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## A Role for PFKFB3/iPFK2 in Metformin Suppression of Adipocyte Inflammatory Responses

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### Abstract

Metformin improves obesity-associated metabolic dysregulation, but has controversial effects on adipose tissue inflammation.

**Objective**—To examine the direct effect of metformin on adipocyte inflammatory responses, and elucidate the underlying mechanisms.

**Methods**—Adipocytes were differentiated from 3T3-L1 cells and treated with metformin at various doses and for different time periods. The treated cells were examined for the proinflammatory responses, as well as the phosphorylation states of AMPK and the expression of PFKFB3/iPFK2. In addition, PFKFB3/iPFK2-knockdown adipocytes were treated with metformin and examined for changes in the proinflammatory responses.

**Results**—Treatment of adipocytes with metformin decreased the effects of lipopolysaccharide on inducing the phosphorylation states of JNK p46 and on increasing the mRNA levels of IL-1 $\beta$  and TNF $\alpha$ . In addition, treatment with metformin increased the expression of PFKFB3/iPFK2, but failed to significantly alter the phosphorylation states of AMPK. In PFKFB3/iPFK2-knockdown

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\*T Qi and Y Chen are contributed equally to this work.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

#### Authors' contribution

C.W. Y.H. and Y.C. designed the research; T.Q., H.L., Y.P., S.L.W, X.G., and J.Z. conducted the research; T.Q., H.L., and C.W. analyzed the data and wrote the manuscript. Y.C., X.Q., and J.A. were involved in scientific discussion. C.W. and Y.H. had primary responsibility for the final content. All authors read and approved the final manuscript.

adipocytes, treatment with metformin did not suppress the proinflammatory responses as did it in control adipocytes.

**Conclusion**—Metformin has a direct effect on suppressing adipocyte proinflammatory responses in an AMPK-independent manner. Also, metformin increases adipocyte expression of PFKFB3/iPFK2, which is involved in the anti-inflammatory effect of metformin.

### Keywords

Inducible 6-phosphofructo-2-kinase; metformin; adipocyte; inflammatory responses; obesity

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## Introduction

Chronic low-grade adipose tissue inflammation is a characteristic of obesity (Lumeng *et al.* 2007, Weisberg *et al.* 2003, Xu *et al.* 2003) and critically contributes to the pathogenesis of type 2 diabetes (Greenberg and Obin 2006, Shoelson *et al.* 2007). As shown in rodent models of obesity, adipose tissue-specific overexpression of monocyte chemoattractant protein-1 causes adipose tissue macrophage accumulation, which is accompanied with increased severity of systemic insulin resistance and metabolic dysregulation (Kamei *et al.* 2006). Similarly, adipocyte-derived Th2 cytokines critically determine the status of adipose tissue inflammation by promoting macrophage alternative activation, which in turn contributes to improvement of systemic insulin sensitivity (Kang *et al.* 2008). These results, together with many other results from both human and rodent studies (Boyle *et al.* 2011, Huo *et al.* 2010, Huo *et al.* 2012, Kamei *et al.* 2006, Ye *et al.* 2007), validate the importance of adipose tissue inflammation in the development of insulin resistance and glucose and fat metabolic dysregulation. Specifically, inflammatory adipose tissue produces a number of pro-hyperglycemic factors that impair insulin signaling in insulin-sensitive tissues including the liver and skeletal muscle, leading to systemic insulin resistance (Berg *et al.* 2001, Cheung *et al.* 2000, Hotamisligil *et al.* 1996, Kabir *et al.* 2005, Trujillo and Scherer 2006). The latter results in increased hepatic glucose production and decreased muscle glucose disposal (Kim *et al.* 2004, Steinberg *et al.* 2006, Wu *et al.* 2005*b*), which are two essential metabolic events responsible for the development of hyperglycemia. Because of this, targeting adipose tissue inflammation to correct obesity-associated insulin resistance is of particular importance in the treatment of type 2 diabetes.

Since it effectively corrects hyperglycemia, metformin has been used as the first-line medicine for the treatment of type 2 diabetes for over several decades (Zheng *et al.* 2015). Mechanistically, metformin acts mainly through suppressing hepatic glucose production to lower plasma levels of glucose. In addition, metformin has been shown to improve fat metabolism in both the liver and muscle, which also contributes to the glucose-lowering effect of metformin (Heishi *et al.* 2006, Perriello *et al.* 1994, Song *et al.* 2001, Stumvoll *et al.* 1995, Woo *et al.* 2014). Following the validation of adipose tissue inflammation as a causal factor of the development of insulin resistance and hyperglycemia, a number of studies also have explored the effects of metformin on adipose tissue inflammation and metabolic functions. Unlike its effect on the liver, the effect of metformin on adipose tissue remains controversial. Specifically, metformin is shown to decrease adipose tissue inflammation in both human subjects and rodent models of obesity and type 2 diabetes (Shin *et al.* 2013,

Zhao *et al.* 2016, Zulian *et al.* 2011), evidenced by decreased proinflammatory signaling and cytokine expression. In contrast, there are studies in which metformin does not alter body weight and/or adipose tissue inflammation, in particular in rodents fed an high-fat diet (HFD) (Anthony *et al.* 2013, Shin *et al.* 2013, Song *et al.* 2001, Woo *et al.* 2014). Given this discrepancy, there is a need to gain mechanistic insights of whether metformin has direct effects on adipocyte inflammatory and metabolic responses.

