行政院國家科學委員會專題研究計畫　成果報告

對非糖尿病代謝症候群患者單獨使用胰島素增敏劑或 statins 或合併使用兩者對代謝症候群各成份、血管內皮細胞功能、及炎性反應指標之影響：兼論患者基因形態對藥物效應之交互作用

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中文摘要：關鍵詞：血脂肪異常，內皮細胞，發炎，胰島素阻抗

背景：許多研究顯示，『胰島素阻抗』可能是造成與其相關的包括高血壓、高血脂、血糖耐受性不良、內皮細胞功能異常等病狀的根本及共通原因。臨床上將這一類病人定義為『代謝症候群』患者。邇來，已有提昇人體對胰島素反應的藥物（rosiglitazone）發展出來。由於『代謝症候群』患者有相當高的心血管疾病發生率，因此許多研究者均欲探究rosiglitazone這類胰島素增敏劑是否能夠降低『代謝症候群』患者的心血管疾病危險性。我們最近的研究首次證明，對於非糖尿病代謝症候群病患使用此類藥物，能夠顯著的提升人體的胰島素靈敏性、改善血管內皮細胞功能及降低發炎因子的濃度，並且不會造成低血糖。然而，這類藥物對於血脂肪數值卻有較不利的影響：它會增加低密度脂蛋白膽固醇及脂原蛋白B（apolipoprotein B）的濃度。由於此往的研究已證明statins類藥物能夠有效降低『代謝症候群』患者的血脂肪數值及心血管疾病危險性。因此，在本研究中，我們將分別比較單獨使用rosiglitazone或simvastatin及同時併用兩者對這類病人的療效；我們將以各種心血管疾病危險因子、血管內皮細胞功能、及各種發炎指標評估作為預測未來心血管疾病發生的指標。此外，我們也要探討不同的基因形態（如PPARγ基因型態多型性C161T和$Pro_{12}Ala$等）對藥物的反應是否有所不同。

實驗方法：年齡在十八至八十歲，且符合美國 NCEP 診斷代謝症候群條件者皆可進入本試驗。受試者先接受八個星期的飲食治療，在此期間內，停止使用所用降血脂肪藥物。在完成了八個星期的藥物清除期後，受試者隨機分配為接受 rosiglitazone（4 mg/day）、simvastatin（20 mg/day）、rosiglitazone (4 mg/day)合併 simvastatin (20 mg/day)、或安慰劑四組。所有的受試者皆接受八個星期的藥物治療。在這段治療期間，受試者及醫師均不知道受試者接受何種治療。在接受藥物治療前，每位受試者均接受兩次血液抽驗（藥物清除期前及隨機分配前一週）；藥物治療後受試者亦接受兩次血液抽驗（七週半及八週）。此外，在隨機分配前一週及接受八週的藥物治療後，進行右肱動脈的血管內皮細胞功能檢驗。我們利用超音波機器評估與內皮細胞功能相關的血流引致血管擴張（flow-mediated vasodilation，FMD）現象及硝化甘油引致的血管擴張（nitroglycerin-induced vasodilation）現象作為內皮細胞功能的指標。

結果及臨床意義：儘管最近發表的 PROactive 研究顯示，對於第二型糖尿病患，在包含 statins 等藥物的標準治療外，使用 pioglitazone 能夠有效降低心血管疾病死亡率。但在本研究中，合併使用 rosiglitazone 及 simvastatin 與單獨使用相較，無法更加改善 FMD；但對於發炎指標（包括 C-反應蛋白及 CD40 ligand）及血脂肪數值則有較單一治療為佳的降低效果。此外，這些藥物對內皮細胞功能（FMD）的改善效果主要乃是經由調控 endothelin-1 濃度而來。基因型態與藥物反應的關聯性並不顯著。

雖然 rosiglitazone 目前的適應症僅限於糖尿病患，然而我們及其他研究者的研究顯示對於非糖尿病患使用 rosiglitazone 不僅沒有低血糖的副作用，尚且能明顯的改善各種代謝症候群的成分及內皮細胞功能。合併使用 simvastatin 似乎亦能提供加成的心血管保護作用。我們亦欲更進一步利用新穎之影像學診斷工具直接探究 glitazone 類藥物對冠狀動脈硬化狀況的效果。

英文摘要:

Keywords: endothelium, inflammation, insulin resistance, insulin sensitizer, statins

Background: The metabolic syndrome consists of insulin resistance, compensatory hyperinsulinemia, hypertension, hypertriglyceridemia, low HDL-C, and obesity. It has been demonstrated that the common denominator of the metabolic syndrome is insulin resistance. Rosiglitazone is a thiazolidinedione (glitazone) developed to reduce insulin resistance in patients with type 2 diabetes. We and other investigators have recently demonstrated that rosiglitazone improved insulin sensitivity and endothelial function, and reduced plasma levels of various inflammatory markers, without causing hypoglycemia, in non-diabetic insulin-resistant subjects. However, we also found that rosiglitazone therapy resulted in untoward changes in lipoprotein metabolism, including increases in LDL-C and apolipoprotein B levels. Given that treatment with rosiglitazone is associated with a worsening of the lipid profile, combination therapy with rosiglitazone plus statins may be an ideal therapeutic option for non-diabetic patients with the metabolic syndrome.

