

Rapid Communication

Aberrant p38 mitogen-activated protein kinase signalling in skeletal muscle from Type 2 diabetic patients

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Abstract

Aims/hypothesis. p38 mitogen activated protein kinase (MAPK) is generally thought to facilitate signal transduction to genomic, rather than metabolic responses. However, recent evidence implicates a role for p38 MAPK in the regulation of glucose transport; a site of insulin resistance in Type 2 diabetes. Thus we determined p38 MAPK protein expression and phosphorylation in skeletal muscle from Type 2 diabetic patients and non-diabetic subjects.

Methods. In vitro effects of insulin (120 nmol/l) or AICAR (1 mmol/l) on p38 MAPK expression and phosphorylation were determined in skeletal muscle from non-diabetic ($n=6$) and Type 2 diabetic ($n=9$) subjects.

Results. p38 MAPK protein expression was similar between Type 2 diabetic patients and non-diabetic subjects.

Insulin exposure increased p38 MAPK phosphorylation in non-diabetic, but not in Type 2 diabetic patients. In contrast, basal phosphorylation of p38 MAPK was increased in skeletal muscle from Type 2 diabetic patients.

Conclusion/interpretation. Insulin increases p38 MAPK phosphorylation in skeletal muscle from non-diabetic subjects, but not in Type 2 diabetic patients. However, basal p38 MAPK phosphorylation is increased in skeletal muscle from Type 2 diabetic patients. Thus, aberrant p38 MAPK signalling might contribute to the pathogenesis of insulin resistance. [Diabetologia (2003) 46:1324–1328]

Keywords 5-AMP activated protein kinase, 5-aminoimidazole-4-carboxamide ribonucleoside, insulin signalling, glucose transport, mitogen-activated protein kinase.

Impaired insulin-mediated whole body glucose uptake in Type 2 diabetes [1, 2, 3, 4, 5], as well as in several other insulin resistant states such as morbid obesity [6, 7], polycystic ovary syndrome [8], gestat-

ional diabetes [9, 10, 11] or pancreatic cancer [12], is associated with defects in insulin signalling to glucose transport in skeletal muscle. Impairments in insulin-stimulated insulin receptor phosphorylation, insulin receptor tyrosine kinase activity, insulin receptor substrate 1 (IRS-1), and phosphatidylinositol (PI) 3-kinase activity have been reported in skeletal muscle from Type 2 diabetic patients [2, 3, 4, 5, 13, 14, 15], as well as in insulin resistant subjects [6, 8, 9, 10, 11, 12], defining a molecular mechanism for whole body insulin resistance on glucose uptake. While insulin signalling to IRS-1 and PI 3-kinase is impaired in skeletal muscle from Type 2 diabetic patients, phosphorylation of the mitogen-activated protein kinase (MAPK) extracellular regulated kinase (ERK1/2) is normal [4, 5], suggesting diversity in insulin action along metabolic and mitogenic signalling pathways in Type 2 diabetes.

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Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, 5-AMP activated protein kinase; ERK 1/2, extracellular regulated kinase; GIR, glucose infusion rate; IRS-1, insulin receptor substrate 1; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; VO_{2max} , maximal oxygen uptake.

MAPK's are part of a large family of related serine/threonine protein kinases that include the ERK1/2, p38 MAPK and c-jun and form a major signalling system to facilitate signal transduction to appropriate genomic, rather than direct metabolic responses [16, 17]. However, p38 MAPK has recently been proposed to play a metabolic role in the regulation of insulin-stimulated glucose transport by either altering the "intrinsic activity of GLUT4" or by facilitating the transition of GLUT4 from an occluded to a fully activated (exposed) state at the plasma membrane [18]. When 3T3-L1 adipocytes and L6 myotubes are co-incubated with insulin and a p38 MAPK inhibitor, glucose transporter translocation to cell surface is normal, whereas the glucose transport is attenuated [19, 20]. Likewise, exposure of Clone 9 cells to the p38 MAPK inhibitor SB203580 or overexpression of dominant negative p38 MAPK mutant, inhibits 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) mediated glucose transport [21]. Since AICAR increases glucose transport via an insulin-independent pathway, presumably involving 5-AMP activated protein kinase (AMPK) [22], p38 MAPK might represent a point of convergence between insulin-dependent and insulin-independent pathways regulating glucose transport. Collectively, these findings have led to the hypothesis that activation of p38 MAPK is a prerequisite for full activation of glucose transport, presumably via activation of GLUT4 [23].