PFKFB3 is the gene that encodes for the inducible 6-phosphofructo-2-kinase (iPFK2). In adipose tissue, the expression of PFKFB3/iPFK2 is at high abundance (Huo *et al.* 2010). As a regulatory enzyme, iPFK2 generates fructose-2,6-bisphosphate, which in turn activates 6-phosphofructo-1-kinase to enhance glycolysis (Okar *et al.* 2004, Rider *et al.* 2004). PFKFB3/iPFK2 has been previously shown to critically regulate adipose tissue functions and systemic insulin sensitivity (Huo *et al.* 2010). For example, PFKFB3/iPFK2 disruption exacerbates diet-induced adipose tissue inflammation and systemic insulin resistance whereas adipose tissue-specific PFKFB3/iPFK2 overexpression decreases adipose tissue inflammation and improves systemic insulin sensitivity (Huo *et al.* 2010, Huo *et al.* 2012). Limited research has suggested that the expression of PFKFB3/iPFK2 is increased in DB-1 melanoma cells upon metformin treatment, likely through a mechanism involving AMP-activated protein kinase (AMPK) (Mendoza *et al.* 2012). However, it is not clear whether metformin alters adipocyte expression of PFKFB3/iPFK2 in the context of adipose tissue inflammatory responses. The present study provides the evidence for the first time to support that metformin stimulates adipocyte expression of PFKFB3/iPFK2. The latter, in turn, is involved in the effect of metformin on suppressing adipocyte inflammatory responses.

## Materials and methods

### Adipocyte differentiation and treatment

3T3-L1 cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin as previously described (Huo *et al.* 2010). To differentiate 3T3-L1 cells, the 2 d post-confluent cells were incubated in DMEM supplemented with 0.5 mM 3-isobutyl-1-methyl-xanthine, 1 µM dexamethasone, and 10 µg/mL insulin for 48 h. Thereafter, the cells were incubated in DMEM supplemented with 10 µg/mL insulin for an additional 8 d. At 10 days post differentiation, adipocytes were used for a dose-response study or a time-course study to examine the anti-inflammatory effect of metformin. In the dose-response study, the differentiated adipocytes were treated with metformin at a dose of 5, 50, or 500 µM (dissolved in phosphate-buffered saline, PBS) or PBS for 24 h in the absence or presence of lipopolysaccharide (LPS, 100 ng/ml) for the last 30 min to harvest protein lysates or LPS (20 ng/mL) for the last 6 h to harvest total RNA samples. In the time-course study, the differentiated adipocytes were treated with metformin (50 µM) for 0, 1, 6, 24, or 48 h in the presence of LPS (100 ng/mL) for the last 30 min to harvest protein lysates or LPS (20 ng/mL) for the last 6 h to harvest total RNA samples. To analyze the proinflammatory signaling, adipocyte lysates were examined for total amount and phosphorylation states of Jun N-terminal kinase (JNK) p46 and nuclear factor kappa B (NF-κB) p65. To analyze the expression of PFKFB3/iPFK2, adipocyte RNA was subjected to reverse transcription and real-time PCR to quantify PFKFB3 mRNA levels whereas

adipocyte lysates were subjected to Western blot analysis to examine iPFK2 amount. In addition, adipocyte RNA was used to examine the mRNA levels of proinflammatory cytokines including IL-1 $\beta$ , IL-6, and TNF $\alpha$ . Details were described in the pertinent assays.

### Adipocyte PFKFB3/iPFK2 knockdown and metformin treatment

Stable PFKFB3/iPFK2 knockdown (iPFK2-KD) and control (iPFK2-Ctrl) 3T3-L1 cells were previously established (Huo et al. 2010). To examine the involvement of PFKFB3/iPFK2 in metformin actions, iPFK2-KD cells and iPFK2-Ctrl cells were differentiated in the same way as described above. After differentiation, the cells were treated with metformin (50  $\mu$ M) or PBS for 24 h in the absence or presence of LPS (100 ng/mL) for the last 30 min to harvest protein lysates or LPS (20 ng/mL) for the last 6 h to harvest total RNA samples. Some differentiated iPFK2-KD cells and iPFK2-Ctrl cells were treated with metformin (50  $\mu$ M) or PBS for 24 h in the absence or presence of insulin (100 nM) for the last 30 min. Protein lysates were harvested and used to examine insulin signaling using Western blot analysis.

### Western blot analysis

Adipocyte lysates were prepared in a lysis buffer containing 50 mM HEPES (pH 7.4), 10 mM EDTA, 50 mM sodium pyrophosphate, 0.1 M sodium fluoride, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin, 2 mM benzamide, and 1% Triton X-100. After protein electrophoresis and transfer, immunoblots were performed using rabbit anti-serum as primary antibody at a 1:1,000 dilution. This dilution was used for each of the primary antibodies used for the present study. After washing, the blot was incubated with a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and followed by a chemiluminescent kit (Immobilon Western; EMD Millipore, Billerica, MA, USA) as previously described (Wu *et al.* 2005a). GAPDH was used as a loading control. The maximum intensity of each band was quantified using ImageJ software. Ratios of P-p46/p46, Pp65/65, P-AMPK/AMPK, or P-Akt/Akt were normalized to GAPDH and adjusted relative to the average of PBS-treated control, which was arbitrarily set as 1 (AU). Also, the amount of iPFK2 was normalized to GAPDH and adjusted similarly. Antibodies against Pp46, p46, Pp65, p65, P-AMPK, and/or AMPK were products of Cell Signaling (Danvers, MA, USA). Antibodies against P-Akt and Akt and anti-rabbit IgG antisera were products of Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

