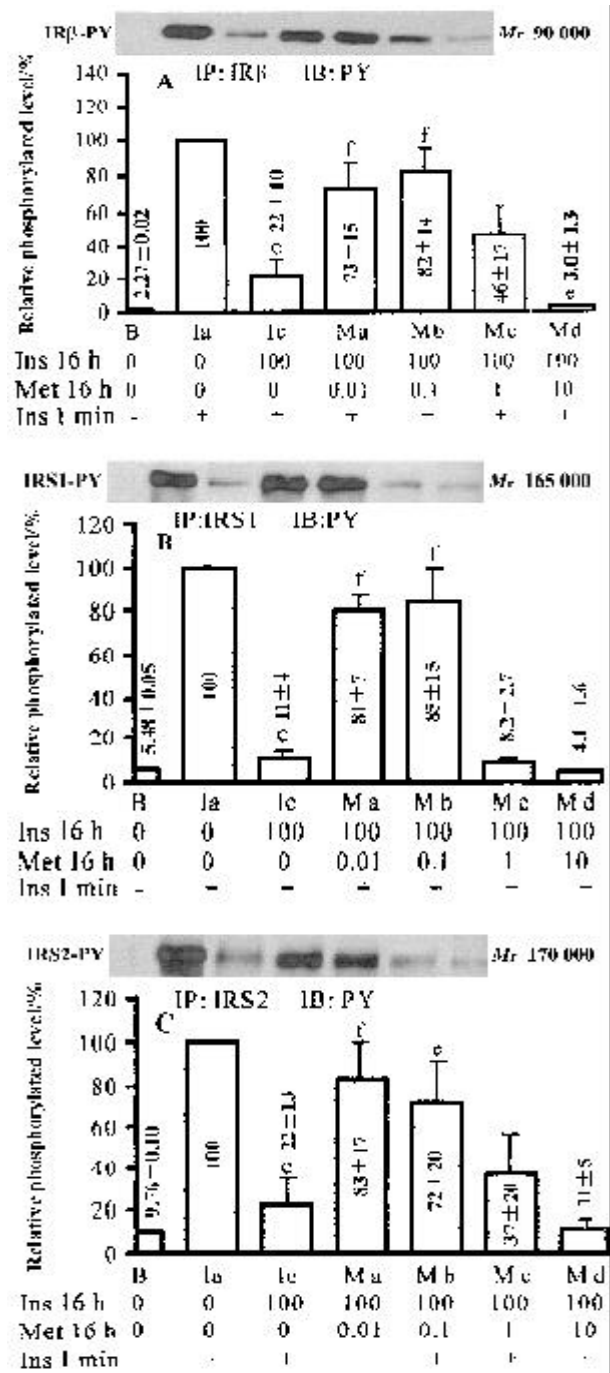


**Fig 1. Effects of metformin on tyrosine phosphorylation of IRb (A), IRS1 (B), and IRS2 (C) after chronic insulin treatment. Hep G2 cells were treated either without insulin (Ia), or with insulin 100 nmol/L (Ic) or with insulin 100 nmol/L and metformin 0.01, 0.1, 1, 10 mmol/L (Ma, Mb, Mc, Md) for 16 h and then were stimulated with insulin 100 nmol/L for 1 min, and cells cultured without these drugs were used to demonstrate the basal level (B). A typical blot for scanning densitometry by Image Quant (Molecular Dynamics) is shown above. *n*=4. Mean±SD. They are expressed as relative to Ia values, which were set at 100 %. ᶜ*P*<0.01 *vs* Ia. ᵉ*P*<0.05, ᶠ*P*<0.01 *vs* Ic. IP, immunoprecipitation; IB, immunoblot; Met, metformin; Ins, insulin.**

($P$<0.01) by exposure to metformin 10 mmol/L (Tab 1).

**Tab 1.  Effects of metformin on protein levels of insulin signaling molecules after chronic insulin treatment.  $n$=4.  Mean±SD.  $^c$$P$<0.01 $vs$ control. $^e$$P$<0.05, $^f$$P$<0.01 $vs$ Ic.**

|         | IRβ | IRS1 | IRS2 | p85 |
|---------|-----|------|------|-----|
| Control | 0.167±0.012 | 0.35±0.03 | 0.241±0.010 | 0.38±0.03 |
| Ia | 0.18±0.06 | 0.34±0.06 | 0.238±0.017 | 0.39±0.05 |
| Ic | 0.07±0.03$^c$ | 0.217±0.021$^c$ | 0.107±0.017$^c$ | 0.36±0.05 |
| Ma | 0.15±0.05$^f$ | 0.27±0.05$^c$ | 0.173±0.023 | 0.37±0.04 |
| Mb | 0.13±0.05$^e$ | 0.26±0.04$^c$ | 0.152±0.017 | 0.35±0.06 |
| Mc | 0.21±0.07$^f$ | 0.29±0.05$^e$ | 0.134±0.021 | 0.37±0.06 |
| Md | 0.19±0.04$^f$ | 0.31±0.03$^f$ | 0.24±0.05$^f$ | 0.38±0.05 |