In this study, we compared the efficacy of rosiglitazone and statin monotherapy (simvastatin), and in combination, on endothelial function and CRP as well as other novel inflammatory markers, as surrogate indicators of future CHD, and components of the metabolic syndrome in non-diabetic patients with the metabolic syndrome. Furthermore, we analyzed the polymorphism status of various candidate genes (PPARγ) and examined whether there is differential response to both study medications.

Methods and Materials: Eligible patients, aged 18 to 80 years conformed to the metabolic syndrome criteria in ATP III, will be instructed to adhere to the AHA Step 1 diet throughout the study and undergo an 8-week run-in period during which previous lipid-lowering therapy will be discontinued. After the run-in phase, patients will be randomized to receive rosiglitazone (4 mg/d)(n = 25), simvastatin (20 mg/d)(n = 25), rosiglitazone (4 mg/d) plus simvastatin (20 mg/d)(n= 25) or matched placebo (n = 25) for the 8-week double-blind phase. The patients will be seen at the screening visit (i.e. before the 8-week run-in), 1 week before randomization, at entry (randomization), and 4 and 8 weeks of treatment. Two fasting blood samples will be obtained at baseline 7 days apart and at the end of the 8-week drug-therapy phase (weeks 7.5 and 8). Endothelium-dependent flow-mediated vasodilation in response to reactive hyperemia and nitroglycerin-induced vasodilation will be evaluated in the right brachial artery 1 week before randomization and after 8 weeks of active treatment.

Results and Clinical Significance: Combination therapy with rosiglitazone and simvastatin did not result in a greater improvement in endothelial function than either agent alone in the present study. On the other hand, combination therapy did result in a greater reduction in hs-CRP and CD40 ligand levels than either agent alone, though not statistically significant. Possible explanations for our observation include small sample size, chance finding, or ceiling effect of rosiglitazone on FMD that made further improvement almost impossible. The anti-atherogenic effect of the two most promising pharmacological agents, statins and glitazones, are additive in view of their effects on inflammatory markers. The improvement in FMD independently correlated with changes in endothelin-1 ($r=-0.64$, $p<0.001$) and apolipoprotein B ($r=-0.34$, $p=0.025$) levels. Genetic polymorphism studied is not associated with differential response to the study drugs.

報告內容

前言：

The metabolic syndrome (syndrome X; insulin resistance syndrome) consists of insulin resistance, compensatory hyperinsulinemia, hypertension, hypertriglyceridemia, low high-density lipoprotein cholesterol (HDL-C), and obesity. It has been demonstrated that the common denominator of the metabolic syndrome is insulin resistance.[1] The metabolic syndrome precedes and predicts the development of type 2 diabetes and coronary heart disease (CHD).[2,3] Because of the associated risk of increased morbidity and mortality, treatment should be introduced at an early stage in individuals with characteristics of the metabolic syndrome.[4] In the post hoc analysis of the Scandinavian Simvastatin Survival Study, patients with combined hyperlipidemia were more likely to have characteristics of the metabolic syndrome, were at an higher risk for CHD events, and received greater benefit from simvastatin therapy compared with patients with isolated low-density lipoprotein cholesterol (LDL-C) elevation.[5] However, treatment with statins did not resolve the fundamental problem of insulin resistance. Furthermore, evidence is accumulating that the metabolic syndrome enhances the risk for CHD at any given LDL-C level, probably through the direct and independent atherogenic effect of insulin resistance.[3,6,7] Therefore, therapeutic options capable of ameliorating or reversing insulin resistance might be considered in the treatment of patients with the metabolic syndrome.