### RNA isolation, reverse transcription, and real-time PCR

The total RNA was isolated from the adipocytes described above. Reverse transcription was performed using the GoScript Reverse Transcription System (Promega) and real-time PCR analysis was performed using SYBR Green (LightCycler 480 system; Roche) (Guo *et al.* 2013, Guo *et al.* 2012, Wu *et al.* 2006). The mRNA levels were analyzed for IL-1 $\beta$ , IL-6, TNF $\alpha$ , and/or PFKFB3. A total of 0.1  $\mu$ g RNA was used for the determination. Results were normalized to 18s ribosomal RNA as plotted as relative expression to the average of PBS-treated control, which was set as 1.

## Statistical methods

Numeric data are presented as means  $\pm$  se (standard error). Two-tailed ANOVA and/or Student's *t* tests were used for statistical analyses. Differences were considered significant at the  $P < 0.05$ .

## Results

### Metformin suppresses adipocyte proinflammatory responses

The effect of metformin on obesity-associated adipose tissue inflammation remains controversial. We sought to examine the direct effect of metformin on adipocyte inflammatory responses. In differentiated 3T3-L1 adipocytes, treatment with LPS, a powerful proinflammatory stimulus, caused significant increases in the phosphorylation states of JNK p46 and NF- $\kappa$ B p65 and in the mRNA levels of IL-1 $\beta$  and TNF $\alpha$  (Fig. 1A–C). Upon treatment with metformin, the effect of LPS on inducing adipocyte proinflammatory responses was significantly weakened. Specifically, treatment with metformin at a dose of 50 or 500  $\mu$ M brought about a significant decrease in LPS-induced JNK p46 phosphorylation compared with metformin treatment at a dose of 5  $\mu$ M (Fig. 1A). At a fixed dose of 50  $\mu$ M, treatment with metformin for 24 or 48 h caused a marked decrease in the phosphorylation states of JNK p46 compared with control treatment or metformin treatment for 6 h (Fig. 1B). Unlike its effects on JNK p46, metformin treatment did not significantly alter adipocyte phosphorylation states of NF- $\kappa$ B p65 in either dose-response or time-course study. When cytokine expression was examined, treatment with metformin at a dose of 50  $\mu$ M for 24 h significantly blunted the effect of LPS on increasing adipocyte mRNA levels of IL-1 $\beta$  and TNF $\alpha$  (Fig. 1C). Of note, treatment of adipocytes with metformin at a relatively low dose, e.g., 5  $\mu$ M, or for a relatively short time, e.g., 6 h, appeared to not suppress or even increase LPS-induced JNK p46 phosphorylation states (Fig. 1A, and B). These effects, however, occurred prior to an increase in iPFK2 amount and/or in the presence of a decrease in iPFK2 amount (below, Fig. 3). Considering that decreased iPFK2 results in an increase in LPS-induced JNK p46 phosphorylation (Huo *et al.* 2010), it is likely that prior to full induction of iPFK2 metformin acts through certain mechanisms to increase LPS-induced JNK p46 phosphorylation. Once iPFK2 is increased, the anti-inflammatory effect of iPFK2 starts to dominate over the proinflammatory effect, thereby decreasing LPS-induced JNK p46 phosphorylation. Regardless, these results suggest that metformin has a direct effect on suppressing adipocyte proinflammatory responses.

### Metformin has limited effect on altering adipocyte AMPK phosphorylation

Metformin is shown to increase AMPK phosphorylation (activation) in various tissues/cells including the liver and muscle (Collier *et al.* 2006, Woo *et al.* 2014). However, the effect of metformin on adipose tissue AMPK phosphorylation remains controversial. Using adipocyte samples of the dose-response and time-course studies, we examined the direct effect of metformin on adipocyte AMPK phosphorylation. Consistent with our previous finding in adipose tissue of obese mice (Woo *et al.* 2014), treatment with metformin displayed limited effects on altering adipocyte AMPK phosphorylation (Fig. 2A, and B). This effect of metformin appeared to be different from that reported by Huypens *et al.* (Huypens *et al.* 2005). However, in the study by Huypens *et al.*, adipocytes were treated with metformin at a

high dose (1 mM) for 48 h. Based on the results of the present study, it appears that metformin, within a relatively low dose range, is not capable of increasing adipocyte AMPK phosphorylation.

