Endocrine pharmacology

Additional effect of metformin and celecoxib against lipid dysregulation and adipose tissue inflammation in high-fat fed rats with insulin resistance and fatty liver

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A B S T R A C T

We investigated the effects of metformin and celecoxib on obesity-induced adipose tissue inflammation, insulin resistance (IR), fatty liver, and high blood pressure in high-fat (HF) fed rats. Male Sprague-Dawley rats were fed with either regular or HF diet for 8 weeks. Rats fed with regular diet were treated with vehicle for further 4 weeks. HF fed rats were divided into 6 groups, namely, vehicle, celecoxib (30 mg/kg/day), metformin (300 mg/kg/day), metformin (150 mg/kg/day), metformin (300 mg/kg/day) with celecoxib (30 mg/kg/day), metformin (150 mg/kg/day) with celecoxib (15 mg/kg/day) for additional 4 weeks. Increased body weight in HF fed rats was significantly reduced by metformin alone and metformin combined with celecoxib. The increases in the HOMA-IR value and the area under the curve of glucose following an oral glucose tolerance test, systolic blood pressure, and adipocyte size were significantly diminished in treated rats, especially rats undergoing combined treatment. Treatments with either celecoxib or in combination with metformin resulted in a reduction in AT macrophage infiltration and decreases in levels of adipose tissue TNF-α, MCP-1, and leptin levels in high-fat (HF) fed rats. Furthermore, the elevated hepatic triglycerides content was significantly decreased in the combined treatment group compared to that of groups of celecoxib or metformin alone. Celecoxib exerts a synergistic beneficial effect with metformin on obesity-associated metabolic and cardiovascular disorders in high-fat fed rats.

1. Introduction

Obesity is a major public health concern with an increasing prevalence worldwide (Flier, 2004). Obesity is associated with insulin resistance (IR) and known as the risk factor for hypertension, dyslipidemia, metabolic syndrome (MS), and type 2 diabetes mellitus (T2DM) (Kopelman, 2000).

Chronic inflammation in fat has been reported to play a crucial role in the development of obesity-induced IR and co-morbidities (Wellen and Hotamisligil, 2003; Winer and Winer, 2012). Adipose tissue macrophage infiltration is considered to contribute to obesity-induced inflammation via secretion of a wide variety of adipose tissue-derived pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) (Chawla et al., 2011; Galic et al., 2011). It has been shown to have local effects on white adipose tissue as well as potential systemic effects on other organs, including liver or skeletal muscle, to promote the development of IR (Guilherme et al., 2008; Shoelson et al., 2006).

Metformin is known as first-line therapy in most clinical practice guidelines for T2DM (American Diabetes, 2015; Nathan et al., 2009) and is recommended for treating obese adolescents with MS (Park et al., 2009). Effects of metformin on hepatic gluconeogenesis are attributed to activation AMP-activated protein kinase (AMPK) (Stephenson et al., 2011). Metformin-induced AMPK activation has resulted in a suppression of acetyl-coA carboxylase (ACC), leading to a reduction in hepatic lipid accumulation and restoration of insulin sensitivity (Shaw, 2013). Metformin is suggested to activate AMPK activity in adipose tissue, through phosphorylation of Thr172 in AMPK in human adipose tissue (Boyle et al., 2011). Moreover, activity of hormone-sensitive lipase (HSL)
that is regulated by reversible phosphorylation of serine residues has been reported to be inhibited by AMPK activation (Arner and Langin, 2014).

Cyclooxygenase-2 (COX-2) is known to contribute to the pathogenesis of obesity-associated inflammation (Ghoshal et al., 2011; Hsieh et al., 2009), a recent research has suggested that COX-2-mediated inflammation contributes to the development of T2DM in some populations (Konheim and Wolford, 2003). Our previous study has also demonstrated that COX-2-mediated inflammation in adipose tissue is crucially involved in obesity-induced IR and fatty liver in high-fat (HF)-diet fed rats (Hsieh et al., 2009). COX-2 upregulation involves activation of nuclear factor-kappa B (NF-kB) along with increased levels of TNF-α which has been reported to trigger lipid peroxidation in non-alcoholic fatty liver disease (NAFLD) (Leclercq et al., 2004; Yu et al., 2006).

