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Local activation of the I κ B-NF- κ B pathway in muscle does not cause insulin resistance

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Polkinghorne E, Lau Q, Cooney GJ, Kraegen EW, Cleasby ME. Local activation of the I κ B-NF- κ B pathway in muscle does not cause insulin resistance. *Am J Physiol Endocrinol Metab* 294: E316–E325, 2008. First published November 20, 2007; doi:10.1152/ajpendo.00537.2007.—Insulin resistance of skeletal muscle is a major defect in obesity and type 2 diabetes. Insulin resistance has been associated with a chronic subclinical inflammatory state in epidemiological studies and specifically with activation of the inhibitor κ B kinase (I κ BK)-nuclear factor- κ B (NF- κ B) pathway. However, it is unclear whether this pathway plays a role in mediating insulin resistance in muscle in vivo. We separately overexpressed the p65 subunit of NF- κ B and I κ BK β in single muscles of rats using in vivo electrotransfer and compared the effects after 1 wk vs. paired contralateral control muscles. A 64% increase in p65 protein ($P < 0.001$) was sufficient to cause muscle fiber atrophy but had no effect on glucose disposal or glycogen storage in muscle under hyperinsulinemic-euglycemic clamp conditions. Similarly, a 650% increase in I κ BK β expression ($P < 0.001$) caused a significant reduction in I κ B protein but also had no effect on clamp glucose disposal after lipid infusion. In fact, I κ BK β overexpression 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 signaling cascade. Interestingly, p65 overexpression 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 I κ BK β -NF- κ B pathway in muscle does not seem to be an important local mediator of insulin resistance.

inhibitor κ kinase-nuclear factor- κ B pathway; skeletal muscle; Toll-like receptors; in vivo electrotransfer

TYPE 2 DIABETES AND OBESITY are intimately related and rapidly increasing human health problems worldwide. Insulin resistance in skeletal muscle is regarded as an essential prerequisite for the development of type 2 diabetes, 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 etiology 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 proinflammatory cytokines, most notably tumor 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 TNF- α impairs glucose uptake into muscle through impairment in insulin signaling 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 κ B kinase β (I κ BK β) (49), and indeed, when the corresponding gene was targeted for knockout, the mice were able to withstand diet-induced insulin resistance (50). I κ BK β is a catalytic subunit of a trimeric serine kinase that also consists of a second catalytic subunit (I κ BK α) and a regulatory subunit (I κ BK γ). This enzyme lies upstream of the transcription factor nuclear factor- κ B (NF- κ B), and together these molecules comprise the principal regulatory pathway for the immune response (5). NF- κ B is typically a heterodimer that can be composed of two of a number of different subunits, including the transactivating unit p65 (5), although artificial overexpression of p65 facilitates the formation of homodimers, which show substantially enhanced activity (31). NF- κ B dimers are retained in a cytoplasmic location by binding an I κ B molecule. When phosphorylated by I κ BK, these inhibitors detach from the complex and are targeted for proteasomal degradation, thus permitting translocation of NF- κ B 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 I κ BK β -NF- κ B 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 I κ BK β -NF- κ B pathway in muscle and local insulin resistance. The pathway may be activated by binding of TNF- α 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 I κ BK β or nuclear translocation of NF- κ B is the more significant event in mediating such an

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effect. Studies of NF- κ B inhibition have suggested that trans-activation 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 I κ BK β , 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 I κ BK β -NF- κ B pathway is sufficient to cause insulin resistance in muscle and whether this is mediated principally through a direct transcriptional effect of NF- κ B or via the kinase activity of I κ BK β . To achieve these aims, we separately overexpressed each protein in single muscles of adult rats by using *in vivo* electrotransfer (IVE) and compared the effects of each manipulation on insulin sensitivity with the contralateral control muscle 1 wk later. This approach has the advantage of minimizing 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 Gateway^R destination vector using the Gateway^R 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'-GGGGACAAGTTTGTACAAAAAAGCAGGCTA-CTACTATAGGGCGAATTG-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAAAGGGAACAAAAGCTG-3' primers (GeneWorks, Hindmarsh, South Australia). pcDNA3 expressing the cDNA for the human p65 subunit of NF- κ B coupled to an NH₃-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 I κ BK β 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-I κ BK β . Molecular reagents were supplied by Promega (Annandale, NSW, Australia), Invitrogen, and New England Biolabs (Genesearch, Arundel, Queensland, Australia).

Animal maintenance and surgery. Male Wistar rats (~150 g) were obtained from the Animal Resources Center (Perth, Australia) and acclimatized to their new surroundings for 1 wk. Animals were maintained at 22 \pm 0.5°C under a 12:12-h day-night cycle and were fed a standard chow diet *ad libitum* (18% fat, 33% protein, and 48% carbohydrate as a percentage of total dietary energy; Norco, Kempsey, Australia). Approximately 1 wk before study, the right and left jugular veins of rats were cannulated as previously described (12). Anesthesia was induced with 5% and maintained with 1–2% halothane in oxygen. The surgical site was irrigated with bupivacaine (0.5 mg/100 g) before closure, and 5 mg/kg ketoprofen was administered to provide post-operative analgesia. Rats were singly housed and handled daily for the following week to minimize separation anxiety. Body weight was recorded daily, and only those rats that had fully recovered their presurgery weight were subsequently studied; $n = 7$ –11 rats per group unless otherwise stated. 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.