Basal phosphorylation of several MAPK's, and most notably that of p38 MAPK, is increased in adipocytes from Type 2 diabetic patients [24], implicating the p38 MAPK signalling pathway in the pathogenesis of insulin resistance. Since skeletal muscle is the major site of insulin-stimulated glucose disposal, the aim of this study was to examine p38 MAPK protein expression and phosphorylation in skeletal muscle from Type 2 diabetic patients and non-diabetic subjects.

Materials and methods

Subjects. The study protocol was reviewed and approved by the institutional ethical committee of the Karolinska Institutet and informed consent was received from all subjects prior to participation. Subjects with a normal resting electrocardiogram, normal blood count, normal kidney, liver and thyroid function were studied (Table 1). Non-diabetic subjects with impaired glucose tolerance, as determined by 2-h plasma glucose concentration greater than or equal to 7.8 mmol/l following the standard 75 g OGTT [25], smokers or subjects using anti-hypertensive medication (β -blocking agent, angiotensin converting enzyme-inhibitors, Ca^{2+} -inhibitors or diuretics) were excluded. The diabetic patients were treated with diet ($n=1$), sulphonylurea ($n=4$), metformin ($n=1$), a combination of sulphonylurea, metformin, acarbose and insulin ($n=1$), a combination of sulphonylurea and metformin ($n=1$), or with insulin only ($n=1$). Their mean duration of diabetes was 5 years (range 2–11 years). All subjects were instructed to avoid strenuous exercise for 72 h before participating in the study. On study days, subjects reported to the laboratory after an overnight fast,

and in case of diabetic patients, before administration of any anti-diabetic medication.

Euglycaemic hyperinsulinaemic clamp. Whole body insulin-stimulated glucose disposal was determined using the euglycaemic hyperinsulinaemic (40 mU/m²/min for 180 min) clamp technique [26]. The glucose infusion rate (GIR) required to maintain euglycaemia during last hour (120–180 min) of the clamp was used as a measure of whole body insulin sensitivity.

Blood chemistry, aerobic capacity and body composition. Plasma glucose concentration was determined using glucose oxidase method (Beckman Instruments, Fullerton, Calif., USA), plasma free insulin and C-peptide concentrations with commercial radioimmunoassays (Pharmacia, Uppsala, Sweden), and HbA_{1c} with an immunological method. Maximal oxygen uptake ($\text{VO}_{2\text{max}}$) was determined on a bicycle ergometer on a separate occasion. $\text{VO}_{2\text{max}}$ was measured continuously with a breath-by-breath data collection technique (Erich Jaeger, Hoechst, Germany). Regional analysis of lean body mass, body fat, and bone mineral content was carried out by dual-energy X-ray absorptionmetry (Lunar, Madison, Wis., USA).

Open muscle biopsy and in vitro incubation of human skeletal muscle. Open biopsies were taken from vastus lateralis muscle under local anaesthesia (mepivacain chloride 5 mg/ml) [27]. Smaller muscle strips were dissected and incubated for 60 min in the absence (basal) or presence of insulin (120 nmol/l), AICAR (1 mmol/l), or a combination of AICAR (1 mmol/l) and insulin (120 nmol/l) as [28]. Thereafter, muscle specimens (15–30 mg wet weight) were homogenised (30 strokes by glass-on-glass homogenisation on ice) in 400 μl HES buffer (255 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l HEPES, pH 7.2) containing 2 $\mu\text{g/ml}$ aprotinin, leupeptin, pepstatin and 400 $\mu\text{mol/l}$ phenylmethylsulfonyl fluoride and subjected to centrifugation at 150 000 g for 1 h at 4°C to obtain total membranes and cytosolic fraction.