In the present study, we hypothesized that COX-2 inhibition in adipose tissue synergistically enhances the therapeutic effect of metformin on obesity-associated lipid dysregulation and adipose tissue inflammation. We examined potential synergistic effect of a COX-2 inhibitor and metformin on improving obesity-associated IR, fatty liver, and high blood pressure in HF fed rats.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (5–6 weeks old) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and housed in the animal center at National Defense Medical Center, which is certificated by the Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animals were handled and housed according to the guidelines and manual of the Committee of the Care and Use of Laboratory Animals in this institute.

The rats were fed with regular chow diet (13.1% fat content) (LabDiet® products Rodent Diet 5010, St. Louis, MO 63144 USA) or a HF diet (45% fat content) (D12451; New Brunswick, NJ 8901 USA) for 8 weeks. Then, over the following 4 weeks, the rats were further divided into 6 groups, namely vehicle, COX-2 inhibitor (Celecoxib [30 mg/kg/day] by gavage; Pfizer, New York, NY, USA), metformin (Met) [300 mg/kg/day by gavage; Char Deh, Taipei, Taiwan], Met [150 mg/kg/day] MC [Celecoxib [15 mg/kg/day]/metformin [150 mg/kg/day]] and 1/2 MC groups [Celecoxib [15 mg/kg/day]+metformin [150 mg/kg/day]].

2.2. Blood pressure measurement

Systolic blood pressure (SBP) was measured as previously described (Hsieh, 2004). In brief, the measurement was carried out in conscious rats at the end of weeks 0, 4, 8, and 12 using an indirect tail-cuff method (volume-oscillometric method) with a fully automatic blood pressure monitoring system (UR-5000; UEDA, Tokyo, Japan).

2.3. Oral Glucose Tolerance Test (OGTT)

Oral glucose tolerance test was conducted without anesthesia in the morning after a 14-h overnight fast at the end of weeks 0, 4, 8, and 12. Rats were habituated to the oral gavage procedure 2–3 times with vehicle in one week before the test and to minimize stress throughout the procedure. On the study day, the certain amount of glucose solution (2 g/ml/kg) was administered by gavage, following a baseline blood sample was obtained from a tail vein. Following glucose administration, blood samples were collected at 30, 60, 90, and 120 min.

2.4. Biochemical analysis

After an overnight fast, whole blood glucose levels were assayed using the glucose oxidase method. Plasma insulin levels were measured using a commercial rat enzyme-linked immunosorbent assay (ELISA) kits (Mercodia AB, Uppsala, Sweden). TNF-α, MCP-1, and leptin levels in tissues were measured with commercial rat ELISA kits (R&D Systems, Minneapolis, MN, USA). Serum alanine transaminase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine, total cholesterol (TC), low-density-lipoprotein cholesterol (LDL-C), and triglyceride levels were assayed using Randox Reagent kits (Randox Laboratories, Ltd., Antrim, UK).

2.5. Calculation

The homeostasis model assessment method (HOMA-IR), an index of systemic IR, was calculated using the following formula before and after treatment in experimental rats: (fasting insulin in μU/ml × fasting glucose in mmol/l)/22.5 (Matthews et al., 1985).

2.6. Immunohistochemistry

Samples of epididymal adipose tissue were fixed in formalin, cut into 4-μm sections, and immunohistochemistry stained using automated stainer (Dako cytometry autostainer; Dako, Glostrup, Denmark). Sections were incubated with ED1 mouse anti-CD68 antibody (Serotec, 1:100; MorPhoSys UK, Ltd., Oxford, UK), followed by incubation with goat anti-mouse secondary antibody and development with HRP substrate (Dako REAL EnVision Detection System). Degree of macrophage infiltration in adipose tissue was quantitated by calculating the ratio of nuclei of CD68-positive cells to total nuclei in 20 fields from 3 slides for each individual rat using 6 rats in each group. Adipocyte cross-sectional area was determined for each adipocyte in each field analyzed using Motic Image Plus 2.0 ml software (Ted Pella, Inc., Redding, CA, USA).