### Metformin stimulates adipocyte PFKFB3/iPFK2 expression

In adipocytes, PFKFB3/iPFK2 has a role in protecting against overnutrition-induced proinflammatory responses (Huo *et al.* 2010, Huo *et al.* 2012). Therefore, we examined the effect of metformin on adipocyte PFKFB3/iPFK2 expression. In the dose-response study detailed above, PFKFB3 expression was increased by LPS stimulation, indicating a defensive response. Upon treatment with metformin at a dose of 5  $\mu$ M for 24 h, the effect of LPS on increasing PFKFB3 expression was blunted. However, treatment with metformin at a higher dose, i.e., 50 or 500  $\mu$ M, for 24 h caused a significant increase in the mRNA levels of PFKFB3 compared with control in the presence of LPS (Fig. 3A). Moreover, treatment with metformin at a dose of 50  $\mu$ M appeared to cause the strongest induction of PFKFB3 mRNA levels. In the time-course study, treatment with metformin at a dose of 50  $\mu$ M for 6 h, 24 h, or 48 h significantly increased the mRNA levels of PFKFB3. Within a period of time between 0 and 48 hr, treatment with metformin for 24 h displayed the strongest effect on stimulating PFKFB3 mRNA levels (Fig. 3B). Consistently, treatment with metformin at a dose of 50  $\mu$ M for 24 h or 48 h significantly increased protein amount of iPFK2 compared with control treatment in the presence of LPS (Fig. 3C); although iPFK2 amount in adipocytes upon metformin treatment for 24 h is not statistically different from that upon metformin treatment for 6 or 48 h (Fig. 3C). Taken together, these results suggest that metformin has a direct effect on stimulating adipocyte expression of PFKFB3/iPFK2.

### PFKFB3/iPFK2 knockdown blunts the effect of metformin on suppressing adipocyte proinflammatory responses

To elucidate a role for PFKFB3/iPFK2 in regulating metformin actions, we examined the effect of metformin on the proinflammatory responses in iPFK2-KD adipocytes. Consistent with the findings in differentiated 3T3-L1 adipocytes (Fig. 3), treatment with metformin increased iPFK2 amount in control cells (iPFK2-Ctrl) (Fig. 4A). However, this effect of metformin was nearly blunted in iPFK2-KD cells. Also, treatment with metformin did not significantly alter the phosphorylation states of AMPK in either iPFK2-KD or iPFK2-Ctrl cells. Although the phosphorylation states of AMPK in iPFK2-KD cells appeared to be increased in relative to those in iPFK2-Ctrl cells, the ratios of P-AMPK to total AMPK (normalized to GAPDH in iPFK2-KD cells) were comparable with those in iPFK2-Ctrl cells in either the presence or absence of metformin treatment. Next, we examined the anti-inflammatory effect of metformin. In iPFK2-Ctrl cells, treatment with metformin caused a significant decrease in the phosphorylation states of JNK p46; although not significantly altering the phosphorylation states of NF- $\kappa$ B p65. However, in iPFK2-KD cells, the phosphorylation states of JNK p46 were significantly higher than those in iPFK2-Ctrl cells, and remained high upon treatment with metformin (Fig. 4B). When proinflammatory cytokine expression was analyzed, the mRNA levels of IL-1 $\beta$ , IL-6, and/or TNF $\alpha$  in iPFK2-Ctrl cells were significantly reduced by treatment with metformin under basal conditions (in the absence of LPS) compared with control (in the absence of metformin). In contrast, the mRNA levels of IL-1 $\beta$ , IL-6, and/or TNF $\alpha$  in iPFK2-KD cells were increased or remained

high upon treatment with metformin under either basal or LPS-stimulated conditions (Fig. 4C). Indeed, the mRNA levels of IL-1 $\beta$ , IL-6, and/or TNF $\alpha$  were much more induced in iPFK2-KD cells upon LPS stimulation in the presence or absence of metformin compared with their respective levels in iPFK2-Ctrl cells (Fig. 4C). Taken together, these results suggest that intact PFKFB3/iPFK2 is required for metformin to fully suppress adipocyte proinflammatory responses.

### **PFKFB3/iPFK2 knockdown blunts the effect of metformin on improving adipocyte insulin signaling**

Adipocyte insulin signaling is reversely correlated with adipocyte proinflammatory status. We examined the involvement of PFKFB3/iPFK2 in metformin actions on improving adipocyte insulin signaling at the level of Akt phosphorylation. In iPFK2-Ctrl cells, treatment with metformin enhanced the effect of insulin on increasing the phosphorylation states of Akt compared with control treatment (Fig. 5A and B). However, this effect of metformin was blunted in iPFK2-KD cells (Fig. 5A and B). Therefore, intact PFKFB3/iPFK2 is needed for metformin to improve adipocyte insulin signaling.

## **Discussion**

The anti-diabetic effect of metformin has been well established. However, it remains controversial whether metformin suppresses adipose tissue inflammation, thereby contributing to the anti-diabetic effect of metformin (Shin *et al.* 2013, Woo *et al.* 2014, Zhao *et al.* 2016, Zulian *et al.* 2011). Because the discrepancy of metformin actions on adipose tissue inflammation appears to be associated with various factors including the strains of rodents used and the doses and paths of metformin delivery, the present study sought to determine the direct effect of metformin on adipocytes. Using *in vitro* systems involving differentiated 3T3-L1 adipocytes, the present study validated a direct effect of metformin on suppressing adipocyte proinflammatory responses. Furthermore, the present study revealed a stimulatory effect of metformin on adipocyte expression of PFKFB3/iPFK2, whose knockdown in adipocytes blunted the anti-inflammatory effect of metformin. Therefore, the present study provided the evidence to support metformin stimulation of adipocyte PFKFB3/iPFK2 as a novel mechanism by which metformin may suppress adipose tissue inflammation.

