Local activation of the IκK-NFκB pathway in muscle does not cause insulin resistance

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Abstract

Insulin resistance of skeletal muscle is a major defect in obesity and type 2 diabetes.

Insulin resistance has been associated with a chronic sub-clinical inflammatory state in epidemiological studies and specifically with activation of the Inhibitor κ-B kinase (IκBK)- Nuclear factor κ-B (NFκB) pathway. However it is unclear whether this pathway plays a role in mediating insulin resistance in muscle in vivo. We separately over-expressed the p65 sub-unit of NFκB and IκBKβ in single muscles of rats using in vivo electrotransfer and compared the effects after one week versus paired contralateral control muscles. A 64% increase in p65 protein (p<0.001) was sufficient to cause muscle fibre atrophy but had no effect on glucose disposal or glycogen storage in muscle under hyperinsulinaemic-euglycaemic clamp conditions. Similarly a 650% increase in IκBKβ expression (p<0.001) caused a significant reduction in Inhibitor κ-B protein but also had no effect on clamp glucose disposal after lipid infusion. In fact, IκBKβ over-expression in particular caused increases in activating tyrosine phosphorylation of Insulin Receptor Substrate-1 (24%; p=0.02) and serine phosphorylation of Akt (23%; p<0.001), implying a moderate increase in flux through the insulin signalling cascade. Interestingly, p65 over-expression resulted in a negative feedback reduction of 36% in Toll-like Receptor (TLR)-2 (p=0.03) but not TLR-4 mRNA. In conclusion activation of the IκBKβ-NFκB pathway in muscle does not seem to be an important local mediator of insulin resistance.

Keywords
IκK-NFκB pathway, skeletal muscle, insulin resistance, Toll-like receptors, in vivo electrotransfer
Introduction

Type 2 diabetes (T2D) and obesity are intimately related and rapidly increasing human health problems world-wide. Insulin resistance in skeletal muscle is regarded as an essential pre-requisite for the development of T2D, manifesting primarily as impaired insulin-stimulated glucose disposal. It is now widely accepted that inappropriate deposition of lipids in skeletal muscle is a principal cause of impaired muscle glucose disposal (21; 34). However the molecular mechanisms linking these phenomena have yet to be fully established.

Several lines of evidence now exist to implicate a subclinical inflammatory state in the aetiology of insulin resistance. Numerous epidemiological associations have been made between insulin resistance and either increased plasma levels of acute phase proteins released by the liver (17; 37) or pro-inflammatory cytokines, most notably Tumour necrosis factor (TNF)-α (45). TNF-α is one of a number of cytokines / adipokines released in increased quantities by adipose tissue in obese humans (24) and in animal models of obesity (25) and has been shown to impair peripheral glucose disposal (30), although whether TNFα impairs glucose uptake into muscle through impairment in insulin signalling is less clear (11; 16).

The observation that administration of salicylates to diabetics has an insulin-sparing effect provided further evidence of a role for the immune system in diabetes (35). The molecular target of salicylates was subsequently discovered to be Inhibitor kappa-B kinase β (IκBKβ) (49), and indeed when the corresponding gene was targeted for knock-
out, the mice were able to withstand diet-induced insulin resistance (50). IκBKβ is a catalytic subunit of a trimeric serine kinase which also consists of a second catalytic subunit (IκBKα) and a regulatory subunit (IκBKγ). This enzyme lies upstream of the transcription factor Nuclear Factor kappa-B (NFκB) and together these molecules comprise the principal regulatory pathway for the immune response (5). NFκB is typically a heterodimer which can be composed of two of a number of different subunits including the transactivating unit p65 (5), although artificial over-expression of p65 facilitates the formation of homodimers, which show substantially enhanced activity (31). NFκB dimers are retained in a cytoplasmic location through binding an Inhibitor kappa-B (IκB) molecule. When phosphorylated by IκBK these inhibitors detach from the complex and are targeted for proteasomal degradation, thus permitting translocation of NFκB to the nucleus and transactivation of its target genes. These classically include cytokines, enzymes and adhesion molecules that mediate the inflammatory process (5).

Activation of the IκBKβ-NFκB pathway in muscle is one way that accumulation of tissue lipids may induce insulin resistance. Evidence supporting this hypothesis has come from studies of both myocyte cell culture systems (26; 42) and the liver in vivo (4; 47) but no causative link has been established between activation of the IκBKβ-NFκB pathway in muscle and local insulin resistance. The pathway may be activated by binding of TNFα to its cognate receptor or as a result of recognition of excess lipids by one or more of the Toll-like Receptors (TLRs) (6; 39; 40). However it is unclear whether this mechanism is significant in skeletal muscle in vivo or indeed whether activation of IκBKβ or nuclear translocation of NFκB are the more significant events in mediating such an effect. Studies
of NFκB inhibition have suggested that transactivation of specific gene targets may be the mechanism whereby activation of the pathway influences insulin sensitivity (8; 26; 47). However the serine phosphorylation of Insulin Receptor Substrate (IRS)-1 by stress kinases including IκBκβ has been more widely proposed as a principal cause of muscle insulin resistance (8; 27; 32; 48). Nevertheless some recent studies have failed to show an association between impaired IRS-1 expression and insulin resistance in muscle in vivo (15; 18).