2.7. Statistical analysis

Statistical analysis was performed according to the repeated measurements of one-way analysis of variance (ANOVA) incorporating with the Bonferroni test. Pearson’s correlation coefficients (r) of the data points from experimental rats were calculated by linear regression. A P value less than 0.05 was considered as a significant difference between groups. Values are expressed as the mean ± S.E.M..

3. Results

3.1. Effects of metformin and/or celecoxib on body weight and food intake, HOMA-IR value, and metabolic parameters

After the drug intervention for 4 weeks, the body weight gain in HF fed rats was reduced in a dose-dependent fashion in presence of metformin and celecoxib (MC/2 and MC; Fig. 1Aa and B). Caloric intake was significantly intervened in the Met, MC/2, and MC groups, whereas no change was found in the Met/2 and Cel groups (Fig. 1C). A significant reduction in the HOMA-IR value was noted in rats treated with metformin and/or celecoxib, especially in MC group (Fig. 1D). The changes of lipid profile were not significant among experimental groups and plasma BUN and Creatinine levels, the index of renal function were improved in rats treated with full-dose metformin (300 mg/kg/day, Met and MC) groups. The increases in plasma insulin levels and systemic
blood pressure in HF-fed group were significantly suppressed in those with metformin and/or celecoxib (Table 1).

3.2. Area under the curve (AUC) of glucose based on the OGTT

The results showed that 4-week drug treatment decreased the...
As shown in Fig. 2C and D, AT treatment with Met and Cel exerted the synergistic effect on HF diet-fed rats for obesity-associated abnormalities is unavailable. We demonstrated that metformin in combination with celecoxib not only significantly attenuated in rats treated with metformin alone, celecoxib alone, and especially the combination of metformin and celecoxib. The elevated levels of adipokines (TNF-α, MCP-1, and leptin) in the conditioned medium derived from epididymal fat (Fig. 3C, D, and E) and subcutaneous fats (Fig. 3F, G, and H) were suppressed in rats co-treated with metformin and/or celecoxib.

3.6. Histopathologic examination, hepatic triglycerides content, and activation of ACC and AMPK in the liver

The hepatic triglycerides content in HF diet-fed rats was significantly lower than that of controls. It was significantly attenuated in rats treated with metformin or celecoxib alone, and further diminished in rats treated with the combination of metformin and celecoxib (Fig. 4A and B). To determine the effects of metformin on AMPK activation, we examined ACC phosphorylation (p-ACC; Ser 79) at the AMPK-specific sites (p-AMPK; Thr172). The augmentation of p-ACC was noted in all Met-treated groups (Fig. 4C, D). In addition, degrees of AMPK phosphorylation in the liver further diminished in rats treated with the metformin and celecoxib combination (Fig. 4A and B). To determine the effects of metformin on AMPK activation, we examined ACC phosphorylation (p-ACC; Ser 79) at the AMPK-specific sites (p-AMPK; Thr172). The augmentation of p-ACC was noted in all Met-treated groups (Fig. 4C, D). In addition, degrees of AMPK phosphorylation in the liver further diminished in rats treated with the metformin and celecoxib combination (Fig. 4A and B).

4. Discussion

Adipose tissue inflammation is suggested to be a crucial risk factor for the development of obesity-associated cardiometabolic disorders, including T2DM; however, effective therapeutic strategy for obesity-associated abnormalities is unavailable. We demonstrated that metformin in combination with celecoxib not only significantly improved obesity-associated dysregulation of energy balance and systemic IR, but also efficiently suppressed obesity-induced AT inflammation. We showed that use of celecoxib in

AUC of glucose in all drug-treated groups, except Met/2 alone (p < 0.05; Fig. 1E and F). Combined treatment resulted in a significant change in AUC of glucose in HF-fed rats (Fig. 1G).