Metformin has a direct effect on suppressing adipocyte proinflammatory responses. In support of this, treatment with metformin at a dose of 50 or 500  $\mu$ M caused a significant decrease in LPS-stimulated adipocyte phosphorylation states of JNK p46 although metformin treatment did not significantly alter LPS-stimulated phosphorylation states of NF- $\kappa$ B p65. In addition, metformin treatment at a fixed dose of 50  $\mu$ M for 24 or 48 h caused significant decreases in LPS-stimulated adipocyte phosphorylation states of JNK p46. Consistently, treatment with metformin brought about a significant decrease in the mRNA levels of IL-1 $\beta$  and TNF $\alpha$ . Of note, metformin appeared to be more effective in adipocytes differentiated from un-transfected 3T3-L1 cells than in adipocytes differentiated from transfected and G418-selected 3T3-L1 cells while exhibiting anti-inflammatory effects. Specifically, metformin treatment caused a significant decrease in the mRNA levels of IL-1 $\beta$

and TNF $\alpha$  in un-transfected cells (Fig. 1C), but not in iPFK2-Ctrl cells (Fig. 4C) under LPS-stimulated conditions. This difference in metformin actions may be due to how adipocytes were prepared. Indeed, adipocytes differentiated from transfected cells exhibited a much stronger response to LPS than did adipocytes differentiated from un-transfected cells. Considering this, it is likely that there is a threshold of adipocyte proinflammatory status for metformin to exhibit anti-inflammatory effects. In other words, metformin may not be effective when LPS-induced proinflammatory responses are too strong. This appeared to be the case. In the present study, under basal conditions where the proinflammatory status was low or mild, metformin treatment significantly decreased the mRNA levels of IL-1 $\beta$ , IL-6, and TNF $\alpha$ , as well as the phosphorylation states of JNK p46. In contrast, in iPFK2-KD cells where the basal proinflammatory status was strong, metformin failed to decrease proinflammatory signaling and cytokine expression. The threshold view, however, needs to be further investigated. Nonetheless, the results argue in favor of a role for iPFK2 in mediating the anti-inflammatory effect of metformin (see below).

Metformin suppression of adipocyte proinflammatory responses does not involve AMPK. Previous results from both human and rodent studies indicate that treatment with metformin stimulates the phosphorylation of states of adipose tissue AMPK (Boyle *et al.* 2011, Lu *et al.* 2016). In contrast, there are studies in which treatment with metformin does not significantly alter the phosphorylation states of adipose tissue AMPK (Woo *et al.* 2014). Similarly, the direct effects of metformin on adipocyte AMPK phosphorylation vary among studies. In the present study, treatment with metformin did not alter adipocyte AMPK phosphorylation as this was supported by the results from both dose-response and time course studies. These results were consistent with the findings of a previous study by Boyle *et al.* (Boyle *et al.* 2011), in which treatment with metformin at a dose of 1 mM for a time period of 0.5 to 24 h did not significantly alter adipocyte AMPK phosphorylation although treatment with metformin for 48 h increased adipocyte AMPK phosphorylation states. Of importance, in the present study, treatment with metformin significantly decreased the phosphorylation states of adipocyte JNK p46 and the mRNA levels of proinflammatory cytokines while exhibiting limited effect on increasing adipocyte AMPK phosphorylation states. Therefore, metformin appears to suppress adipocyte proinflammatory responses in an AMPK-independent manner. This is different from the effect of metformin on hepatocytes, in which treatment with metformin increases the phosphorylation states of AMPK and suppresses the proinflammatory responses (Woo *et al.* 2014). The mechanisms underlying the differential effects of metformin on AMPK phosphorylation states in hepatocytes versus in adipocytes are not clear, but may be due to the differences in the isoforms of the catalytic subunit of AMPK. In adipocytes, the  $\alpha$ 1 catalytic subunit is the predominant isoform and accounts for the major part of AMPK activity (Daval *et al.* 2005) whereas in hepatocytes  $\alpha$ 2-subunit is the main functional isoform of AMPK (Andreelli *et al.* 2006, Jelenik *et al.* 2010). As shown in muscles, treatment with metformin increases the activities of AMPK $\alpha$ 2, but not AMPK $\alpha$ 1 (Collier *et al.* 2006). Considering this, lacking responses in AMPK $\alpha$ 1 to metformin likely explains the observation of the present study. Alternatively, a relatively low dose, e.g., 50  $\mu$ M, is sufficient for metformin to exert anti-inflammatory effects whereas at a relatively high dose, e.g., 1 mM, is needed for metformin to increase AMPK phosphorylation as reported by Huypens *et al.* (Huypens *et al.* 2005). In this case, metformin



would still act to suppress adipocyte proinflammatory responses independent of AMPK phosphorylation.