HepG2 cells were treated either without addition of insulin (Ia), or with insulin 100 nmol/L (Ic) or with insulin 100 nmol/L plus metformin 0.01, 0.1, 1, 10 mmol/L (Ma, Mb, Mc, Md) for 16 h and then stimulated with insulin 100 nmol/L for 1 min, cells cultured without these drugs were used to demonstrate the basal control.  The data are OD values of Scanning densitometry by Image Quant (Molecular Dynamics).

**Effect of metformin on the interaction of IRS1 and IRS2 with PI3K following chronic insulin treatment**  To determine the effect of reduced IRS1 and IRS2 expression and phosphorylation after chronic insulin treatment on their interaction with the p85 subunit of PI3K, blots with immunopresipitates for IRS1 and IRS2 were immunoblotted with anti-p85 antibodies.  As described above, cells pretreated in the absence of insulin responded to an acute maximal insulin stimulation with an increase in IRS-associated p85 of more than 10-fold.  In contrast, after the cells were exposed to insulin 100 nmol/L for 16 h, immunodetectable p85 was significantly decreased, which was consistent with changes in phosphorylation of IRS1 and IRS2.  However, in the presence of metformin 0.01 mmol/L, these effects of chronic insulin treatment on the association of IRS with p85 were reserved.  Immunode-tectable p85 was increased from 34 % to 86 % ($P$<0.01) of control levels in anti-IRS1 immunoprecipitates and from 30 % to 92 % ($P$<0.01) in anti-IRS2 immunoprecipitates.  When the dose of metformin was further increased to the pharmacological concentration 10 mmol/L, association of IRS1 with p85 was further decreased by 58 % ($P$>0.05) and of IRS2 by 30 % ($P$<0.05) of chronic treatment with insulin 100 nmol/L alone (Fig 2).
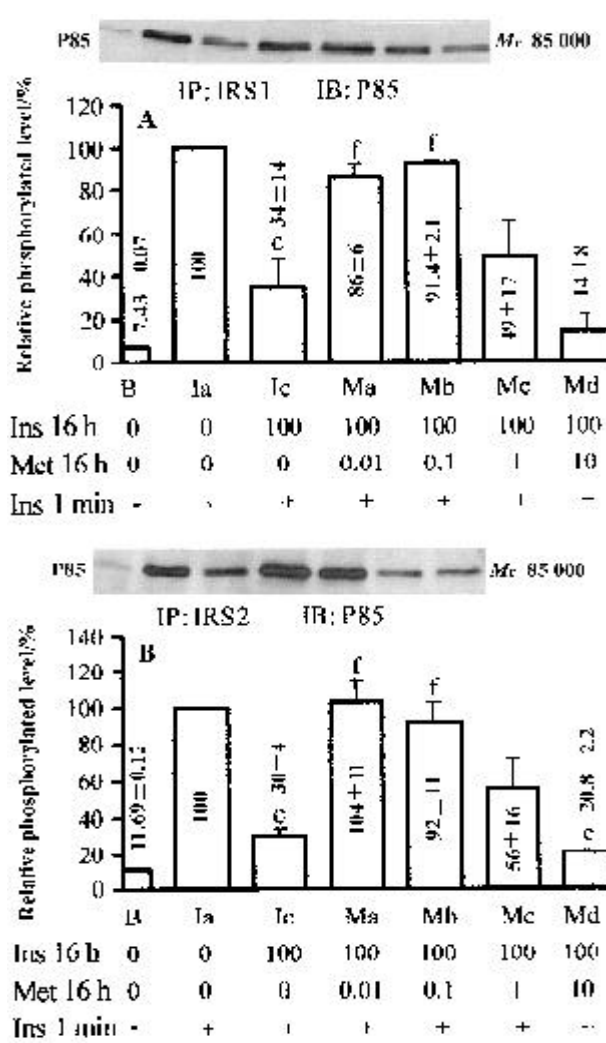


**Fig 2.  Effects of metformin on association of p85 subunit of PI3K with IRS1 (A) and IRS2 (B) after chronic insulin treatment. HepG2 cells were treated either without insulin (Ia), or with insulin 100 nmol/L (Ic) or with insulin 100 nmol/L and metformin 0.01, 0.1, 1, 10 mmol/L (Ma, Mb, Mc, Md) for 16 h and then were stimulated with insulin 100 nmol/L for 1 min, and cells cultured without these drugs were used to demonstrate the basal level (B).  A typical blot for scanning densitometry by Image Quant (Molecular Dynamics) is shown above.  $n$=4.  Mean±SD.  They are expressed as relative to Ia values, which were set at 100 %.  $^c$$P$<0.01 $vs$ Ia. $^e$$P$<0.05, $^f$$P$<0.01 $vs$ Ic. IP, immunoprecipitation; IB, immunoblot; Met, metformin; Ins, insulin.**

The protein expression of p85 was not significantly changed, either therapeutic or pharmacological concentrations of metformin added to the media (Tab 1).