3.3. Average cell size of adipocytes and macrophage infiltration in epididymal fat

We observed that the HF diet-fed rats exhibited a marked increase in average adipocyte size compared with that of controls (Fig. 2A and B). The mean cell size of epididymal adipocytes was reduced in rats treated with metformin and/or celecoxib. Treatment with Met and Cel exerted the synergistic effect on HF diet-associated adipocyte hypertrophy. As shown in Fig. 2C and D, AT macrophage infiltration was significant in HF diet-fed rats, which was ameliorated in presence of celecoxib alone or in combination with metformin.

3.4. Phosphorylation of AMPK and HSL in epididymal fat

To determine the effects of metformin on AMPK pathway, we examined HSL phosphorylation (p-HSL; Ser565) at the AMPK-specific sites (p-AMPK; Thr172). In HF diet-fed rats, metformin alone or in combination with celecoxib markedly induced AMPK activation and HSL phosphorylation (Fig. 2E, F, and G). Augmentation of AMPK and HSL phosphorylation by metformin alone or in combination with celecoxib in HF diet-fed rats were comparable.

3.5. Adipokine levels in plasma and adipose tissue-conditioned medium

Elevated plasma MCP-1 and leptin levels were observed in HF diet-fed rats (Fig. 3A and B). The increased plasma MCP-1 level was restored in the MC group, whereas no change was observed in the other treatment groups (P < 0.05). In addition, the elevated plasma leptin level in HF diet-fed rats was significantly attenuated in rats treated with metformin alone, celecoxib alone, and especially the combination of metformin and celecoxib. The elevated levels of adipokines (TNF-α, MCP-1, and leptin) in the conditioned medium derived from epididymal fat (Fig. 3C, D, and E) and subcutaneous fats (Fig. 3F, G, and H) were suppressed in rats co-treated with metformin and/or celecoxib.
synergy enhanced anti-diabetic effect of metformin. Of note, half-strength combination of metformin and celecoxib exerted favorable therapeutic effect on obesity-induced cardiometabolic disorders than full-dose metformin with less hepato-renal toxicity.

Current treatment options for patients with obesity-related MS and diabetes include lifestyle adjustment and medications. The limitations of the currently recommended stepwise treatment for obesity-related cardiometabolic disorders, especially failure of monotherapy, have led to the use of aggressive combination therapies (Ji et al., 2013). Metformin is thought to pharmacologically act as an insulin sensitizer in patients with MS and diabetes, resulting in improved glucose uptake in muscle and suppress hepatic glucose production (Musso et al., 2012). The main challenges in the management of T2DM are failed blood glucose control and development of chronic complications. The class of Sulfonylureas is commonly used as add-on medication on the basis of efficacy and cost (1998). Metformin has been shown to have a relatively low drug interaction in patients with obesity-related diabetes (Gross et al., 2011).

Based on the current findings about the effects of metformin and celecoxib on obesity-related inflammation, it is hypothesized that combined therapy with metformin and a COX-2 inhibitor effectively improve adipose tissue inflammation and systemic metabolic abnormalities in the obese state. Our results demonstrated that combined therapy synergistically decreases body weight, food intake, IR, and adipocyte size, attenuates adipose tissue macrophage infiltration, and reduces the inflammatory adipokine levels derived from plasma and adipose tissue-conditioned medium compared to rats treated with metformin or COX-2 inhibitor alone. The high blood pressure in HF-diet fed rats was significantly attenuated after combination treatment, even in the half-dose combination group. Our findings suggest that co-treatment with a
COX-2 inhibitor synergistically enhances the therapeutic effect of metformin on cardiometabolic disorders in this diet-induced obese model.