Thus in this study we aimed to determine whether local activation of the IκBκβ-NFκB pathway is sufficient to cause insulin resistance in muscle and whether this is mediated principally through a direct transcriptional effect of NFκB or via the kinase activity of IκBκβ. To achieve these aims we separately over-expressed each protein in single muscles of adult rats using in vivo electrotransfer (IVE) and compared the effects of each manipulation on insulin sensitivity with the contralateral control muscle one week later. This approach has the advantage of minimising the potential confounding effects of developmental or whole body physiological compensation seen in traditional germ-line genetic manipulation techniques (13).

Materials and Methods

Vector Construction

The muscle specific mammalian expression vector EH114 has been described previously (13). EH114 was converted to a GatewayR destination vector using the GatewayR Vector Conversion System (Invitrogen, Mount Waverley, Victoria, Australia) by insertion of the
cassette into the *EcoRV* site. The Donor vector pDONR201-BSIIMCS was created by recombination of pDONR201 with the multiple cloning site of pBluescriptII KS(+) prepared by PCR using 5'-

GGGGACAAGTTTGTACAAAAAAGCAGGCTACTCACTATAGGGCGAATTG-3’

and 5’-

GGGGACCACTTTGTACAAGAAGGCTGGGTCTAAAGGGAACAAAAGCTG-3’

primers (GeneWorks Pty Ltd, Hindmarsh, South Australia). pcDNA3 expressing the cDNA for the human p65 subunit of NFκB coupled to an n-terminal c-myc tag was kindly donated by Shane Grey, Garvan Institute (43). The *HindIII* –*XbaI* excised fragment was blunt-ended and ligated into EH114 to make EH114-p65. pCMV vector containing human cDNA corresponding to IκBκ was a generous gift from Steven Shoelson, Harvard Medical School, Boston, MA. A *SacII-NotI* excised fragment was subcloned into the corresponding sites of pDONR201-BSIIMCS and then recombined into EH114-GW using LR Clonase II to make EH114-GW- IκBκ. Molecular reagents were supplied by Promega Corp (Annandale, NSW, Australia), Invitrogen and New England Biolabs (Genesearch Pty Ltd, Arundel, Queensland, Australia).

**Animal maintenance and surgery**

Male Wistar rats (ca.150g) were obtained from the Animal Resources Centre (Perth, Australia) and acclimatised to their new surroundings for one week. Animals were maintained at 22 ± 0.5°C under a 12-h day/12-h night cycle and were fed a standard chow diet (Norco, Kempsey, Australia) (18% fat, 33% protein and 48% carbohydrate as a percentage of total dietary energy) *ad libitum*. Approximately one week before study, the
right and left jugular veins of rats were cannulated as previously described (12).

Anaesthesia was induced with 5% and maintained with 1–2% halothane in oxygen. The surgical site was irrigated with bupivicaine (0.5mg/100g) prior to closure and 5mg/kg ketoprofen was administered to provide post-operative analgesia. Rats were single-housed and handled daily for the following week to minimise separation anxiety. Body weight was recorded daily, and only those rats which had fully recovered their pre-surgery weight were subsequently studied. All experimental procedures were approved by the Garvan Institute/St. Vincent’s Hospital Animal Experimentation Ethics Committee and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation. n = 7-11 per group unless otherwise stated.

**In vivo Electro-transfer**

Preparation and injection of DNA and electro-transfer was carried out as previously described (13). Paired tibialis cranialis muscles (TCMs) were injected percutaneously with six spaced 50µl aliquots of DNA prepared in endotoxin-free sterile saline (Qiagen Maxi/ Mega-Prep kits, Doncaster, Victoria, Australia) at 0.5mg/ml. In each case right TCMs from each animal were injected with test constructs and left TCMs were injected with empty EH114 vector as within-animal control. One 800V/cm 100µs electrical pulse and four 80V/cm 100ms pulses at 1Hz were administered sequentially via tweezer electrodes attached to an ECM-830 electroporator (BTX, Holliston, MA) immediately afterwards. Empty vector was used as control in preference to the enhanced green fluorescence protein vector previously described (13), as expression of GFP alone was
shown in preliminary experiments to increase p65 protein expression (data not shown). This method has previously been shown by our group (13; 15) and others (29) to alter expression of signalling molecules sufficiently to cause altered glucose disposal into muscle.