In vivo electrotransfer. Preparation and injection of DNA and electrotransfer was carried out as previously described (13). Paired tibialis cranialis muscles (TCMs) were injected percutaneously with six spaced 50- μ l aliquots of DNA prepared in endotoxin-free sterile saline (Qiagen Maxi/Mega-Prep kits; Doncaster, Victoria, Australia) at 0.5 mg/ml. In each case, the right TCMs from each animal were injected with test constructs and left TCMs were injected with empty EH114 vector as within-animal controls. One 800 V/cm, 100- μ s electrical pulse and four 80 V/cm, 100-ms pulses at 1 Hz were administered sequentially via tweezer electrodes attached to an ECM-830 electroporator (BTX, Holliston, MA) immediately afterward. Empty vector was used as a control in preference to the enhanced green fluorescent protein vector previously described (13), because expression of green fluorescent protein 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 signaling molecules sufficiently to cause altered glucose disposal into muscle.

Assessment of in vivo glucose metabolism in rats under euglycemic-hyperinsulinemic clamp conditions. Conscious rats were studied after 5–7 h of fasting. One jugular cannula was connected to an infusion line and the other to a sampling line between 8:30 and 9:30 AM, and the rats were then allowed to acclimatize to the study cage for 30–40 min. Hyperinsulinemic-euglycemic clamps were conducted as described previously (12), involving a variable infusion of 30% glucose and a rate of insulin infusion commensurate with the generation of normal postprandial plasma levels. A combined bolus injection of 2-deoxy-D-[2,6-³H]glucose and D-[U-¹⁴C]glucose (Amersham Biosciences, Little Chalfont, UK) was administered 45 min before the end of the clamp. In rats that had been electroporated with I κ BK β , the clamp was preceded by a 1-h infusion of 2% Intralipid (Travenol, Sydney, Australia)/0.9% saline containing 40 IU/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 I κ BK (3) but not to induce insulin resistance in muscle, which might disguise the effects of the genetic manipulation (14). At the end of each study, rats were euthanized by intravenous injection of pentobarbitone sodium (Nembutal; Abbott Laboratories, Sydney, Australia), and their muscles were 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-[2,6-³H]glucose together with the disintegrations per minute of phosphorylated [³H]deoxyglucose from individual muscles was used to calculate the insulin-stimulated glucose metabolic index (R'_g), 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 analyzer (YSI 2300; Yellow Springs, OH). The remaining plasma was frozen in liquid nitrogen and subsequently used for plasma insulin determination by radioimmunoassay (Linco Research, St. Charles, MO). Muscle glycogen was analyzed as described previously (10). Glucose incorporation into glycogen was determined from the D-[U-¹⁴C]glucose tracer disappearance curve and counts of ¹⁴C in muscle as previously described (28).

Muscle lysates, SDS-PAGE, and immunoblotting. Protein expression and phosphorylation of molecules present in muscle was assessed using SDS-PAGE and quantification of Western blots of cell lysates. Whole tissue lysates were prepared from dismembranated muscle (Mikro-dismembrator II; B. Braun Biotech, Melsungen, Germany) by manual homogenization in RIPA buffer [65 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4, 1% (vol/vol) Nonidet P-40 detergent, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, and 10% (vol/vol) glycerol, containing 25 μ g/ml leupeptin, 10 μ g/ml aprotinin,

2 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 10 mM NaF, and 1 mM PMSF], followed by incubation for 90 min at 4°C and centrifugation for 10 min at 12,000 g. Protein content of supernatants was quantified using the Bradford method (protein assay kit; Bio-Rad Laboratories, Regents Park, NSW, Australia), and aliquots containing 10–60 μ g of protein were denatured in Laemmli buffer for 5 min at 95°C or 10 min at 65°C.

Proteins were resolved by SDS-PAGE electrophoresis and electrotransferred as previously described (14). Immunoblotting using 1:500–1:1,000 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 (Santa Cruz, CA), and all other antibodies from Cell Signaling Technology (Beverly, MA).

Muscle sections and immunohistochemistry. TCMs were mounted on cork using Tissue-tek (Sakura Finetech, 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 4 μ m, deparaffinized, and rehydrated. Tissue slides were retrieved and blocked with hydrogen peroxide followed by serum-free protein block (DAKO, Carpinteria, CA) and then incubated in primary antibody (anti NF- κ B-p65 rabbit polyclonal, 1:100; Santa Cruz Biotechnology). Detection was achieved using EnVision+ (DAKO) with immunocomplexes visualized using DAB+ chromagen. Slides were examined using a Zeiss Axiovert 200M microscope (North Ryde, NSW, Australia) under a $\times 20$, 0.45 LD Achromat objective. Pictures were captured using a Zeiss AxioCam HR camera. The cross-sectional area of 25 randomly selected muscle fibers was measured in each of 10 fields covering test and control TCMs. The area and mean gray value (MGV) of each cell was calculated using ImageJ (<http://www.uhnresearch.ca/facilities/wcif/fdownload.html>). Fibers in the test muscles were defined for intensity of staining using MGV and were placed in one of three groups: light (MGV > 150), medium (125 < MGV < 150), or dark staining (MGV < 125) corresponding to the level of p65 expression. A conservative estimate of fiber transfection rate was made in each test muscle by calculating the percentage of fibers with MGV less than the lowest fiber MGV value in the corresponding control muscle.