p38 MAP kinase phosphorylation and expression. Equal amounts of total cytosolic protein (13.3 μg) was separated on 7.5% SDS-PAGE. Following electrophoresis, proteins were transferred to polyvinylidenedifluoride membranes (Millipore, Bedford, Mass., USA). Membranes were blocked in TBST (10 mmol/l Tris, 100 mmol/l NaCl, 0.02% Tween-20) containing 7.5% non-fat milk for 2 h at room temperature, washed with TBST for 10 min, and then incubated with appropriate primary antibody overnight at 4°C. To determine p38 MAPK phosphorylation, membranes were subjected to immunoblot analysis with a phosphospecific p38 MAPK antibody that recognises p38 MAPK phosphorylated at Thr¹⁸⁰ and Tyr¹⁸² (Cell Signaling Technology, Beverly, Mass., USA). Following the primary antibody incubation, membranes were washed with TBST and incubated with appropriate secondary antibody for 1 h at room temperature, followed by washing in TBST. Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Amersham, Arlington Heights, Ill., USA) and quantified by densitometric scanning. Membranes were then incubated in stripping buffer (62.5 mmol/l Tris, pH 6.7, 2% SDS, and 100 mmol/l β -mercaptoethanol) for 30 min at 60°C, washed extensively in TBST, and subjected to immunoblot analysis to determine p38 MAPK protein expression (Cell Signaling Technology). Immunoreactive proteins were detected and quantified as described above.

Statistical analysis. Data are presented as means \pm SEM. Wilcoxon's test and Mann-Whitney U-test were used in the analysis of paired and unpaired data, respectively. A p value of less than 0.05 was considered statistically significant.

Table 1. Subject characteristics

	Non-diabetic (n=6)	Type 2 diabetic (n=9)
Age (yrs)	57±3	60±1
BMI (kg/m ²)	27.3±0.7	27.1±0.6
Body fat (%)	23.0±2.9	24.0±1.5
VO _{2max} (ml×kg ⁻¹ ×min ⁻¹)	33.3±3.8	28.1±2.4
GIR (μmol×kg ⁻¹ ×min ⁻¹)	35.4±4.5	17.7±2.7 ^a
Plasma glucose (mmol/l)	5.5±0.1	9.1±0.5 ^a
Insulin (mU/l)	24±6	54±6 ^a
C-peptide (nmol/l)	0.62±0.06	0.95±0.12
HbA _{1c} (%)	4.6±0.1	6.1±0.4 ^a
Triglyceride (mmol/l)	1.0±0.1	1.6±0.2 ^b
Cholesterol (mmol/l)	5.1±0.3	4.7±0.3
HDL (mmol/l)	1.28±0.07	1.12±0.08
Diabetes Duration (yrs)	–	5±1

GIR=glucose infusion rate during 120–180 min of the euglycaemic hyperinsulinaemic clamp period. ^a*p*<0.01, ^b*p*=0.05

Results

Subject characteristics. Type 2 diabetic patients and non-diabetic subjects were matched with respect to age, adiposity and physical fitness (Table 1). Plasma glucose, insulin and HbA_{1c} concentrations were higher, and glucose disposal was lower in Type 2 diabetic patients compared to non-diabetic subjects.

p38 MAPK protein expression and phosphorylation. p38 MAPK protein expression was similar in skeletal muscle from non-diabetic and Type 2 diabetic patients (Fig. 1A). p38 MAPK protein expression was not modified by 60 min exposure to insulin, AICAR or combination of AICAR and insulin. Exposure to insulin increased p38 MAPK phosphorylation 126% in skeletal muscle from non-diabetic subjects (*p*<0.05; Fig. 1B), but not in Type 2 diabetic patients. In contrast, p38 MAPK phosphorylation tended to decrease 42% following exposure to insulin in Type 2 diabetic subjects (*p*=0.05). Basal p38 MAPK phosphorylation was increased 213% in skeletal muscle from Type 2 diabetic patients (*p*<0.05; Fig. 1B) compared to non-diabetic subjects. Exposure of skeletal muscle to AICAR and a combination of AICAR and insulin increased p38 MAPK phosphorylation in non-diabetic subjects; however, this did not reach statistical significance.