Metformin induces phosphorylation of AMPK together with an increment in the phosphorylation of ACC in liver, and moderately reduces the release of pro-inflammatory cytokines in the epididymal and subcutaneous fat of this diet-induced obese model. Activation of AMPK in primary mouse adipocyte results in the phosphorylation of HSL on serine 565, leading to decreased HSL activity and lipolysis (Djouder et al., 2010). In addition, activated AMPK has been reported to inhibit ACC activity in an association with modulation of liver fatty acid metabolism (Fullerton et al., 2013). In rodents, there is also a general consensus to describe activation of AMPK in adipose tissue following induction of lipolysis (Gauthier et al., 2008). It is suggested that metformin primarily alleviates obesity-induced systemic IR and metabolic abnormalities by improving the energy metabolism in liver and adipose tissue via AMPK-mediated signaling (Galic et al., 2011). Metformin-mediated reduction in adipocyte hypertrophy can subsequently improve adipose tissue inflammation.

We have shown that the COX-2 mediated inflammation in adipose tissue plays a crucial role in IR and fatty liver in a HF-diet-induced obese animal model and a selective COX2 inhibitor significantly reversed adipocyte hypertrophy, macrophage

![Fig. 3. Plasma concentration of (A) MCP-1, (B) leptin. Epididymal adipose tissue conditional medium concentration of (C) TNF-α, (D) MCP-1, and (E) leptin. Subcutaneous adipose tissue conditional medium concentration of (F) TNF-α, (G) MCP-1, and (H) leptin. N=6 per group. Data are the mean ± S.E.M. *P < 0.05 vs. Cont in the corresponding period, #P < 0.05 vs. HFa in the corresponding period, †P < 0.05 vs. MC in the corresponding period.](image-url)
infiltration, and reversed the diminished markers of adipocyte differentiation, especially in visceral fat (Hsieh et al., 2010). Furthermore, a recent report has demonstrated that the reduced adiposity in COX-2-deficient mice is attributed to suppression of adipocyte differentiation and inflammation (Choshal et al., 2011). COX-2 inhibition suppressed tissue MCP-1 content to reduce the macrophage chemotaxis into the visceral fat and also attenuated the inflammatory adipokine production from inflamed adipose tissue resulting in improvement of systemic IR and related metabolic disorders in this HF diet-induced obese model (Hsieh et al., 2010). In addition, a recent study has demonstrated that the reduced adiposity in COX-2-deficient mice is attributed to suppression of adipocyte differentiation and inflammation (Hsieh et al., 2010). COX-2 inhibition suppressed tissue MCP-1 content to reduce the macrophage chemotaxis into the visceral fat and also attenuated the inflammatory adipokine production from inflamed adipose tissue resulting in improvement of systemic IR and related metabolic disorders in this HF diet-induced obese model (Hsieh et al., 2010). Furthermore, phosphatidylserine exposure of erythrocytes has been shown to foster blood clotting, suggesting a toxic effect upon celecoxib treatment (Chan, 2005). In addition, the stimulation of eryptosis detected with metformin has been shown to facilitate the release of pro-inflammatory cytokines (Kang et al., 2014). Therefore, the dose and duration of metformin and celecoxib treatment requires further studies to elucidate possible toxic effects prior to its use in clinical practice.

There were some limitations that need to be addressed. First, a clinical study should be essential to further verify the beneficial effects of COX-2 inhibitors and also their additional action with metformin on IR and adipose tissue inflammation in obese human subjects. Second, it remains controversial whether or not combined treatment with metformin and celecoxib would increase metformin-related lactic acidosis (Audia et al., 2008).

In conclusion, the present study demonstrates the synergistic effect of metformin in combination with celecoxib in the treatment of obesity-related adipose tissue inflammation, IR, and fatty liver, as well as high blood pressure to a greater extent than individual monotherapy. The metformin and celecoxib combination represents a potential novel and powerful new strategy to treat obesity-associated metabolic abnormalities, such as MS and T2DM.

Conflicts of interest statement

The authors declare that they have no conflicting interests.

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