Real-time RT-PCR. Real-time RT-PCR was used to quantify relative expression of mRNAs for TLR-2 and TLR-4 and TNF- α in muscles electrotransferred with p65. Total RNA was extracted using TRI reagent (Sigma-Aldrich, Sydney, Australia), and the yield was 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. DNase RNA then underwent reverse transcription for 60 min at 37°C using the Omniscript RT kit (Qiagen, Clifton Hill, Victoria, Australia) 1 \times buffer containing 0.5 μ M dNTPs, 1 μ M oligo(dT), 0.5 U/ μ l RNase inhibitor (Promega), and 0.2 U/ μ l RT enzyme, with the final product containing 20 ng/ μ l cDNA.

Primers for rat TLR-2, TLR-4, and muscle-specific RING finger protein (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 as follows: TLR-2, 5'-CGAAAAGAGCCACAAAAGTGT-3' and 5'-CATTATCTTGC-GCAGTTTGC-3'; TLR-4, 5'-ACAGCAGAGGAGAAAGCATCT-3' and 5'-GCAATGGCTACACAGGAAT-3'; MuRF-1, 5'-ACAAC-CTGTGCCGCAAGTG-3' and 5'-AGGACAACCTGTGCCGCAAGTG-3'; and 36B4, 5'-CGACCTGGAAGTCCAACACTAC-3' and 5'-ATCTGCTGCATCTGCTTG-3'.

These primers were utilized in 45 cycles of conventional PCR, with annealing at 55°C, to synthesize DNA standards for relative quantification of targets. PCR was carried out using GoTaq green master mix (Sigma). Agarose electrophoresis was conducted subsequently to

demonstrate single DNA species of the expected length, which were excised and gel purified using Wizard Plus SV minipreps DNA purification system (Promega). Two microliters of cDNA, serial standard DNA dilution, or no template control were mixed with primer-optimized concentrations of MgCl₂, primer pairs, and SYBR green JumpStart Taq ReadyMix (Sigma, St. Louis, MO). Samples underwent 35–45 cycles of 95°C denaturation for 5 s, annealing for 15 s, and 72°C extension for 20 s using a Rotorgene thermal cycler (RG3000; Corbett Research Australia, NSW, Australia). Amplification and melting curves were followed in each case to confirm that 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 means \pm SE. 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 fiber cross-sectional area and degree of p65 immunostaining were made using one-Way analysis of variance (ANOVA) followed by Holm-Sidak post hoc analysis. Analyses were conducted using Sigma Stat version 3.00 (SPSS, Chicago, IL) with *P* < 0.05 regarded as significant.

RESULTS

Overexpression of the p65 subunit of NF- κ B in skeletal muscle. To establish whether specific activation of the NF- κ B signaling pathway in skeletal muscle is capable of causing local insulin resistance, we aimed to overexpress the transcriptionally active NF- κ B 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 muscles by Western blotting. As shown in Fig. 1A, a $64 \pm 17\%$ increase in p65 expression was achieved in test compared with paired contralateral control muscles (*P* < 0.001).

To verify that the p65 overexpression was of functional significance and resulted in an alteration in an established endpoint, we next considered the effects of the manipulation on muscle fiber size, since increased activation of the classic NF- κ B signaling 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 fibers in transverse section was examined at the 1-wk time point in a subset of rats (*n* = 4). Typical photomicrographs (Fig. 1, B and C) show variable levels of p65 overexpression between fibers in the test muscle and a uniform lack of detectable p65 immunostaining in the control muscle. Mean fiber transfection rate was $\geq 71 \pm 7\%$. The photomicrographs and the accompanying summary graph (Fig. 1D) demonstrate that there was an inverse relationship between the level of p65 expression and fiber cross-sectional area (*P* < 0.001 overall). Light-, medium-, and dark-staining fibers were 89, 58, and 37% of the size of control fibers, respectively (all at least *P* < 0.05 vs. control). This finding implies that the degree of p65 overexpression induced resulted in fiber atrophy, consistent with that previously observed (2). Consistent with this there was a $44 \pm 17\%$ increase in MuRF-1 mRNA in test muscles (*P* = 0.022). MuRF-1 is an E3 ligase shown to be an NF- κ B target gene and to mediate muscle atrophy (7). Thus we were

