Akt, AS160, metabolic risk factors and aerobic fitness in middle-aged women

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ABSTRACT

Aims: *This study investigated the association between the basal (rest) insulin-signaling proteins, Akt, and the Akt substrate AS160, metabolic risk factors, inflammatory markers and aerobic fitness, in middle-aged women with varying numbers of metabolic risk factors for type 2 diabetes.* ***Methods:*** *Sixteen women (n=16) aged 51.3±5.1 (mean ±SD) years provided muscle biopsies and blood samples at rest. In addition, anthropometric characteristics and aerobic power were assessed and the number of metabolic risk factors for each participant was determined (IDF criteria).* ***Results:*** *The mean number of metabolic risk factors was 1.6±1.2. Total Akt was negatively correlated with IL-1β (r = −0.45, p = 0.046), IL-6 (r = −0.44, p = 0.052) and TNF-α (r = −0.51, p = 0.025). Phosphorylated AS160 was positively correlated with HDL (r = 0.58, p= 0.024) and aerobic fitness (r = 0.51, p=0.047). Furthermore, a multiple regression analysis revealed that both HDL (t=2.5, p=0.032) and VO_{2peak} (t=2.4, p=0.037) were better predictors for phosphorylated AS160 than TNF-α or IL-6 (p>0.05).* ***Conclusions:*** *Elevated inflammatory markers and increased metabolic risk factors may inhibit insulin-signaling protein phosphorylation in middle-aged women, thereby increasing insulin resistance under basal conditions. Furthermore, higher HDL and fitness levels are associated with an increased AS160 phosphorylation, which may in turn reduce insulin resistance.*

Key words: insulin signaling, inflammation, cytokines, aerobic fitness

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INTRODUCTION

Individuals with clusters of metabolic risk factors for metabolic syndrome and type 2 diabetes (T2DM) present with increased inflammation (16). Increased inflammation, indicated by elevated plasma concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and C-reactive protein (CRP) may lead to abnormal glucose homeostasis, insulin resistance and type 2 diabetes (T2DM) (5, 7, 11). A possible contributor for the increase in inflammatory markers in middle-aged individuals is poor fitness level and increased visceral fat (2). The mechanism by which inflammatory markers affect insulin resistance is not fully understood. TNF-α (10, 11), CRP (5) and other low grade inflammatory markers, such as, IL-6 and IL-8, may lead to insulin resistance by inhibiting the phosphorylation of the insulin receptor substrate (IRS)-1, which in turn may impair downstream signaling (5). Akt and its 160 kDa (AS160) substrate are important downstream proteins in the phosphatidylinositol 3-kinase (PI3-K) pathway, and are proposed to form a link between the insulin-signaling cascade, and glycogen and protein synthesis (14). AS160 may also represent a common convergence point between the pathways regulating insulin and contraction stimulated GLUT-4 translocation (4). Insulin stimulated phosphorylation of both Akt (13) and AS160 (12) is reduced in individuals with T2DM during hyperinsulineamic clamp, compared to healthy controls. In addition, it has been reported that TNF-α treatment completely blocked insulin action on both Akt and AS160 leading to insulin resistance (3) Whether an association exists between metabolic risk factors, inflammatory markers, aerobic fitness and the phosphorylation of Akt and AS160 under basal conditions in middle-aged women is uncertain. Such associations are potentially important, because modern day sedentary lifestyles involve long periods of rest (including sleeping) or low-intensity activities of daily living. During these periods, food is not consumed, and therefore insulin levels are relatively low. We examined the association between metabolic risk factors, inflammatory markers, aerobic fitness and the phosphorylation of Akt and AS160 under basal conditions in women with varying numbers of metabolic risk factors. We hypothesized that elevated plasma concentrations of inflammatory markers and metabolic risk factors would be associated with reduced basal levels of Akt and AS160 (total and phosphorylation forms).