## DISCUSSION

Chronic hyperinsulinism can induce insulin resistance[4].  Cells cultured exposed to the high concentrations of insulin is an established model to induce

insulin resistance *in vitro*[4,5]. Thereby, in the present study, we first set up an insulin resistant model by using chronic treatment of Hep G2 cells with high doses of insulin. Such *in vitro* allows direct assessment of the effect of an additional treatment on the biological response to insulin stimulation. Since metformin exerts its beneficiary antihyperglycemic effect primarily in liver, this should also involve an alteration of the insulin resistant state in this organ. Therefore, it appeared interesting to focus on determining relevant molecules which transmit the effects of metformin within the insulin signaling cascade in a liver cell model system, in which insulin resistance had been induced. The data described here have suggested that impaired insulin signal transduction linked to IRβ, IRS1, IRS2, and PI3K is associated with insulin resistance, which was caused by chronic insulin treatment.

Metformin could interact with insulin at many potential steps, including the increased binding of insulin to its receptor[6], the intensified of insulin receptor tyrosine kinase activity[7-9], the elevated inositol-1,4,5-trisphosphate production, the augmented glycogen synthesis, and the inhibition of PEPCK as a key enzyme of gluconeogenesis[10]. In present experiment, the effect of chronic insulin treatment can be reversed by metformin. It suggests that metformin's action site is most likely located at an early post-receptor level and may directly or indirectly interact with the intracellular insulin signaling cascade.

Up to now, there were only a few reports regarding the effect of metformin on intracellular insulin signaling system and there has been a controversy in different experiments. Metformin was reported to increase insulin signaling transduction in cholesterol-treated Hep G2 cells[11] and to reverse chronic insulin effects on insulin signaling in rat adipocytes[5]. However, metformin treatment had no effect on insulin signaling cascade in human adipoctey[12] and skeletal muscle[13]. These different observations may be due to difference of tissues and cultured conditions. In the present study, therapeutic doses of metformin have reversed the reduction in phosphorylation of IRβ , IRS1, and IRS2 induced by chronic insulin treatment. Namely, through elevation of tyrosine phosphorylation of the insulin receptor metformin can increase further tyrosine phosphorylation of IRS1 and IRS2, as well as the association of IRS1 and IRS2 with PI3K. PI3K has been shown to play a critical role in many insulin-regulated metabolic processes, including stimulation of glucose transport, activation of glycogen synthase, and inhibition of PEPCK as the key enzyme of gluconeogenesis. Since the effect of metformin on insulin signaling processes was observed at concentrations reached in the serum of metformin-treated patients, these data are somewhat suggestive for the actual situation in humans. Thus, the primary mechanism of metformin's action to restore insulin sensitivity in hepatocytes might be related to the increased insulin post-receptor signal transduction linked to tyrosine phosphorylation of IRβ, IRS1 and IRS2 and the activation of PI3K.

In contrast to the effect of metformin at therapeutical concentrations, pharmacological concentrations of metformin inhibit further phosphorylation of signaling proteins and association of IRS with PI3K. These inhibitory effects may reflect the fact that higher metformin concentrations inhibit insulin action[9]. It should be noted that the changes in tyrosine phosphorylation associated with metformin treatment were not parallel to the alterations of protein expression level, which were increased in the presence of pharmacological concentrations of metformin. This suggests that effect of metformin on tyrosine phosphorylation of signaling protein could not be explained by the changes in the level of protein expression. Whether inhibitory effects of pharmacological concentrations of metformin are correlated with either the alteration in insulin receptor, IRS1 and IRS2 serine phosphorylation, or the direct action of metformin on insulin signal transduction or other regulatory event, remains to be investigated in future studies.

In conclusion, the present data suggest that chronic insulin exposure of Hep G2 cells results in down-regulation of insulin signal transduction via PI3K pathway. Therapeutic concentrations of metformin can reverse the effect of chronic hyperinsulinism on expression and activation of insulin signaling molecules, and pharmacological concentrations of metformin inhibit insulin signal transduction. The effect of metformin on insulin signal transduction represents a primary mechanism of metformin action in insulin resistant state.

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