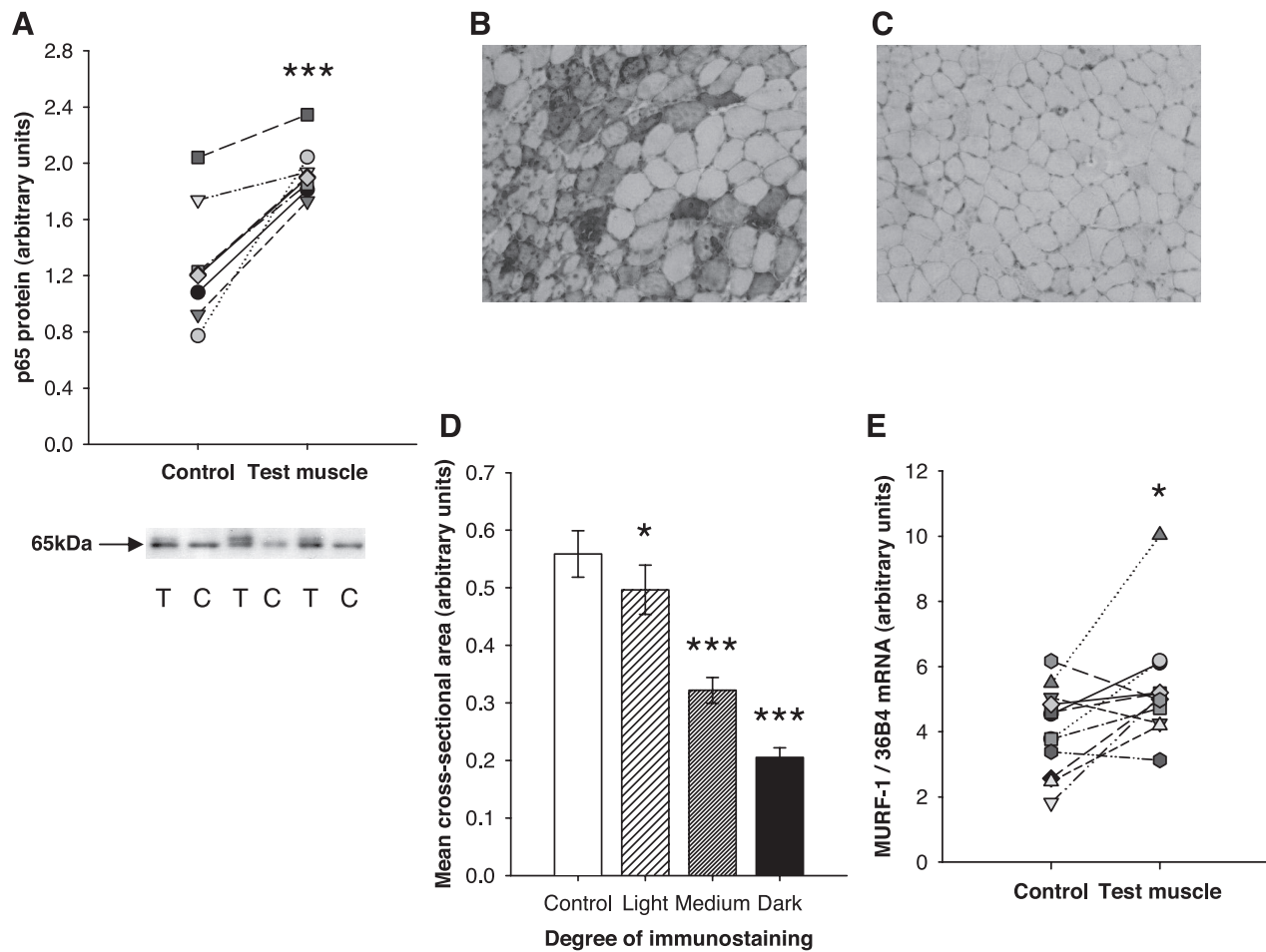


Fig. 1. Overexpression of p65 causes muscle fiber atrophy. A summary graph of paired data and a sample Western blot (A) demonstrate that the p65 subunit of NF- κ B was overexpressed in tibialis cranialis muscles (TCMs) by $64 \pm 17\%$. Each pair of symbols represents control and contralateral test muscles removed from one animal. On the blot, a doublet is apparent in test lysate lanes (T) because the exogenous p65 species has an epitope tag that renders it 2 kDa larger than the endogenous species. C, control muscle lysates. Transverse sections through test (B) and control (C) muscles immunostained (black) for p65 show variable degrees of p65 overexpression in fibers of the former. A conservative estimate of fiber transfection rate in test muscles was $71 \pm 7\%$. A summary graph (D) indicates that there was an inverse relationship between p65 expression and fiber size in muscle fibers. $P < 0.001$ (ANOVA). Muscle-specific RING finger (MuRF)-1 mRNA corrected for 36B4 mRNA expression (E) was measured using real-time PCR, showing a $44 \pm 17\%$ increase in test muscles. * $P < 0.05$, *** $P < 0.001$ vs. control.

able to successfully overexpress p65 in TCMs to an extent that resulted in measurable downstream effects.

p65 overexpression does not impair muscle glucose disposal. To assess whether activation of NF- κ B has an impact on insulin sensitivity and glucose disposal in muscle, we measured uptake of radiolabeled glucose and 2-deoxy-D-[2,6- 3 H]glucose tracer into p65-overexpressing and control muscles under hyperinsulinemic-euglycemic clamp conditions 1 wk

after IVE. Rats weighed 234 ± 5 g at the time of study and had plasma glucose and insulin concentrations of 7.2 ± 0.3 mM and 81 ± 3 mU/L, respectively, during the clamp. Clamp GIR, R_d , and EGO values for these animals were 41.7 ± 3.3 , 38.9 ± 3.3 , and -2.8 ± 2.6 mg \cdot kg $^{-1}\cdot$ min $^{-1}$, 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

Table 1. Effects of p65 and I κ BK β overexpression on physiological parameters during hyperinsulinemic-euglycemic clamp

Variable	p65 Overexpression			I κ BK β Overexpression		
	Test	Control	P Value	Test	Control	P Value
Glucose disposal into TCM, mg \cdot kg $^{-1}\cdot$ min $^{-1}$	29.7 ± 3.7	29 ± 3.2	0.85	26.6 ± 2.5	28.2 ± 1.7	0.47
Glucose incorporation into TCM glycogen, mg \cdot kg $^{-1}\cdot$ min $^{-1}$	10.5 ± 1.7	10.3 ± 1.4	0.85	9.5 ± 1.0	11.7 ± 1.2	0.056
Stored TCM glycogen, nmol/mg	51 ± 3	50 ± 2	0.50	61 ± 4	61 ± 3	0.94

Data are means \pm SE for test vs. control muscle groups and demonstrate no effect of either I κ BK β or p65 overexpression in tibialis cranialis muscle (TCM) for 1 wk on glucose disposal or glycogen accumulation.