**Assessment of in vivo glucose metabolism in rats under euglycaemic-hyperinsulinaemic clamp conditions**

Conscious rats were studied after 5–7h of fasting. One jugular cannula was connected to an infusion line and the other to a sampling line between 8:30 and 9:30am and the rats were then allowed to acclimatise to the study cage for 30–40 min. Hyperinsulinaemic-euglycaemic clamps were conducted as described (12), involving a variable infusion of 30% glucose and a rate of insulin infusion commensurate with the generation of normal post-prandial plasma levels. A combined bolus injection of 2-deoxy-D-[2,6-³H]-glucose and D-[U-¹⁴C]-glucose (Amersham Biosciences, Little Chalfont, Bucks, UK) was administered 45 minutes before the end of the clamp. In rats which had been electroporated with IκBKβ the clamp was preceded by a one hour infusion of 2% Intralipid (Travenol, Sydney, Australia)/ 0.9% saline containing 40iu/ml heparin, which was continued for the duration of the clamp. This short term infusion has been shown to be sufficient to activate total muscle IκBK (3) but not to induce insulin resistance in muscle that might disguise the effects of the genetic manipulation (14). At the end of each study, rats were euthanased by intravenous injection of pentobarbitone sodium (Nembutal; Abbott Laboratories, Sydney, Australia) and their muscles rapidly dissected and freeze-clamped using liquid nitrogen-cooled tongs. Plasma glucose tracer
disappearance was used to calculate whole body glucose disposal (\(R_d\)). Endogenous glucose output (EGO) was derived from the difference between \(R_d\) and the net Glucose Infusion rate (GIR). The area under the tracer disappearance curve of 2-deoxy-D-[2,6,\(^3\)H] glucose together with the disintegrations per minute of phosphorylated \([\^3\text{H}]\) deoxyglucose from individual muscles were used to calculate insulin-stimulated glucose metabolic index (Rg'), an estimate of tissue glucose uptake (28).

During clamps plasma was immediately obtained from withdrawn blood by centrifugation and glucose was determined immediately using a glucose analyser (YSI Inc. 2300, Yellow Springs, OH). The remaining plasma was frozen in liquid nitrogen and subsequently used for plasma insulin determination by radioimmunoassay (Linco Research Inc., St. Charles, MO). Muscle glycogen was analysed as previously (10). Glucose incorporation into glycogen was determined from the D-[U-\(^{14}\text{C}\)]-glucose tracer disappearance curve and counts of \([^{14}\text{C}]\) in muscle as previously described (28).

**Muscle lysates, SDS-PAGE and immunoblotting**

Protein expression and phosphorylation of molecules present in muscle was assessed by SDS-PAGE and quantification of western blots of cell lysates. Whole tissue lysates were prepared from dismembranated muscle (Mikro-dismembranator II, B. Braun Biotech, Melsungen, Germany) by manual homogenisation in RIPA buffer (65mmol/l tris, 150mmol/l NaCl, 5mmol/l EDTA, pH 7.4, 1% (v/v) NP-40 detergent, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, containing 25\(\mu\)g/ml leupeptin, 10\(\mu\)g/ml aprotinin, 2mmol/l sodium orthovanadate, 1mmol/l sodium pyrophosphate, 10mmol/l NaF and 1mmol/l polymethylsulphonyl fluoride (PMSF))
followed by incubation for 90 minutes at 4°C and centrifugation for 10 minutes at 12000g. Protein content of supernatants was quantified using the Bradford method (Protein Assay kit, Bio-Rad laboratories, Regents Park, NSW, Australia), and aliquots containing 10-60µg protein were denatured in Laemmli buffer for 5 minutes at 95°C or 10 minutes at 65°C. Proteins were resolved by SDS-PAGE electrophoresis and electro-transferred as previously described (14). Immunoblotting using 1:500-1:1000 dilutions of primary antibody and quantitation were also as previously described (14). pY612-IRS-1 antibody was purchased from Biosource International (Camarillo, CA), total IRS-1 from Upstate Cell Signaling Solutions (Waltham, MA), p65 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and all other antibodies from Cell Signaling Technology (Beverley, MA).