It is a novel finding that treatment with metformin increased adipocyte expression of PFKFB3/iPFK2. Notably, treatment with metformin caused an increase in iPFK2 amount in both dose-response and time-course studies. In addition, treatment with metformin significantly increased the mRNA levels of PFKFB3 in the presence of LPS. Previously, AMPK activation upon lowering the pH values of the culture media is accompanied with increased PFKFB3/iPFK2 expression in glioblastoma cells (Mendoza *et al.* 2012). In the present study, metformin increased adipocyte PFKFB3/iPFK2 expression in the absence of stimulating AMPK phosphorylation. Considering this, metformin is likely capable of stimulating PFKFB3/iPFK2 expression in both AMPK-dependent and AMPK-independent manners. Regardless, increased amount of iPFK2 was correlated with the effect of metformin on decreasing adipocyte proinflammatory responses, suggesting the involvement of PFKFB3/iPFK2 in the anti-inflammatory effect of metformin. In adipocytes, PFKFB3/iPFK2 has been demonstrated as a metabolic regulator that links glucose and fatty acid metabolism and inflammatory responses. Specifically, PFKFB3/iPFK2, at an increase in its activity, is able to channel free fatty acids away from excessive oxidation to fat synthesis/storage, thereby reducing the generation of reactive oxygen species (Guo *et al.* 2010, Huo *et al.* 2012). Because of this, it is conceivable that metformin stimulation of PFKFB3/iPFK2 serves as a mechanism by which metformin suppresses adipocyte proinflammatory responses.

The involvement of PFKFB3/iPFK2 in the anti-inflammatory effect of metformin was further supported by the results from adipocytes upon PFKFB3/iPFK2 knockdown. In the present study, increased proinflammatory responses were confirmed in PFKFB3/iPFK2-knockdown adipocytes compared with those in control adipocytes. Of importance, metformin did not effectively suppress the proinflammatory responses in PFKFB3/iPFK2-knockdown adipocytes as did it in control adipocytes. As previously demonstrated by Guo *et al.*, PFKFB3/iPFK2 knockdown increased adipocyte proinflammatory responses, which was due to, in large part, increased adipocyte oxidative stress (Guo *et al.* 2010). In contrast, PFKFB3/iPFK2 over-expression promoted adipocyte fat deposition, leading to decreased adipocyte proinflammatory responses (Huo *et al.* 2012). Based on this, it is very likely that intact PFKFB3/iPFK2 is needed for a metabolic environment in which metformin is able to effectively suppress the proinflammatory responses. As additional evidence, metformin treatment increased insulin signaling, indicated by insulin-induced Akt phosphorylation, in control adipocytes, but not in PFKFB3/iPFK2-knockdown adipocytes.

In summary, the present study provided the evidence to support a direct effect of metformin on suppressing adipocyte proinflammatory responses. This effect of metformin appeared to be AMPK-independent. Moreover, the present study reported for the first time that metformin stimulated adipocyte expression of PFKFB3/iPFK2, which also was AMPK-independent. In PFKFB3/iPFK2-knockdown adipocytes, the proinflammatory responses were much exacerbated and were not suppressed upon metformin treatment compared with those in control adipocytes. Taken together, metformin appears to act through stimulating PFKFB3/iPFK2 expression to suppress adipocyte proinflammatory responses.

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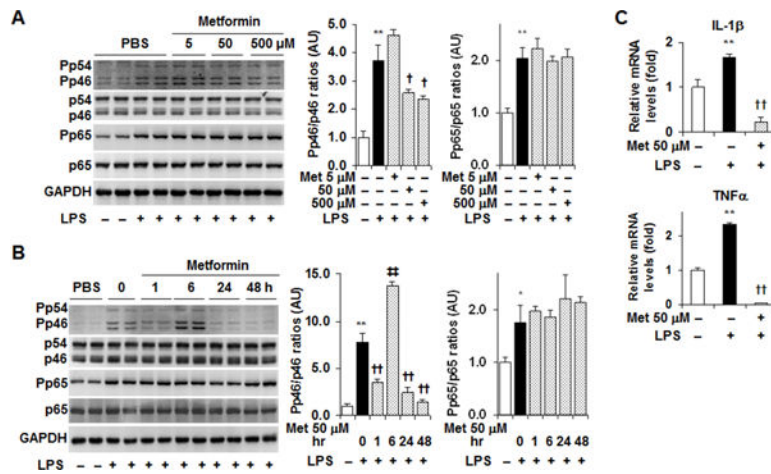
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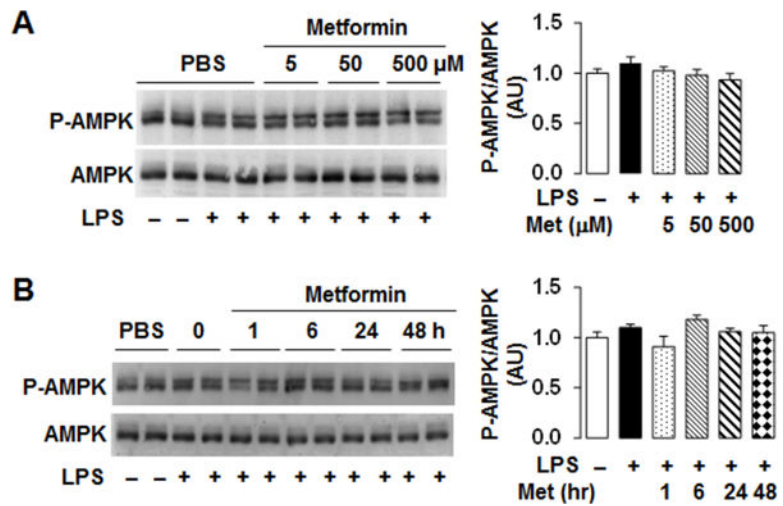
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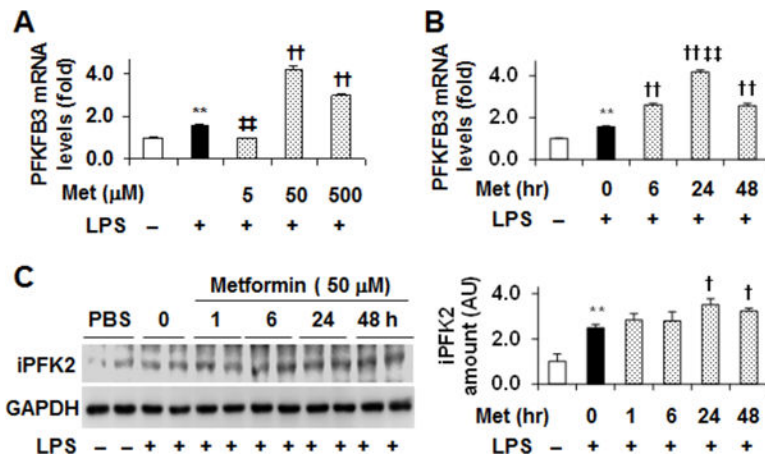