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 overexpression, we measured activating phosphorylation (15) and protein expression of key phosphatidylinositol 3-kinase (PI3-kinase) pathway signaling intermediates by Western blotting in lysates derived from test and control muscles removed at the end of the clamp procedure. As shown in Fig. 2, *A* and *B*, expression of IRS-1 and Akt was unaltered, as was Ser⁴⁷³ phosphorylation of Akt, whereas Tyr⁶¹² phosphorylation of IRS-1, which facilitates binding of the p85 subunit and thus PI3-kinase and Akt activation, was in fact increased by $28 \pm 8\%$ ($P = 0.011$). Thus analysis of glucose disposal and activation of the insulin signaling cascade together provide no evidence that local NF- κ B activation is deleterious for muscle insulin sensitivity.

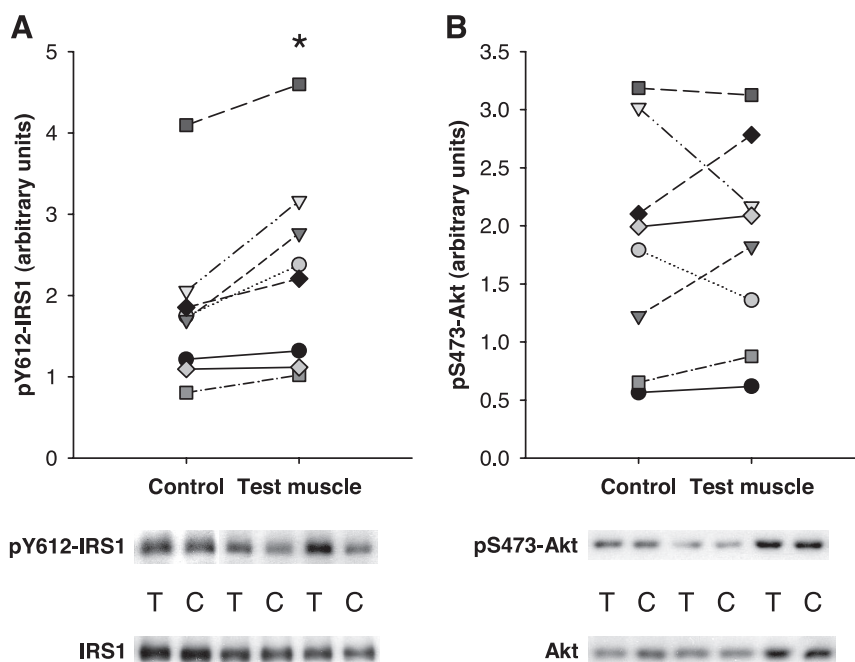
p65 overexpression in muscle has specific negative feedback effects on the classic NF- κ B pathway. We were also interested in what effect overexpression of p65 might have on upstream regulators of activation of the classic NF- κ B activation pathway, since this has not been established in skeletal muscle. To this end, we measured mRNA expression of TLR-2 and TLR-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 $36 \pm 9\%$ ($P = 0.022$) reduction in TLR-2 mRNA in p65-overexpressing muscles, with TLR-4 mRNA being unaffected by the manipulation (Fig. 3, *A* and *B*), implying a specific negative feedback effect to attenuate NF- κ B activation. Protein levels of I κ B α were also quantified by Western blotting and found to be increased by $49 \pm 17\%$ ($P = 0.023$) in test muscles (Fig. 3C), suggesting that a counterregulatory retention of NF- κ B dimers in the cytoplasm may be induced. These data together imply a contrasting mechanism of regulation of the NF- κ B pathway in muscle with the well-recognized feed-forward cycle of TNF-

NF- κ B activation occurring in inflammatory cells as part of the classic pathway of activation (5).

I κ BK β overexpression in skeletal muscle. Although we did not detect an effect of NF- κ B activation to attenuate insulin sensitivity in skeletal muscle, the above-described experiment did not rule out the possibility that upstream activation of the I κ BK-NF- κ B pathway could be implicated. Indeed, direct Ser³⁰⁷ phosphorylation of IRS-1 by I κ BK β 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-described experiment whereby we used IVE to introduce EH114-GW-I κ BK β into single rat TCMs, electroporated contralateral TCMs with empty vector, and compared the resulting protein expression after 1 wk. As shown in Fig. 4A, total I κ BK β protein was increased by $655 \pm 54\%$ in test compared with paired control muscles ($P < 0.001$). This resulted in a $470 \pm 97\%$ increase in total Ser^{180/181}-phosphorylated I κ BK α/β (Fig. 4B; $P < 0.001$) and a consequent $12 \pm 5\%$ reduction in I κ B α protein (Fig. 4C; $P = 0.033$), commensurate with increased NF- κ B translocation to the nucleus and transactivation of target genes (5). I κ BK α expression was unaffected by this manipulation, suggesting that there was no compensatory down-regulation of this catalytic subunit, and p65 protein expression was also unchanged (data not shown). Thus we were able to successfully and specifically overexpress I κ BK β in rat muscle and demonstrate an appropriate downstream effect of this manipulation.

I κ BK β overexpression does not impair muscle glucose disposal. In rats electroporated with I κ BK β , we compared glucose disposal and glycogen storage in test and control muscles under hyperinsulinemic-euglycemic clamp conditions. Clamps in this experiment were preceded by an additional 1-h moderate infusion of Intralipid sufficient to activate I κ BK β in the muscle but not to induce insulin resistance when continued for a maximum of 3.25 h (3). Rats weighed 234 ± 4 g at the time

Fig. 2. Effect of p65 overexpression on intracellular signaling. Summary graphs and sample Western blots show that p65 overexpression in TCMs caused a $28 \pm 8\%$ increase in Tyr⁶¹²-phosphorylated insulin receptor substrate (IRS)-1 (*A*) and had no effect on Ser⁴⁷³-phosphorylated Akt (*B*). Protein expression of IRS-1 and Akt were unchanged by the manipulation. * $P < 0.05$ vs. control.