**Muscle sections and immunohistochemistry**

TCMs were mounted on cork using Tissue-tek (Sakura Finetechnical, Tokyo, Japan) in a transverse orientation and were snap-frozen in liquid nitrogen-cooled isopentane after dissection. Transverse sections of formalin fixed paraffin-embedded TCMs were cut at 4µm, deparaffinised and rehydrated. Tissue slides were retrieved and blocked with hydrogen peroxide, followed by serum-free protein block (DAKO, Carpenteria, CA) then incubated in primary antibody (anti NFκB-p65 rabbit polyclonal, Santa Cruz 1:100). Detection was achieved using Envision + (DAKO) with immunocomplexes visualised by DAB+ Chromagen. Slides were examined using a Zeiss Axiovert 200M microscope (North Ryde, NSW, Australia) under a 20x 0.45 LD Achromplan objective. Pictures were
captured using a Zeiss Axiocam HR camera. The cross-sectional area of 25 randomly-selected muscle fibres was measured in each of 10 fields covering test and control TCMs. The area and mean grey value (mgv) of each cell was calculated using Image J (http://www.uhnresearch.ca/facilities/wcif/fdownload.html). Fibres in the test muscles were defined for intensity of staining using mgv and were placed in one of three groups: light (mgv>150), medium (mgv>125, <150) or dark staining (mgv<125) corresponding to the level of p65 expression. A conservative estimate of fibre transfection rate was made in each test muscle by calculating the percentage of fibres with mgv less than the lowest fibre mgv value in the corresponding control muscle.

**Real-time RT-PCR**

Real-time RT-PCR was used to quantify relative expression of mRNAs for TLRs 2 and 4 and Transforming Nuclear Factor (TNF)-α in muscles electrotransferred with p65. Total RNA was extracted using Tri-Reagent (Sigma-Aldritch, Sydney, Australia) and the yield quantified by spectrophotometry (DU-600, Beckman Instruments, Fullerton, CA) and agarose gel electrophoresis. Contaminating genomic DNA was removed by digestion with RNAse-free DNase (Promega), followed by heat/EDTA inactivation of the enzyme. DNAsed RNA then underwent reverse transcription for 60 minutes at 37°C using the Omniscript RT kit (Qiagen Ltd, Clifton Hill, Victoria, Australia) 1x buffer containing 0.5µM dNTPs, 1µM OligodT, 0.5units/µl RNAses inhibitor (Promega Corp, Annandale, NSW, Australia) and 0.2units/µl RT enzyme, the final product containing 20 ng/µl cDNA.
Primers for rat TLR-2, TLR-4 and MURF-1 were designed using the Primer 3 program (http://frodo.wi.mit.edu) and primers for acidic ribosomal phosphoprotein 36B4 were as published (41). They were obtained from Sigma-Proligo, Lismore, NSW, Australia. Their sequences were TLR-2: 5’-CGAAAAGAGCCACAAAACTGT-3’ and 5’-CATTATCTTGCGCAGTTTGC-3’, TLR-4: 5’-ACAGCAGAGGAGAAAGCATCT-3’ and 5’-GCAATGGGCTACACCAGGAAT-3’, and MURF-1: 5’-ACAAACCTGTGCCGCAAGTG-3’ and 5’-AGGACAACCTGTGCCGCAAGTG-3’ and 36B4: 5’-CGACCTGGAAGTCCAACTAC-3’ and 5’-ATCTGCTGCATCTGCTTG-3’.

These were utilised in 45 cycles of conventional PCR, annealing at 55°C, to synthesise DNA standards for relative quantification of targets. PCR was carried out using Go Taq Green Master Mix (Sigma). Agarose electrophoresis was conducted subsequently to demonstrate single DNA species of the expected length, which was excised and gel purified using Wizard Plus SV Minipreps DNA purification System (Promega, NSW Australia). 2µL of cDNA, serial standard DNA dilution or no template control was mixed with primer-optimised concentrations of MgCl2, primer pairs and SYBR Green Jumpstart Taq ReadyMix (Sigma, St Louis, MI). Samples underwent 35-45 cycles of 95°C denaturation for 5sec, annealing for 15sec and 72°C extension for 20sec using a Rotorgene thermal cycler (RG3000, Corbett Research Australia, NSW). Amplification and melting curves were followed in each case to confirm profiles were consistent with the production of the expected amplicon, and relative quantification was achieved with reference to curves of crossing point versus dilution of DNA standard with respect to each mRNA species.
Statistics

All data are quoted as mean ± standard error. Comparisons between treated and control muscles were made using the paired Student’s t-test or the Signed Rank test for non-normally distributed data. Comparisons between mean fibre cross-sectional area and degree of p65 immunostaining were made by One-Way analysis of variance (ANOVA) followed by Holm-Sidak post-hoc analysis. Analyses were conducted using Sigma Stat v3.00 (SPSS Inc, Chicago, IL), with p<0.05 regarded as significant.