Rosiglitazone is a thiazolidinedione (glitazone) developed to reduce insulin resistance in patients with type 2 diabetes.[8] We and other investigators have even demonstrated that rosiglitazone improved insulin sensitivity, without causing hypoglycemia, in non-diabetic insulin-resistant subjects.[9,10] Moreover, in vitro and animal studies suggest that glitazones have beneficial effects on the endothelium and vascular inflammation,[11] both of which play important roles in the pathogenesis of atherosclerosis. However, we also found that rosiglitazone therapy resulted in untoward changes in lipoprotein metabolism, including increases in LDL-C and apolipoprotein B levels.[10] Given that treatment with rosiglitazone is associated with a worsening of the lipid profile, combination therapy with rosiglitazone plus statins may be an ideal therapeutic option for non-diabetic patients with the metabolic syndrome.

In patients at high risk of CHD, endothelial dysfunction is observed in morphologically intact vessels before the onset of clinically manifested vascular disease. As a consequence, assessment of endothelial function, by measuring the flow-mediated dilation (FMD) of the brachial artery, is currently being regarded as a potential tool for prediction of CHD risks.[12,13] We for the first time demonstrated that treatment with rosiglitazone improved endothelium-dependent vascular reactivity in non-diabetic patients with the metabolic syndrome.[10] However, it is still uncertain whether the co-administration of rosiglitazone and statin, compared with either agent alone, may achieve additional benefits in improving endothelium-dependent FMD of the brachial artery in insulin-resistant subjects.[14]

C-reactive protein (CRP), a sensitive marker of vascular inflammation, has been shown to be a powerful independent predictor of future CHD in numerous prospective epidemiological

studies.[15] It has been demonstrated that treatment with rosiglitazone significantly reduced CRP levels in patients with type 2 diabetes and non-diabetic patients with the metabolic syndrome.[8,10] Recent studies have demonstrated that the multipotent immunomodulator CD40 ligand and its receptor, CD40, play an important role in the various stages of atherogenesis.[16] In addition to the 39-kDa, cell-associated form, CD40 ligand also occurs in a soluble, biologically active form. It has been shown that elevations of circulating soluble CD40 ligand (sCD40L) predict future CHD in healthy individuals and patients with acute coronary syndromes, irrespective of plasma lipid and CRP levels.[17,18] We also demonstrated that both statin and thiazolidinedione could reduce plasma levels of sCD40L to a similar extent.[10,19]

研究目的：

In this study, we compared the efficacy of rosiglitazone and statin monotherapy (simvastatin), and in combination, on endothelial function and CRP as well as other novel inflammatory markers, as surrogate indicators of future CHD, and components of the metabolic syndrome in non-diabetic patients with the metabolic syndrome. We also examined the relationships between changes in various novel cardiovascular risk markers and FMD to elucidate the pathogenic mechanisms underlined. Furthermore, we analyzed the polymorphism status of various candidate genes and examine whether there is differential response to both study medications.

研究方法：

Subjects and Study Design

Patients will be recruited from the Clinics at the National Taiwan University Hospital, without restriction to sex or socioeconomic status. Inclusion criteria included the following: age 18 to 80 years and the presence of metabolic syndrome. The metabolic syndrome was determined by criteria as defined by the National Cholesterol Education Program Adult Treatment Panel III,[6] modified to use WHO proposed waist circumference cut-points for Asians.[10] Therefore, this required subjects to have three or more of the following criteria: 1) waist circumference of >90 cm in men and >80 cm in women; 2) serum triglycerides of ≥150 mg/dl; 3) HDL-C levels of <40 mg/dl in men and <50 mg/dl in women; 4) impaired fasting glucose of 110 to 125 mg/dl; or 5) blood pressure of ≥130/85 mmHg or treated hypertension. Major exclusion criteria are acute coronary event, stroke, or coronary revascularization within the preceding 3 months; insulin-dependent diabetes mellitus or poorly controlled non-insulin-dependent diabetes mellitus (HbA_{1c} >8%); severe obesity; overt liver disease; chronic renal failure; hypothyroidism; myopathy; alcohol or drug abuse; several other significant diseases; or use of other lipid-lowering therapy, immunosuppressants, erythromycin and/or neomycin, ketoconzaole, and hormone-replacement therapy. All subjects will give written informed consent.

Eligible patients will be instructed to adhere to the American Heart Association Step 1 diet throughout the study and undergo an 8-week run-in period during which previous lipid-lowering therapy will be discontinued. After the run-in phase, patients will be randomized to receive rosiglitazone (4 mg/d)(n = 25), simvastatin (20 mg/d)(n = 25), rosiglitazone (4 mg/d) plus

simvastatin (20 mg/d)(n = 25) or matched placebo (n = 25) for the 8-week double-blind phase. Patients assigned to the simvastatin group will be arranged to take placebo with breakfast and simvastatin at bedtime; those assigned to the rosiglitazone group will be arranged to take rosiglitazone with breakfast and placebo at bedtime; those assigned to the rosiglitazone plus simvastatin group will be arranged to take rosiglitazone with breakfast and simvastatin at bedtime; and those assigned to the placebo group will be arranged to take placebo both with breakfast and at bedtime. Both doctors and participants are blinded to the medications studied. The patients will be seen at the screening visit (i.e. before the 8-week run-in), 1 week before randomization (baseline laboratory and vascular studies), at entry (randomization), and 4 and 8 weeks of treatment. At week 8, physical examinations, laboratory assessments, and vascular studies will be repeated.