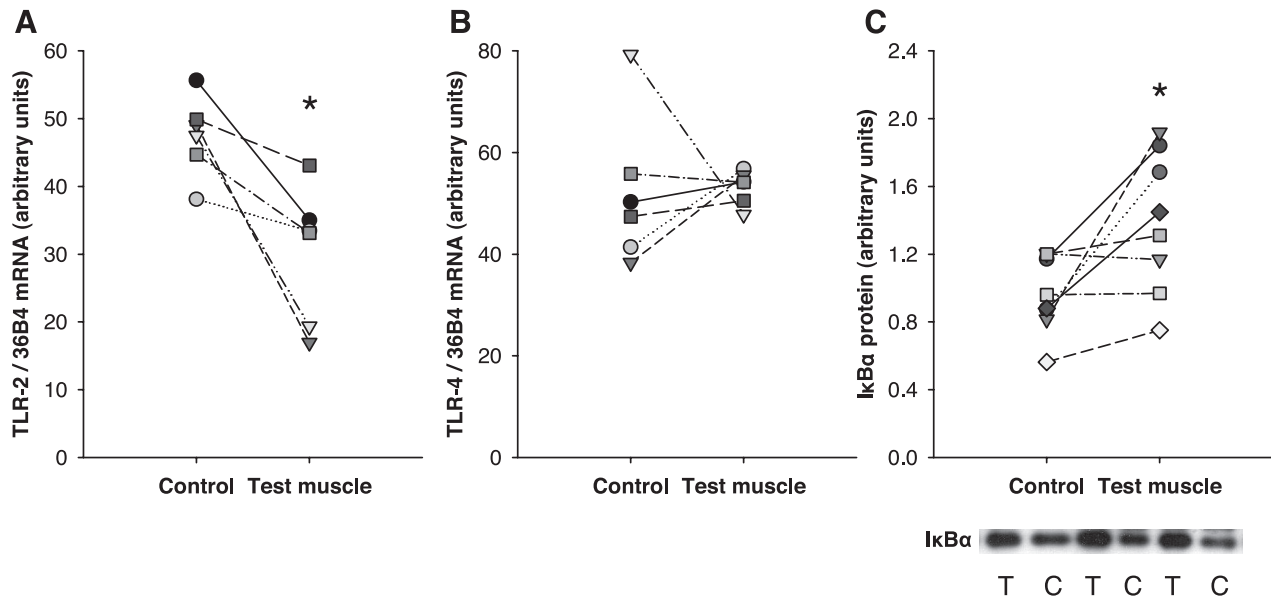


Fig. 3. p65 overexpression has negative feedback effects on the classic NF- κ B signaling pathway. Graphs show the effects of p65 overexpression in muscle on Toll-like receptor (TLR)-2 (A) and TLR-4 mRNA expression (B) as measured using real-time PCR and corrected for 36B4 mRNA. A specific reduction of $36 \pm 9\%$ in TLR-2 mRNA resulted. A summary graph and blot (C) demonstrate a $49 \pm 17\%$ increase in I κ B α protein expression in test vs. control muscles. $*P < 0.05$ vs. control.

of study and had plasma glucose and insulin concentrations of 8.5 ± 0.2 mM and 301 ± 31 mU/l, respectively, during the clamp. Clamp GIR, R_d , and EGO values for these animals were 28.0 ± 1.2 , 33.3 ± 1.8 , and 5.4 ± 1.6 mg \cdot kg $^{-1}\cdot$ min $^{-1}$, respectively. Similarly to results obtained with p65 overexpression, there was no significant difference in R_g' between paired muscles (Table 1), although there was a small, nonsignificant 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,

since 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 I κ BK β overexpression, because these studies were not carried out simultaneously, the differences in glucose and insulin concentrations measured during the clamps may be due, at least in part, to differences in basal concentrations between cohorts (24 ± 2 vs. 12 ± 1 mU/l plasma insulin and 4.7 ± 0.1 vs. 4.3 ± 0.1 mM blood glucose for I κ BK β and p65 rats, respectively).