Results

Over-expression of the p65 subunit of NFκB in skeletal muscle

In order to establish whether specific activation of the NFκB signalling pathway in skeletal muscle is capable of causing local insulin resistance we aimed to over-express the transcriptionally active NFκB subunit p65 (38) in muscle. To this end we used IVE to introduce a muscle-specific p65-expressing vector (EH114-p65) into the right TCM of a cohort of rats, while the contralateral TCM was electroporated with an equivalent amount of empty EH114 vector. One week after IVE, the optimum time to examine the effects of this manipulation in muscle (13), we measured p65 protein in paired muscle by western blotting. As can be seen in Figure 1A, a 64±17% increase in p65 expression was achieved in test versus paired contralateral control muscles (p<0.001).

To verify that the p65 over-expression was of functional significance and resulted in an alteration in an established end-point, we next considered the effects of the manipulation on muscle fibre size, as increased activation of the classical NFκB signalling pathway has
been previously associated with muscular atrophy (2, 7). The relationship between the intensity of p65 immunostaining and the cross-sectional area of muscle fibres in transverse section was examined at the one week time point in a sub-set of rats (n=4). Typical photomicrographs (Figures 1B and 1C) show variable levels of p65 over-expression between fibres in the test muscle and a uniform lack of detectable p65 immunostaining in the control muscle. Mean fibre transfection rate was ≥ 71±7%. The photomicrographs and the accompanying summary graph (Figure 1D) demonstrate that there was an inverse relationship between the level of p65 expression and fibre cross-sectional area (p<0.001 overall). Light, medium and dark-staining fibres were 89%, 58% and 37% of the size of control fibres respectively (all at least p<0.05 versus control). This finding implies that the degree of p65 over-expression induced here resulted in fibre atrophy consistent with that previously observed (2). Consistent with this there was a 44±17% increase in MURF-1 mRNA in test muscles (p=0.022). MURF-1 is an E3 ligase shown to be an NFκB target gene and to mediate muscle atrophy (7). Thus we were able to successfully over-express p65 in TCM to an extent which resulted in measurable downstream effects.

**p65 over-expression does not impair muscle glucose disposal**

In order to assess whether activation of NFκB has an impact on insulin sensitivity and glucose disposal in muscle we measured uptake of radiolabelled glucose and 2-DG tracer into p65 over-expressing and control muscles under hyperinsulinaemic-euglycaemic clamp conditions one week after IVE. Rats weighed 234 ± 5g at the time of study and had plasma glucose and insulin concentrations of 7.2 ± 0.3mM and 81 ± 3mU/l respectively
during the clamp. Clamp GIR, R_d and EGO for these animals were 41.7 ± 3.3, 38.9 ± 3.3 and -2.8 ± 2.6mg/kg.min respectively. There were no differences between paired muscles in R_g’ or glycogen synthesis measured by incorporation of tracer into glycogen during the clamp (Table 1). Furthermore, glycogen content of muscles, reflecting glucose disposal and glycogen usage over a longer period of time, was also unaffected by the manipulation (Table 1).

To further establish whether muscle insulin sensitivity was altered by p65 over-expression we measured activating phosphorylation (15) and protein expression of key PI-3 kinase pathway signalling intermediates by western blotting in lysates derived from test and control muscles removed at the end of the clamp procedure. As can be seen from Figure 2A and B, expression of IRS-1 and Akt were unaltered, as was Serine-473 phosphorylation of Akt, while Tyrosine-612 phosphorylation of IRS-1 which facilitates binding of the p85 subunit and thus PI-3 kinase and Akt activation, was in fact increased by 28±8% (p=0.011). Thus, analysis of glucose disposal and activation of the insulin signalling cascade together provide no evidence that local NFkB activation is deleterious for muscle insulin sensitivity.

**p65 over-expression in muscle has specific negative feedback effects on the classical NFkB pathway**

We were also interested in what effect over-expression of p65 might have on upstream regulators of activation of the classical NFkB activation pathway, as this has not been established in skeletal muscle. To this end we measured mRNA expression of TLRs 2 and 4, both of which have been suggested to play a role in mediating the effects of raised
plasma fatty acids in peripheral insulin sensitive tissues (39; 44). We found a 36±9% reduction in TLR-2 mRNA in p65 over-expressing muscles, with TLR-4 mRNA being unaffected by the manipulation (Figure 3A and B), implying a specific negative feedback effect to attenuate NFκB activation. Protein levels of IκBα were also quantified by western blot and found to be increased by 49±17% in test muscles (Figure 3C), suggesting that a counter-regulatory retention of NFκB dimers in the cytoplasm may be induced. These data together imply a contrasting mechanism of regulation of the NFκB pathway in muscle with the well-recognised feed-forward cycle of TNF-NFκB activation occurring in inflammatory cells as part of the classical pathway of activation (5).