PATIENTS AND METHODS

Participants

Sixteen women (n=16) aged 51.3±5.1 (mean ±SD) years participated in the study. After initial medical assessment each participant was classified as having 0, 1, 2, 3 or 4 risk factors for metabolic syndrome as published by the International Diabetes Federation (18). These criteria include: waist circumference ≥80 cm for women, triglycerides ≥ 1.7mmol·L^{-1}, high density lipoprotein (HDL) <1.29 mmol·L^{-1} for women, systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg (or hypertensive medications) and fasting blood glucose level ≥5.6 mmol·L^{-1}. Participants also provided muscle samples and additional blood to measure the plasma concentrations of cytokines and CRP. One partici-

pant was using a beta-blocker and five were undergoing hormone replacement therapy. Each participant received written and verbal explanations about the nature of the study before giving informed consent. The study protocol was approved by the Human Research Ethics Committees of both Victoria University and Austin Health.

Procedures

Blood samples: Details of the blood collection and analysis have been reported previously (16). In brief, blood samples were collected after 12 hours of fasting. The blood was centrifuged and plasma was analysed for high sensitivity CRP using an automated analyser (SYNCHRON LX ® System/Lxi725, Beckman, USA). IL-1β, IL-6, IL-8 and TNFα were analysed using high sensitivity multiplex assays (Human High-Sensitivity Cytokine assay, LincoPlex, Millipore, Billerica, MA, USA), according to the manufacturer's protocol. Insulin resistance was estimated by the homeostasis model assessment (HOMA-IR). HOMA-IR was calculated from fasting glucose and insulin:

Insulin resistance = fasting glucose ($mmol \cdot L^{-1}$) × fasting insulin ($\mu U \cdot ml^{-1}$)/22.5 (9).

Aerobic power (VO_{2peak}): Complete details of the VO_{2peak} assessment have been reported previously (15). In brief, VO_{2peak} was assessed during a symptom-limited graded exercise test on a Cybex MET 100 cycle ergometer (Cybex Metabolic Systems, Ronkonkoma, NY, USA). The protocol consisted of an initial intensity of 25 W, with increments of 20 W.min-1 for males and 10 $W.min^{-1}$ for females. The tests were terminated when participants' ratings of perceived exertion reached "very hard" (Borg scale = 17). VO_2 for each 15 sec interval was measured by gas analysis (Medgraphics, Cardio2 and CPX/D System with Breezeex Software, 142090-001, Revia, MN, USA) that was calibrated before each test.

Muscle biopsy: Resting muscle samples were taken from the vastus lateralis under local anesthesia with Xylocaine 1%, utilizing the percutaneous needle biopsy technique with suction (8). The samples were immediately frozen in liquid nitrogen and were then stored at –80ºC until analysis.

Muscle Sample Preparation: Muscle (25–47 mg) was homogenised (Kinematica Polytron, Brinkmann, CT) in ice-cold lysis buffer consisting of 20 mmol•L-1 Tris-HCl, 5 $mmol \bullet L^{-1}$ EDTA, 10 $mmol \bullet L^{-1}$ Na-pyrophosphate, 100 $mmol \bullet L^{-1}$ NaF, 2 $mmol \bullet L^{-1}$ Na_3VO_4, 1% NP-40, and protease inhibitor tablet (Roche, Lewes, Sussex, UK). Homogenised muscle was incubated for 30 min at 4ºC and then centrifuged at 13000 g for 30 min. The supernatant was stored at –80ºC.

Total protein determination: Total protein concentration was determined using BCA Pearce protein assay kit (Pierce Biotechnology, IL, USA) using BSA as standard.

Antibodies: The phospho-Akt substrate (Ser/Thr) antibody, phospho-Akt (Ser473) antibody and Akt antibody were supplied by Cell Signaling Technology (Beverly, MA), and Anti-AS160 (Rab-GAP) was supplied from Upstate (NY, USA).

Blotting: Proteins were separated using 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in Tris-buffered saline with 0.5% Tween (TBST) at room temperature for 1 h. Membranes were incubated overnight at 4ºC with antibodies, diluted (1:2000) in blocking buffer. Membranes were washed

with TBST and protein was detected using secondary rabbit HRP-conjugated antibody (Cell Signaling Technology, MA, USA). Proteins were viewed by chemiluminescence (Western Lighting Chemiliuminescence Reagent Plus, Perkin Elmer, Boston, MA) and bands were quantified using Kodak Imaging software (Kodak ID 3.5, Boston, MA).