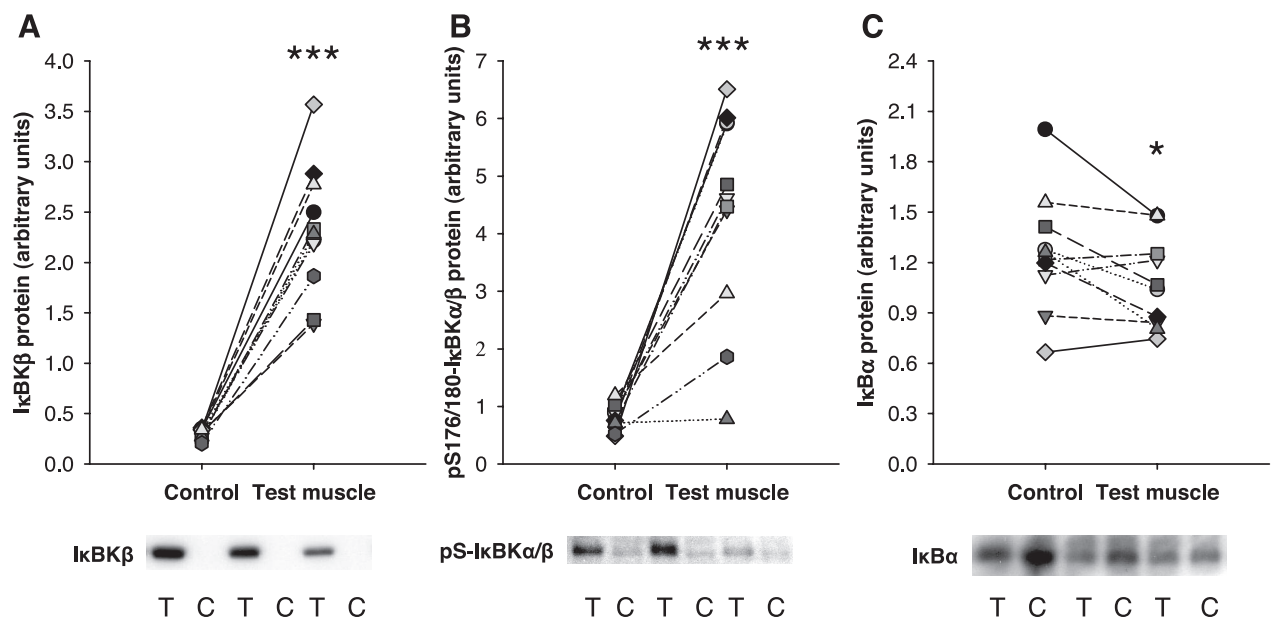


Fig. 4. I κ BK β overexpression in muscle. Summary graphs and representative Western blots confirm successful overexpression of I κ BK β in TCM: I κ BK β protein was increased by $655 \pm 54\%$ (A) and total serine-phosphorylated I κ BK (pS-I κ BK α/β) was increased by $470 \pm 97\%$ (B) in test muscles, resulting in a $12 \pm 5\%$ reduction in I κ B α expression (C), implying activation of NF- κ B. $*P < 0.05$; $***P < 0.001$ vs. control.

I κ BK β overexpression increases phosphorylation of insulin signaling intermediates in muscle. To further investigate whether increased activation of I κ BK β would result in reduced insulin sensitivity, we measured expression and phosphorylation of signaling intermediates in the PI3-kinase cascade in muscles removed from rats at the end of the clamp procedure. We found increases in Tyr⁶¹² phosphorylation of IRS-1 ($24 \pm 11\%$, $P = 0.023$; Fig. 5A), Ser⁴⁷³ phosphorylation of Akt ($23 \pm 3\%$, $P < 0.001$; Fig. 5B), and Ser⁹ phosphorylation of glycogen synthase kinase-3 β ($12 \pm 4\%$, $P = 0.007$; Fig. 5C) as determined 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 signaling pathway in I κ BK β -overexpressing muscles that 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 signaling 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 I κ BK β overexpression (Fig. 5D), confirming that there was no compensatory increase in activity of alternative serine kinases.

DISCUSSION

In the studies described presently, we aimed to determine whether increased activity of the I κ BK β -NF- κ B pathway is sufficient to cause insulin resistance in muscle and to determine which component of the pathway might be more important in this role. To this end, we separately overexpressed I κ BK β and the p65 subunit of NF- κ B in single muscles of normal adult