**IκBκβ over-expression in skeletal muscle**

Although we did not detect an effect of NFκB activation to attenuate insulin sensitivity in skeletal muscle, the above experiment did not rule out the possibility that upstream activation of the IκBK-NFκB pathway could be implicated. Indeed direct Serine-307 phosphorylation of IRS-1 by IκBκβ has been proposed as the principal mechanism whereby tissue inflammation has an impact on insulin sensitivity (1; 8). We therefore adopted a complementary approach to the above experiment whereby we used IVE to introduce EH114-GW-IκBκβ into single rat TCMs, electroporated contralateral TCMs with empty vector and compared the resulting protein expression after one week. As can be seen in Figure 4A, total IκBκβ protein was increased by 655±54% in test versus paired control muscles (p<0.001). This resulted in a 470±97% increase in total Serine-180/181-phosphorylated IκBα/β (Figure 4B; p<0.001) and a consequent 12±5% reduction in IκBα protein (Figure 4C; p=0.033), commensurate with increased NFκB
translocation to the nucleus and transactivation of target genes (5). IκBKα expression was unaffected by this manipulation, suggesting that there was no compensatory down-regulation of this catalytic sub-unit, while p65 protein expression was also unchanged (data not shown). Thus we were able to successfully and specifically over-express IκBKβ in rat muscle and demonstrate an appropriate downstream effect of this manipulation.

**IκBKβ over-expression does not impair muscle glucose disposal**

In rats electroporated with IκBKβ we compared glucose disposal and glycogen storage in test and control muscles under hyperinsulinaemic-euglycaemic clamp conditions. Clamps in this experiment were preceded by an additional one hour moderate infusion of Intralipid sufficient to activate IκBKβ in the muscle but not to induce insulin resistance when continued for a maximum of 3.25 hours (3). Rats weighed 234 ± 4g at the time of study and had plasma glucose and insulin concentrations of 8.5 ± 0.2mM and 301 ± 31mU/l respectively during the clamp. Clamp GIR, R_d and EGO for these animals were 28.0 ± 1.2, 33.3 ± 1.8 and 5.4 ± 1.6mg/kg.min respectively. Similar to the results obtained with p65 over-expression there was no significant difference in R_g’ between paired muscles (Table 1), although there was a small non-significant reduction in incorporation of glucose into glycogen during the clamp (p=0.056; Table 1). However, this effect was not mirrored by muscle glycogen content, as values in test and control muscles were identical (Table 1). Although it is not appropriate to statistically compare glucose turnover between cohorts of animals used for p65 and IκBKβ over-expression as these studies were not carried out simultaneously, the differences in glucose and insulin concentrations measured during the clamps may at least in part be due to differences in
basal concentrations between cohorts (24±2 versus 12±1 mU/l plasma insulin and 4.7±0.1 versus 4.3±0.1 mM blood glucose for IκBκ and p65 rats respectively).

**IκBκ over-expression increases phosphorylation of insulin signalling intermediates in muscle**

To further investigate whether increased activation of IκBκ would result in reduced insulin sensitivity we measured expression and phosphorylation of signalling intermediates in the PI-3 kinase cascade in muscles removed from rats at the end of the clamp procedure. We found increases in Tyrosine-612 phosphorylation of IRS-1 (24±11%, p=0.023; Figure 5A), Serine-473 phosphorylation of Akt (23±3%, p<0.001; Figure 5B) and Serine-9 phosphorylation of Glycogen synthase kinase-3β (12±4%, p=0.007; Figure 5C) by western blotting in the absence of any effect on total protein levels of IRS-1 or Akt. This consistent set of data implies a moderate increase in flux through the insulin signalling pathway in IκBκ over-expressing muscles, which was perhaps insufficient to be reflected in changes in glucose disposal but is in marked contrast to the predicted effect of activation of this kinase to impair signalling via serine phosphorylation of IRS-1. Furthermore, neither activating phosphorylation nor protein expression of p70S6 kinase, c-Jun terminal kinase (JNK) or p38 Mitogen-activated protein kinase (MAPK) were significantly altered by IκBκ over-expression (Figure 5D), confirming that there was no compensatory increase in activity of alternative serine kinases.
Discussion

In the studies described herein we aimed to determine whether increased activity of the IκBKβ-NFκB pathway is sufficient to cause insulin resistance in muscle and which component of the pathway might be more important in this role. To this end we separately over-expressed IκBKβ and the p65 subunit of NFκB in single muscles of normal adult rats for one week using IVE and compared the effects on insulin sensitivity with paired control muscles. Despite these manipulations resulting in increased degradation of IκBα and muscle fibre atrophy respectively, consistent with increased activity of each of these molecules (7), we found no change in acute glucose disposal or glycogen storage as a result. This lack of effect occurred despite a similar degree of over-expression of target genes to that previously achieved using this method with other signalling molecules which did result in increased glucose disposal into muscle (13; 15; 29). In fact we found evidence for a moderate increase in flux through the PI-3 kinase/insulin signalling cascade, especially following IκBKβ over-expression. In addition we found evidence for a negative feedback effect of p65 over-expression on activation of the IκBKβ-NFκB pathway in muscle, in contrast to the feed-forward effect of NFκB activation seen as part of the immune response in inflammatory cells.