Statistics

Spearman Rho correlations were used to assess the relationship between Akt and AS160 and inflammatory markers and metabolic risk factors. Correlations were also controlled for age and the number of metabolic risk factors for each person (0, 1, 2, 3 or 4). Data was analyzed with SPSS (Version 16). Data are reported as mean ± SD and all statistical analyses were conducted at the 95% level of significance.

Table 1. Participant's characteristics

Variable	Women (n=16)
Age (yrs)	51.3±5.1
Height (cm)	161.2±6.2
Mass (kg)	72.0±14.6
Waist (cm)	87.2±12.7
BMI ($kg \cdot m^{-2}$)	27.6±5.5
Fasting Glucose ($mmol \cdot L^{-1}$)	5.3±0.6
HOMA-IR	1.21±1.0
Triglyceride ($mmol \cdot L^{-1}$)	1.1±0.6
LDL ($mmol \cdot L^{-1}$)	3.4±0.8
HDL ($mmol \cdot L^{-1}$)	1.8±0.5
SBP (mmHg)	126.8±14.5
DBP (mmHg)	83.5±7.5
VO_{2peak} ($ml \cdot kg^{-1} \cdot min^{-1}$)	21.6±4.1
IL-1β ($pg \cdot mL^{-1}$)	6.0±4.2
IL-6 ($pg \cdot mL^{-1}$)	16.8±13.9
IL-8 ($pg \cdot mL^{-1}$)	8.2±8.6
TNFα ($pg \cdot mL^{-1}$)	6.0±3.6
CRP ($mg \cdot L^{-1}$)	2.8±1.8
N of risk factors (IDF criteria)	
0	3
1	5
2	4
3	3
4	1
Total	1.6±1.2

BMI (body mass index), HOMA-IR (homeostasis model assessment of insulin resistance), LDL (low-density lipoprotein), HDL (high-density lipoprotein), SBP (systolic blood pressure), DBP (diastolic blood pressure), VO_{2peak} (peak oxygen consumption), IL (interleukin), TNFα (tumor necrosis factor alpha), CRP (C-reactive protein), IDF (International Diabetes Federation).

RESULTS

Participants' characteristics are presented in Table 1. Participants presented with a range of metabolic risk factors including obesity, impaired fasting glucose, hypertension and dyslipidaemia.

Total Akt was correlated with total AS160 ($r = 0.70$, $p < 0.002$) and phosphorylated Akt was correlated with phosphorylated AS160 ($r = 0.70$, $p < 0.002$).

Total Akt was negatively correlated with IL-1β, IL-6 and TNF-α (Figure 1A, B and C). In addition, when data were adjusted for age, total AS160 was negatively correlated with IL-6 ($r = -0.58$, $p = 0.025$), IL-8 ($r = -0.51$, $p = 0.045$), and TNF-α ($r = -0.56$, $p = 0.03$). Phosphorylated AS160 was positively correlated with HDL ($r = 0.58$, $p = 0.019$) and aerobic fitness ($r = 0.58$, $p = 0.018$). A multiple regression analysis revealed that both HDL ($t=2.5$, $p=0.032$) and VO_{2peak} ($t=2.4$, $p=0.037$) were better predictor for phosphorylated AS160 than TNF-α or IL-6 ($p>0.05$).

DISCUSSION

The main finding of the current study was that middle-aged women inflammatory markers correlated negatively with basal

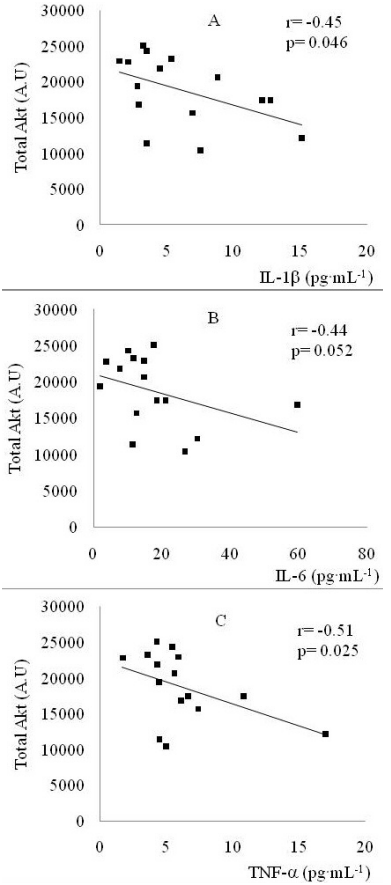

Figure 1. Correlation between total Akt and IL-1β, IL-6 and TNF-α in middle-aged women.