Discussion

Insulin-stimulated glucose transport and translocation of insulin sensitive glucose transport protein, GLUT4, to cell surface is impaired in skeletal muscle from Type 2 diabetic patients [28, 29, 30]. Recent studies in cultured cells and animal models have provided evi-

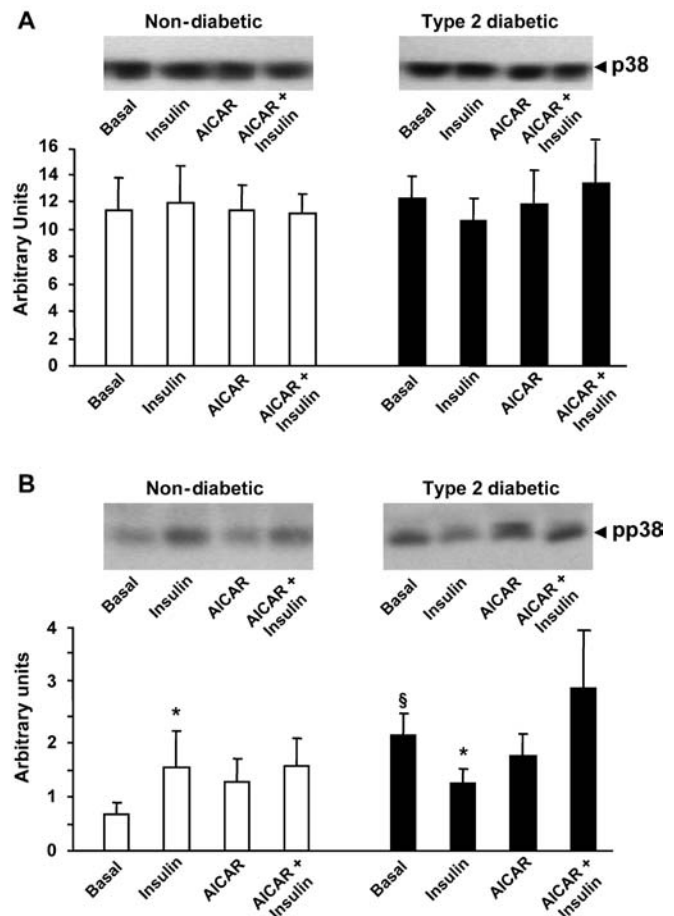


Fig. 1A, B. p38 MAPK protein expression and phosphorylation in skeletal muscle from Type 2 diabetic and control subjects. p38 MAPK protein expression (A) and phosphorylation (B) was determined in skeletal muscle after *in vitro* incubation in the absence (basal) or presence of 120 nmol/l insulin, 1 mmol/l AICAR (*n*=9 Type 2 diabetic and *n*=6 control subjects) or a combination of AICAR and insulin (*n*=6 Type 2 diabetic and *n*=5 control subjects). Muscles were homogenised, and lysates were subjected to immunoblot analysis. Representative immunoblot and graph for means ± SEM arbitrary densitometric units are shown. **p*≤0.05 vs basal, §*p*<0.05 vs non-diabetic subjects

dence to suggest that p38 MAPK phosphorylation is required for full activation of glucose transport [19, 20, 21, 31]. Thus, p38 MAPK could be important for the regulation of glucose uptake in insulin-sensitive tissues. Basal phosphorylation of p38 MAPK is increased in adipocytes from Type 2 diabetic patients, and this is linked to decreased GLUT4 expression. [24]. Here we report p38 MAPK phosphorylation is increased in skeletal muscle from Type 2 diabetic patients. The observation of increased basal p38 MAPK phosphorylation in two important insulin target tissues, namely skeletal muscle and adipose tissue, supports the hypothesis that p38 MAPK could play a role in the pathogenesis of insulin resistance in Type 2 diabetes. Alternatively, increased p38 MAPK phosphorylation in muscle could be a consequence of diabetes,