Metabolism and innate immunity are two of the most evolutionarily conserved systems in the animal kingdom. Indeed the anatomical basis and molecular underpinning of each are based upon common ancestral features (23). However the molecular basis for the recently recognised relationship between inflammation and insulin sensitivity of tissues is not well characterised. In particular it is unclear whether activation of inflammatory signalling
pathways is of relevance only within cells of a specific immune lineage or additionally within cells traditionally thought of as insulin sensitive, including myofibres. In the studies described we have introduced cDNA constructs under the control of skeletal muscle specific promoters by IVE. This has permitted us to examine the significance of activation of the IκBKβ-NκFB pathway specifically in skeletal myocytes in the absence of the confounding factors of developmental compensation or whole body physiological adaptation to germ-line manipulation (13).

In finding no effect of IκBKβ activation on insulin-stimulated glucose disposal into muscle, our results corroborate those obtained by Cai et al (7), who saw no effect of transgenic over-expression of IκBKβ on whole body glucose tolerance or ex vivo uptake of glucose into extensor digitorum longus muscles, despite a marked atrophic effect. Furthermore Rohl et al showed that a muscle specific deletion of IκBKβ did not prevent obesity- induced insulin resistance in mice (36). These chronic studies, together with the relatively acute manipulations described herein, suggest that the earlier in vitro findings (26; 42) are of limited physiological relevance. Thus the effects on muscle insulin sensitivity of pharmacological intervention or knockout of IκBKβ (47) or its downstream targets such as inducible nitric oxide synthase (9; 33) seem to result from a primary effect in another cell type, and both liver (1; 8) and myeloid cells (1; 22) have been implicated in mouse studies. A rationale for the link between increased plasma fatty acids, systemic sub-clinical inflammation and muscle insulin resistance might therefore be as follows: binding of lipid derivatives by TLRs on macrophage membranes results in activation of the intracellular IκBKβ-NκFB pathway and release of cytokines such as TNFα, which
cause NκFB-independent signalling impairments in muscle, such as reduced AMPK activity (46) and thus attenuated glucose disposal. In support of this, recent studies by Hevener et al have shown that macrophage infiltration of rodent muscle is increased by high fat feeding and that PPARγ expression in macrophages is necessary to suppress the activation of the IκBKβ-NκFB pathway and preserve muscle insulin sensitivity (22).

One of the proposed mechanisms whereby increased IκBKβ activity might cause insulin resistance is through Ser307 phosphorylation of IRS-1, resulting in reduced tyrosine phosphorylation of this molecule and hence impaired recruitment of PI-3 kinase (34). However we found evidence for moderately increased flux through the insulin signalling pathway in muscles electroporated with IκBKβ especially. Whereas it may be that the magnitude of the effect may not have been sufficient to be reflected in an enhancement in insulin-stimulated muscle glucose disposal, previous work has also demonstrated that the effects of altering IRS-1 abundance or activation on glucose disposal may not be clear-cut (15; 18). These findings do not rule out the possibility that activation of alternative stress kinases including novel Protein kinases C, p70S6kinase and c-Jun n-terminal kinase (27; 32; 48) may have a local impact on muscle insulin sensitivity.

We also found evidence that p65 over-expression results in negative feedback effects on activation of NFκB which have not been documented in muscle to date. Specifically, TLR-2 but not TLR-4 expression was down-regulated, implying a reduction in sensitivity of myofibres to activators related chemically to peptidoglycans rather than lipopolysaccharide (6). Recent data suggests a key role for TLR-2 in mediating palmitate-
induced insulin resistance in myotubes (39), hence this may imply the existence of a protective mechanism in muscle against excessive activation of NFκB target genes. Furthermore, up-regulation of IκBα under the same circumstances would tend to sequestrate NFκB in the cytoplasm and also limit the inflammatory response. These findings are in marked contrast to the feed-forward activation of the IκBβ-NFκB pathway normally expected in myeloid cells as part of the innate immune response (5). However recent publications have identified mechanisms for negative feedback regulation of NFκB in endothelial cells (20) and macrophages (19) which confirm the feasibility of this hypothesis for muscle.