**Figure 1. Metformin suppresses adipocyte proinflammatory responses**

After differentiation, 3T3-L1 adipocytes were used to examine the effects of metformin on the proinflammatory responses. **(A)** Dose-response study. Adipocytes were treated with metformin (Met) at a dose of 5, 50, or 500  $\mu$ M (dissolved in phosphate-buffered saline, PBS) or PBS for 24 hr. **(B)** Time-course study. Adipocytes were treated with metformin (50  $\mu$ M) for 0, 1, 6, 24, or 48 hr. **(C)** Adipocyte cytokine expression. Adipocytes were treated with metformin (50  $\mu$ M) for 24hr. For A – C, prior to harvest, the cells were also treated with lipopolysaccharide (LPS, 100 ng/ml) for 30 min to analyze inflammatory signaling or LPS (20 ng/ml) for 6 hr to analyze cytokine expression. For A and B, adipocyte lysates were subjected to Western blot analysis. Blots were quantified using densitometry. AU, arbitrary unit. For C, cytokine expression was examined using real-time PCR. For bar graphs (A – C), data are means  $\pm$  S.E. n = 4. \*,  $P < 0.05$  and \*\*,  $P < 0.01$  LPS in the presence or absence of Met vs. PBS (empty bar); †,  $P < 0.05$  and ††,  $P < 0.01$  Met at 50 or 500  $\mu$ M in the presence of LPS vs. LPS alone; ‡,  $P < 0.01$  Met treatment for 6 hr vs. LPS alone (in B, left bar graph).

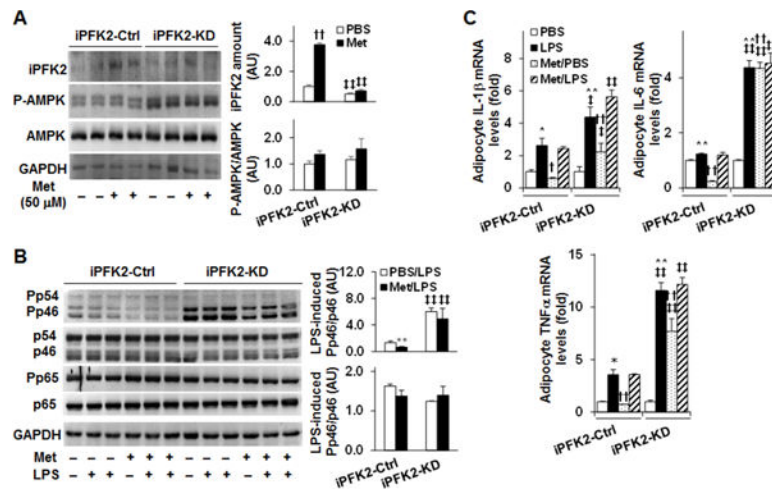


**Figure 2. Metformin does not significantly alter adipocyte AMPK phosphorylation**  
 Adipocytes were differentiated and treated as described in Fig. 1. (A) AMPK phosphorylation in the dose-response study. (B) AMPK phosphorylation in the time-course study. For A and B, the amount and phosphorylation states of AMPK were examined using Western blot analysis. Blots were quantified using densitometry. AU, arbitrary unit.