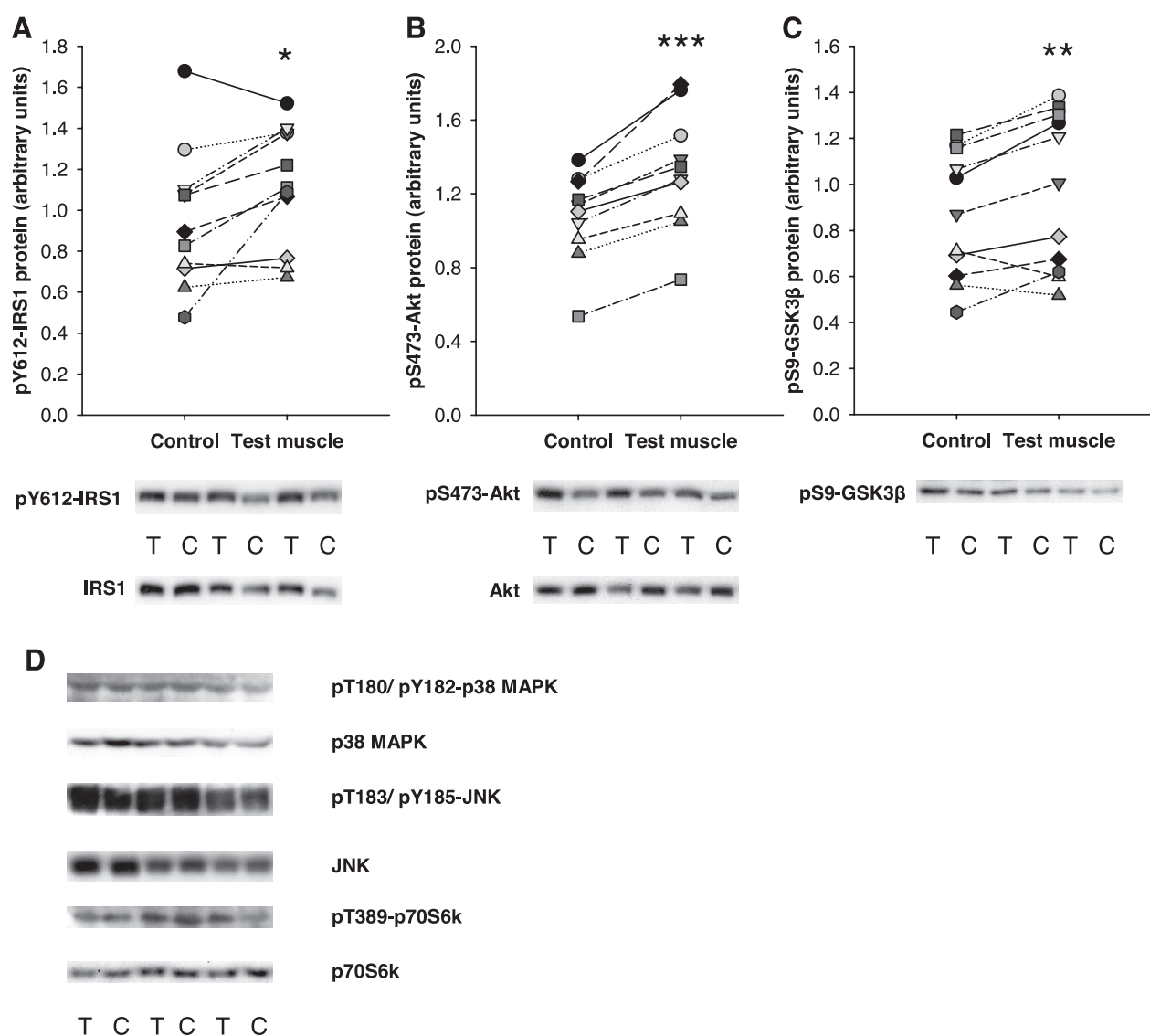


Fig. 5. I κ BK β overexpression in muscle does not impair signaling through the phosphatidylinositol 3-kinase (PI3-kinase) cascade. Summary graphs and representative blots show that overexpression of I κ BK β results in increases in phosphorylation of signaling intermediates IRS-1 (pY⁶¹², by $24 \pm 11\%$; A), Akt (pS⁴⁷³, by $23 \pm 3\%$; B), and glycogen synthase kinase (GSK)-3 β (pS⁹, by $12 \pm 4\%$; C), implying increased flux through the PI3-kinase cascade. However, there was no compensatory change in expression or phosphorylation of alternative serine kinases (p38 MAPK, JNK, or p70S6 kinase; D). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control.

rats for 1 wk using IVE, and we compared the effects on insulin sensitivity with those in paired control muscles. Despite these manipulations resulting in increased degradation of I κ B α and muscle fiber 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 overexpression of target genes to that previously achieved using this method with other signaling molecules that did result in increased glucose disposal into muscle (13, 15, 29). In fact, we found evidence for a moderate increase in flux through the PI3-kinase/insulin signaling cascade, especially following I κ BK β overexpression. In addition, we found evidence for a negative feedback effect of p65 overexpression on activation of the I κ BK β -NF- κ B pathway in muscle, in contrast to the feed-forward effect of NF- κ B 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 on common ancestral features (23). However, the molecular basis for the recently recognized relationship between inflammation and insulin sensitivity of tissues is not well characterized. In particular, it is unclear whether activation of inflammatory signaling pathways is of relevance only within cells of a specific immune lineage or, additionally, within cells traditionally thought of as insulin sensitive, including myofibers. In the studies described presently, we 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 I κ BK β -NF- κ B 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 I κ BK β activation on insulin-stimulated glucose disposal into muscle, our results corroborate those obtained by Cai et al. (7), who saw no effect of transgenic overexpression of I κ BK β 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. (36) showed that a muscle-specific deletion of I κ BK β did not prevent obesity-induced insulin resistance in mice. These chronic studies, together with the relatively acute manipulations described presently, 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 I κ BK β (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 among increased plasma fatty acids, systemic subclinical 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 I κ BK β -NF- κ B pathway and release of cytokines such as TNF- α , which cause NF- κ B-independent signaling impairments in muscle, such as reduced AMP kinase activity (46) and thus attenuated glucose disposal. In support of this, recent studies by Hevener et al. (22) have shown that macrophage infiltration of rodent muscle is increased by high fat feeding and that peroxisome proliferator-

activated receptor- γ expression in macrophages is necessary to suppress the activation of the I κ BK β -NF- κ B pathway and preserve muscle insulin sensitivity.

One of the proposed mechanisms whereby increased I κ BK β 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 PI3-kinase (34). However, we found evidence for moderately increased flux through the insulin signaling pathway in muscles electroporated with I κ BK β especially. Although 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, p70S6 kinase, and JNK (27, 32, 48) may have a local impact on muscle insulin sensitivity.

We also found evidence that p65 overexpression results in negative feedback effects on activation of NF- κ B that have not been documented in muscle to date. Specifically, TLR-2 but not TLR-4 expression was downregulated, implying a reduction in sensitivity of myofibers to activators related chemically to peptidoglycans rather than lipopolysaccharide (6). Recent data suggest 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 NF- κ B target genes. Furthermore, upregulation of I κ B α under the same circumstances would tend to sequester NF- κ B in the cytoplasm and also limit the inflammatory response. These findings are in marked contrast to the feed-forward activation of the I κ BK β -NF- κ B 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 NF- κ B in endothelial cells (20) and macrophages (19) that confirm the feasibility of this hypothesis for muscle.

In conclusion, our data do not provide evidence for a role of activation of the I κ BK β -NF- κ B 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.

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