In conclusion, our data do not provide evidence for a role of activation of the IκBβ-NFκB pathway in muscle in the initiation of insulin resistance in muscle in vivo. Instead, activation of this pathway in adipose or hepatic macrophages may be more important in generating muscle insulin resistance via secondary means.
Acknowledgements

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References


17. **Freeman DJ, Norrie J, Caslake MJ, Gaw A, Ford I, Lowe GD, O'Reilly DS, Packard CJ and Sattar N**. C-reactive protein is an independent predictor of risk


Figure legends

Figure 1  Over-expression of p65 causes muscle fibre atrophy
Summary graph of paired data and sample western blot demonstrating that the p65 sub-unit of NFκB was over-expressed in TCM by 64±17% (A). Each pair of symbols represents control and contralateral test muscles removed from one animal. On the blot a doublet is apparent in test lysate lanes as the exogenous p65 species has an epitope tag which renders it 2kDa larger than the endogenous species. Transverse sections through Test (B) and Control (C) muscles immunostained (black) for p65 showing variable degrees of p65 over-expression in fibres of the former. A conservative estimate of fibre transfection rate in test muscles was 71±7%. Summary graph indicating that there was an inverse relationship between p65 expression and fibre size in muscle fibres (D); ANOVA p<0.001. MURF-1 mRNA corrected for 36B4 mRNA expression measured by Real-time PCR showing a 44±17% increase in test muscles (E). ⋆ p<0.05, ⋆⋆⋆ p<0.001 versus control. T-test, C-control muscle lysates.

Figure 2  Effect of p65 over-expression on intracellular signalling
Summary graphs and sample western blots showing that p65 over-expression in TCM caused a 28±8% increase in Tyrosine-612-phosphorylated IRS-1 (A) and had no effect on Serine-473-phosphorylated Akt (B). Protein expression of IRS-1 and Akt were unchanged by the manipulation. ⋆ p<0.05 versus control. T-test, C-control muscle lysates.
**Figure 3**  
*p65 over-expression has negative feedback effects on the classical NFκB signalling pathway*

Graphs showing the effects of p65 over-expression in muscle on Toll-like Receptor (TLR)-2 (A) and TLR-4 (B) mRNA expression as measured by Real-time PCR and corrected for 36B4 mRNA. A specific reduction of 36±9% in TLR-2 mRNA resulted. Summary graph and blot demonstrating a 49±17% increase in IκBα protein expression in test versus control muscles (C). * p<0.05 versus control. T-test, C-control muscles.

**Figure 4**  
*IκBKβ over-expression in muscle*

Summary graphs and representative western blots confirming successful over-expression of IκBKβ in TCM. IκBKβ protein was increased by 655±54% (A) and total phosphorylated IκBK (α and β) was increased by 470±97% (B) in test muscles, resulting in a 12±5% reduction in IκBα expression (C), implying activation of NFκB. * p<0.05, ** p<0.001 versus control. T-test, C-control muscles.

**Figure 5**  
*IκBKβ over-expression in muscle does not impair signalling through the PI-3 kinase cascade*

Summary graphs and representative blots showing that over-expression of IκBKβ results in increases in phosphorylation of signalling intermediates IRS-1 (Tyrosine 612, by 24±11%, A), Akt (Serine 473, by 23±3%, B) and GSK3β (Serine 9, by 12±4%, C), implying increased flux through the PI-3 kinase cascade. However there was no compensatory change in expression or phosphorylation of alternative serine kinases (p38 MAPK, JNK, p70S6 kinase, D). * p<0.05, ** p<0.01, *** p<0.001 versus control. T-test, C-control muscles.
Table 1  Effects of p65 and IκBβ over-expression on physiological parameters during hyperinsulinaemic-euglycaemic clamp

Data for test versus control muscles demonstrate no effect of either IκBβ or p65 over-expression in tibialis cranialis muscle (TCM) for one week on glucose disposal or glycogen accumulation.
# Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>p65 over-expression</th>
<th>IkBKB over-expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test</td>
<td>Control</td>
</tr>
<tr>
<td>Glucose disposal into TCM (mg / kg.min)</td>
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<td>29 ± 3.2</td>
</tr>
<tr>
<td>Glucose incorporation into TCM glycogen (mg / kg.min)</td>
<td>10.5 ± 1.7</td>
<td>10.3 ± 1.4</td>
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<tr>
<td>Stored TCM glycogen (nmol / mg)</td>
<td>51 ± 3</td>
<td>50 ± 2</td>
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</table>
Figure A shows the expression levels of IkBβ protein (arbitrary units) in control and test muscle, with significant differences indicated by ***. Figure B displays the pS176/180-IkBα/β protein expression, also showing significant differences with ***. Figure C illustrates the IkBα protein levels, with a trend indicated by *.