Laboratory Assays

Two fasting blood samples will be obtained at baseline 7 days apart and at the end of the 8-week drug-therapy phase (weeks 7.5 and 8). Venous blood samples are placed into tubes containing EDTA. Samples are centrifuged within 30 minutes at 2000 rpm for 10 minutes. The plasma will then be separated and stored at -70℃ until analysis. Levels of total cholesterol, total triglycerides, LDL-C and HDL-C are assayed by routine laboratory techniques with the use of methodology of the Lipid Research Clinics, as reported previously. If plasma triglycerides are ≥400 mg/dL, LDL-C will be assessed by a direct method. Plasma concentrations of interleukin (IL)-1β, IL-6, CD40, and sCD40L are determined in duplicate using commercially available immunosorbent kits (IL-1β and IL-6, R&D Systems; CD40 and sCD40L, Bender MedSystems). High-sensitivity CRP will be assayed by rate nephelometry (Dade Behring, Newark, Del.). High-sensitivity CRP will be assayed by rate nephelometry (Dade Behring, Newark, Del.). Routine chemical clinical analyses will be performed by standard methods subjects to strict quality control. The coefficients of variation are <5% for every type of measurement.

Vascular Studies

Endothelium-dependent flow-mediated vasodilation in response to reactive hyperemia and endothelium-independent nitroglycerin-induced vasodilation will be evaluated in the right brachial artery 1 week before randomization and after 8 weeks of active treatment. Ultrasound measurements are performed using a high-resolution ultrasound machine (Hewlett Packards, 5500) equipped with an L11-3 linear array transducer, as previously described by us. Arterial diameters are measured at rest, during reactive hyperemia, again at rest (after vessel recovery), and after administration of 0.6 mg sublingual nitroglycerin. The condition of reactive hyperemia is induced by inflation of a pneumatic cuff on the upper arm to suprasystolic pressure, followed by cuff deflation after 4.5 minutes. The brachial artery is scanned in longitudinal section 2 to 8 cm above the elbow, and the arterial diameter is measured on B-mode images with the use of ultrasonic calipers. The end-diastolic arterial diameter is measured from one media-adventitia interface to the other at the clearest section 3 times at baseline, every 20 seconds after reactive

hyperemia, and after administration of nitroglycerin. The maximum vessel diameter is taken as the average of the 3 consecutive maximum diameter measurements after hyperemia and nitroglycerin, respectively. Vasodilation will then be calculated as the percent change in diameter compared with baseline. In our laboratory, the measurements are performed by a single experienced operator in a temperature-controlled room (21 to 24℃) at the same time of day on patients fasted overnight. The intraobserver variation is 1.5%. Medications are omitted on the morning of the visit, and nitrates are withheld for 24 hours before studies.

Genetic Studies

Genomic DNA was prepared from samples of whole blood by standard methods. The first polymorphism of the peroxisome proliferators activated receptor-gamma (PPARγ) gene studied in this study is a silent C161→T substitution at exon 6. The polymerase chain reaction (PCR) was used to detect the C161→T at exon 6 of the PPARγ gene.[20] The forward and reverse primers were 5'–CAA GAC AAC CTG CTA CAA GC–3' and 5'–TCC TTG TAG ATC TCC TGC AG–3'. The amplification was performed in a 25 μl volume containing 100 ng DNA, 20 pmol of each primer, 2.0 mmol/l MgCl , 50 mmol/l KCl, 25 μmol/l dNTP, 5 mmol/l Tris–HCl (pH 8.3) and 1 Unit Taq polymerase. Samples were subjected to denaturing at 94℃ for 1 min followed by 34 cycles of 94℃ for 30 s, 56℃ for 30 s and 72℃ 1 min. The thermal cycles finish with 72℃ for 5 min. The 200 bp PCR products were digested with a *Pml*I restriction enzyme and run in 8% polyacrylamide gel for 30 min and silver-stained. This resulted in two fragments (120bp and 80bp) for the wild-type and one fragment (200bp) when the restriction site was eliminated by the C161→T transition. The genotypes were identified as CC, CT and TT. The second polymorphism studied is a missense mutation at codon 12 of PPARγ2 (CCG^{Pro}→GCG^{Ala}).[21] A 270 bp fragment of the PPARγ2 gene encompassing the site of the polymorphism was generated from genomic DNA by PCR using upstream primer 5'–GCCAATTCAAGCCCAGTC–3' and mutagenic downstream primer 5'–GATATGTTTGCAGACAGTGTATCAGTGAAGGAATCGCTTTCCG–3' which introduces a BstU-I restriction site (CG\CG) only when the C→G substitution at nucleotide 34 is present. The PCR products were digested with BstU-I, electrophoresed on a 2.5% agarose gel and stained with ethidium bromide. The expected products after digestion with BstU-I are 270 bp for normal homozygotes, 227 bp and 43 bp for Pro12Ala homozygotes, and 270 bp, 227 bp and 43 bp for heterozygotes.