levels of both total and phosphorylated Akt and AS160 levels. Furthermore, HDL and aerobic fitness correlated positively with the basal level of phosphorylated AS160.

Metabolic risk factors, insulin resistance and T2DM are associated with increased inflammation (7, 16). Inflammation may alter insulin-signaling such as IRS-1 (11), which in turn may impair insulin action, and therefore, lead to insulin resistance (10, 11). In addition, Bouzakri and Zierath (3) have reported the TNF-α induced insulin resistance as it blocks insulin action on Akt and AS160. In the present study we found an association between the plasma concentrations of IL-6 and IL-8 and downstream insulin-signaling proteins (total and phosphorylated Akt and AS160) in women with varying number of metabolic risk factors. These findings support those of Bouzakri and Zierath (3) and indicate that low-grade inflammation inhibits not only upstream proteins (e.g. IRS-1) but also down-stream proteins that regulate GLUT-4 translocation and glucose homeostasis.

Inflammatory markers IL-1β, IL-6 and TNF-α were also associated with a reduction in total Akt and total AS160. It is important to acknowledge that some females were on hormone replacement therapy (HRT) which may influence the interactions between insulin-signaling proteins and inflammatory cytokines. Some studies have reported that HRT can increase inflammatory markers (such as plasma CRP concentration) by almost 60%,whereas other studies have reported that HRT reduces in the plasma concentrations of IL-6 and TNF-α (for review see reference (17).

In this study, higher fitness levels and plasma HDL concentrations were associated with phosphorylated AS160. In addition, a multiple regression analysis showed that both HDL and aerobic fitness, but not TNF-α or IL-6, were predictors of phosphorylated AS160 in this population. This finding is important because it suggests that middle-aged women in general, and particularly those with metabolic risk factors, should participate in aerobic exercise and other lifestyle interventions such as dietary modification. When combined, these interventions improve insulin resistance, glucose control and metabolic risk factors (for review see (1). Arsenault et al (2) have also reported that there is a negative association between

aerobic fitness and inflammatory markers (CRP and IL-6) in middle-aged women. They concluded that people with low fitness levels are characterized by increased inflammatory markers. We have provided further evidence that systemic inflammation is also associated with reduced expression of insulin-signaling proteins in skeletal muscle. Collectively, these findings may suggest that low aerobic fitness is associated with increased low-grade inflammation, both of which may have negative influence insulin-signaling proteins, leading to increased insulin resistance.

Drew et al. (6) have recently reported that intravenous HDL treatment markedly increases phosphorylation of acetyl-CoA carboxylase β phosphorylation (an indicator of AMPK activation) in skeletal muscle of patients with T2DM. This finding suggests that increased HDL can reduce insulin resistance in T2DM. In the current study, we found that HDL correlates with phoshorylated AS160, which also suggests that higher levels of HDL increase insulin-signaling phosphorylation, which in turn may improve insulin resistance. As suggested by Drew et al, it is possible that the mechanism involves the AMP-activated protein kinase pathway. Larger, more detailed studies are needed to further explore the mechanisms behind the relationship between insulin resistance, HDL levels and skeletal muscle AMPK activation in this population.

In conclusion, the present data indicate that elevated systemic markers of inflammation, and increased metabolic risk factors may inhibit insulin-signaling protein phosphorylation, thereby increasing insulin resistance under basal conditions. Furthermore, higher HDL and fitness levels are associated with an increase AS160 phosphorylation, which may in turn reduce insulin resistance.

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Declaration of Competing Interests: Nothing to declare.

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