**Figure 3. Metformin increases adipocyte expression of PFKFB3/iPFK2**

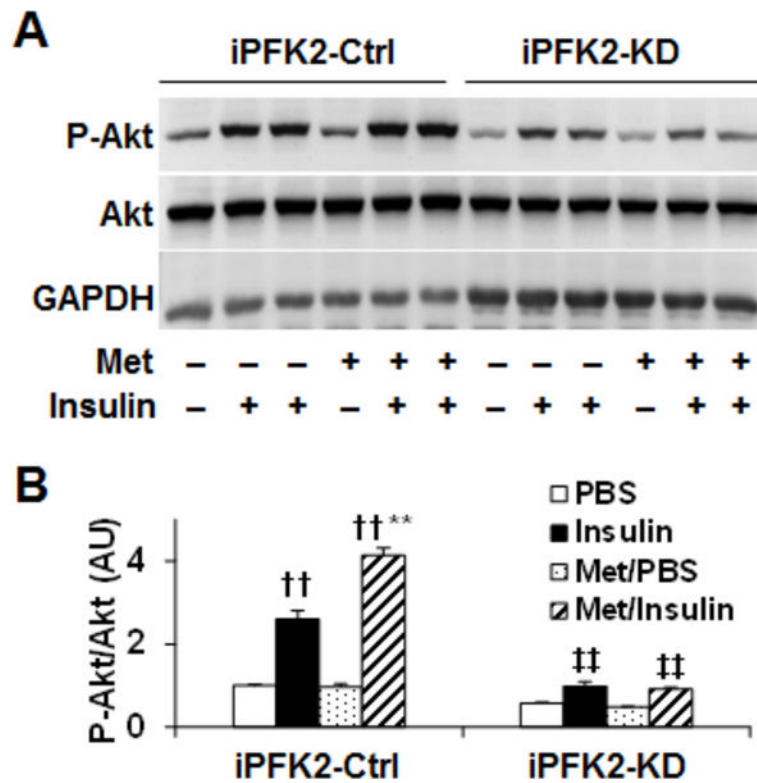
Adipocytes were differentiated and treated as described in Fig. 1. (A) PFKFB3 expression in the dose-response study. (B) PFKFB3 expression in the time-course study. For A and B, the mRNA levels of PFKFB3 were quantified using real-time PCR. (C) The amount of iPFK2 in time-course study was examined using Western blot analysis. Blots were quantified using densitometry. AU, arbitrary unit. Left panel, representative blots; right panel, quantification of blots. For bar graphs (A – C), data are means  $\pm$  S.E.  $n = 4$ . \*\*,  $P < 0.01$  LPS alone vs. PBS (empty bar); ††,  $P < 0.01$  Met at 5  $\mu$ M in the presence of LPS vs. LPS alone (in A) or Met for 24 hr vs. Met for any time period other than 24 hr (in B); †,  $P < 0.05$  and ††,  $P < 0.01$  Met at 50 or 500  $\mu$ M in the presence of LPS vs. LPS alone (in A – C).



**Figure 4. PFKFB3/iPFK2 knockdown impair the effect of metformin on suppressing adipocyte proinflammatory responses**

PFKFB3/iPFK2 knockdown (iPFK2-KD) adipocytes and control (iPFK2-Ctrl) adipocytes were generated as described in Methods. **(A)** Adipocyte iPFK2 amount and AMPK phosphorylation states. After differentiation, iPFK2-KD adipocytes and iPFK2-Ctrl adipocytes were treated with or without metformin (50  $\mu$ M) for 24 hr. **(B)** Adipocyte proinflammatory signaling. Differentiated iPFK2-KD adipocytes and iPFK2-Ctrl adipocytes were treated with or without metformin (50  $\mu$ M) for 24 hr in the absence or presence of LPS (100 ng/ml) for the last 30 min. For A and B, protein lysates were subjected to Western blot analysis. Blots were quantified using densitometry. AU, arbitrary unit. **(C)** Adipocyte expression of proinflammatory cytokines. Differentiated iPFK2-KD adipocytes and iPFK2-Ctrl adipocytes were treated with or without metformin (50  $\mu$ M) for 24 hr in the absence or presence of LPS (20 ng/ml) for the last 6 hr. The mRNA levels of proinflammatory cytokines were quantified using real-time PCR. For C, data are means  $\pm$  S.E.  $n = 4$ . For all bar graphs, \*,  $P < 0.05$  and \*\*,  $P < 0.01$  Met/LPS vs. PBS/LPS for iPFK2-Ctrl cells (in B) or LPS alone vs. PBS for the same type of cells (iPFK2-KD or iPFK2-Ctrl cells in C); †,  $P < 0.05$  and ††,  $P < 0.01$  iPFK2-KD vs. iPFK2-Ctrl under the same condition (PBS or Met in A; PBS/LPS or Met/LPS in B; PBS, Met, PBS/LPS, or Met/LPS in C); ‡,  $P < 0.05$  and ‡‡,  $P < 0.01$  Met vs. PBS for iPFK2-Ctrl cells (in A) or Met/PBS vs. PBS for the same type of cells (iPFK2-KD or iPFK2-Ctrl cells in C).





**Figure 5. PFKFB3/iPFK2 knockdown blunts the effect of metformin on improving adipocyte insulin signaling**  
 PFKFB3/iPFK2 knockdown (iPFK2-KD) adipocytes and control (iPFK2-Ctrl) adipocytes were generated as described in Methods. (A, B), After differentiation, iPFK2-KD adipocytes and iPFK2-Ctrl adipocytes were treated with or without metformin (50 μM) for 24 hr in the absence or presence of insulin (100 ng/ml) for the last 30 min. Adipocyte lysates were examined for the amount and phosphorylation states of Akt using Western blot analysis. Blots were quantified using densitometry. AU, arbitrary unit. A, representative bolts; B, quantification of blots. For B, data are means ± S.E. n = 4. \*\*,  $P < 0.01$  Met/Insulin vs. Insulin (iPFK2-Ctrl cells); ††,  $P < 0.01$  iPFK2-KD vs. iPFK2-Ctrl under the same condition (Insulin or Met/Insulin); †††,  $P < 0.01$  Insulin vs. PBS or Met/Insulin vs. Met/PBS for iPFK2-Ctrl cells.