Statistical Analysis

The data were analyzed by nonparametric methods to avoid assumptions about the distribution of measured variables. Comparisons between groups were made using the Mann-Whitney U test. The differences between baseline and post-treatment values were analyzed using the Wilcoxon signed-rank test. Mann-Whitney analysis was used for comparison of the percentage changes between baseline and post-treatment values in patients receiving fenofibrate versus those receiving simvastatin. The association of these measurements with other baseline biochemical

parameters was assessed by the Spearman rank correlation test. Multivariate regression analysis was performed to test the independent association between indexes of endothelial function and various baseline characteristics. Statistical significance was set at $P < 0.05$.

結果與討論：

We previously demonstrated that treatment with rosiglitazone was associated with improvement in endothelial function in non-diabetic insulin resistant individuals, without causing hypoglycemia.[10] However, treatment with rosiglitazone resulted in a worsening of lipid profiles as well. It is therefore rationale to consider that combination therapy with rosiglitazone and statins will provide additional benefits in reducing coronary risk in non-diabetic patients with the metabolic syndrome. Interestingly, despite our previous observation revealed that both rosiglitazone and simvastatin achieved significant improvements in endothelial function to a similar extent,[10,19] combination therapy with rosiglitazone and simvastatin did not result in a greater improvement in endothelial function than either agent alone in the present study. On the other hand, combination therapy did result in a greater reduction in hs-CRP and CD40 ligand levels than either agent alone, though not statistically significant. This intriguing finding is in fact contrary to the recently published prospective pioglitazone clinical trial in macrovascular events (PROactive) study,[22] in which treatment with pioglitazone reduced the composite of all-cause mortality, non-fatal myocardial infarction, and stroke, on top of optimal medical therapy including statins, in patients with type 2 diabetes and evidence of pre-existing macrovascular disease. Possible explanations for our observation include small sample size, chance finding, or ceiling effect of rosiglitazone on FMD that made further improvement almost impossible. The anti-atherogenic effect of the two most promising pharmacological agents, statins and glitazones, are additive in view of their effects on inflammatory markers.

Furthermore, we found that combination treatment with rosiglitazone and simvastatin resulted in a significant reduction in fasting plasma levels of endothelin-1 (-23%), soluble CD40 ligand (-40%), high-sensitivity C-reactive protein (-44%), resistin (-26%), and insulin (-38%), and systolic and diastolic blood pressure. There were no significant changes in fasting plasma glucose, intercellular adhesion molecule-1, or vascular cell adhesion molecule-1 levels with either treatment. The improvement in FMD with rosiglitazone treatment independently correlated with changes in endothelin-1 ($r=-0.64$, $p<0.001$) and apolipoprotein B ($r=-0.34$, $p=0.025$) levels. The present study demonstrated the safety and benefits of rosiglitazone use in reversing insulin resistance and ameliorating inflammation and endothelial dysfunction, which is primarily mediated by its effect on endothelin-1, in non-diabetic patients with the metabolic syndrome. However, it should be noted that the detrimental effect of rosiglitazone on apolipoprotein B did influence FMD adversely.

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計畫成果自評：

The anti-atherogenic effect of glitazone in patients with type 2 diabetes has been demonstrated in the recently published PROactive study. Our study extended this finding and proved that glitazone could be safely administrated in non-diabetic insulin-resistant patients. We also for the first time showed that endothelin-1 may be the prime pathogenic mediator of the anti-atherogenic effect of glitazones. In the future, we will examine the effect of glitazone on coronary vasculature by direct imaging modalities to further clarify its anti-atherogenic effect. This finding has been reported in Abstract from in the annual meeting of the European Society of Cardiology and will be published in high-ranking international medical journal.