without any causal role in its pathogenesis. However in contrast to adipocytes [24], increased p38 phosphorylation in skeletal muscle cannot be linked to changes in either IRS-1 or GLUT4 protein expression [5, 32, 33]. Furthermore, p38 MAPK protein expression is not altered in skeletal muscle from Type 2 diabetic patients. While increased p38 MAPK phosphorylation could be a common feature of skeletal muscle and adipose tissue in Type 2 diabetes, tissue-specific responses to increased p38 MAPK signalling are envisaged.

Insulin increased p38 MAPK phosphorylation in skeletal muscle from healthy subjects. This finding is in contrast to studies in adipocytes, whereby insulin stimulation did not alter p38 MAPK phosphorylation in either healthy control subjects or Type 2 diabetic patients [24] and probably reflects cell-specific differences in the regulation of p38 MAPK. When skeletal muscle from Type 2 diabetic patients was incubated with insulin, p38 MAPK phosphorylation tended to decrease. The mechanism for the reduction in p38 MAPK signalling in skeletal muscle from Type 2 diabetic patients under insulin-stimulated conditions is not clear, but could involve increased protein phosphatase activity to repress p38 MAPK signalling. In absolute terms, the phosphorylation of p38 MAPK under insulin-stimulated conditions was comparable to control subjects. Thus, differences in insulin-mediated p38 MAPK signalling are unlikely to play any major role in insulin resistance of muscle glucose transport in Type 2 diabetes. While the insulin-response of p38 MAPK phosphorylation was altered in skeletal muscle from Type 2 diabetic patients, phosphorylation of another MAPK, namely ERK 1/2, is normal [4, 5]. Thus, there is divergence in insulin responses along MAPK kinase cascades in skeletal muscle from Type 2 diabetic subjects.

Increased glucose and insulin concentration in Type 2 diabetic subjects might contribute to increased basal p38 MAPK phosphorylation, as sustained exposure to high glucose and insulin concentrations has been observed to increase both phosphorylation and activity of p38 MAPK in cultured L6 myotubes [34]. Increased basal p38 MAPK phosphorylation might be a compensatory mechanism for hyperglycaemia to increase glucose transport. Yet the direct link between p38 MAPK and GLUT4 has yet to be revealed. GLUT4 is not likely to represent a direct substrate of p38 MAPK, because Ser⁴⁸⁸, the major phosphorylated residue in GLUT4, does not lie within a p38 MAPK consensus phosphorylation site [18], thus other targets are likely to be involved in this putative pathway. Nevertheless, when 3T3-L1 adipocytes and L6 myotubes are co-incubated with insulin and a p38 MAPK inhibitor, glucose transporter translocation to cell surface is normal, whereas the glucose transport is attenuated [19, 20]. This would imply that p38 MAPK is required for full insulin-stimulated glucose transport.

Glucose transport in skeletal muscle can also be mediated by an insulin-independent mechanism involving AMPK [22]. Exposure of skeletal muscle from non-diabetic subjects to AICAR, an activator of AMPK [28], or a combination of AICAR and insulin, tended to increase p38 MAPK phosphorylation, although this was not statistically significant. Thus, p38 MAPK could be a point of convergence in insulin-dependent and insulin-independent signalling cascades to glucose transport since p38 MAPK inhibition also impairs AICAR-mediated glucose transport [21].

In conclusion, p38 MAPK protein expression is normal in skeletal muscle from Type 2 diabetic patients. Insulin increases p38 MAPK phosphorylation in skeletal muscle from healthy subjects, but not in Type 2 diabetic patients. In contrast, basal phosphorylation of p38 MAPK is increased in skeletal muscle from Type 2 diabetic subjects. Thus, altered p38 MAPK signalling could contribute to the pathogenesis of insulin resistance.

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