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

Emma Polkinghorne,1,2 Quintin Lau,1 Gregory J. Cooney,1,3 Edward W. Kraegen,1,2,3 and Mark E. Cleasby1

1Diabetes and Obesity Research Program, Garvan Institute of Medical Research, Sydney; 2School of Medical Sciences, Faculty of Medicine, and 3St. Vincent’s Clinical School, Faculty of Medicine, University of New South Wales, Sydney, Australia

Submitted 17 August 2007; accepted in final form 19 November 2007

Polkinghorne E, Lau Q, Cooney GJ, Kraegen EW, Cleasby ME. Local activation of the IκK-NF-κB 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 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 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 κB kinase (IκK)-nuclear factor-κB (NF-κB) 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-κB and IκBκB 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 IκBκB expression (P < 0.001) caused a significant reduction in IκB protein but also had no effect on clamp glucose disposal after lipid infusion. In fact, IκBκB 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 IκBκB-NF-κB pathway in muscle does not seem to be an important local mediator of insulin resistance.

inhibitor κ kinase-nuclear factor-κB 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 κB kinase β (IκBκB) (49), and indeed, when the corresponding gene was targeted for knockout, the mice were able to withstand diet-induced insulin resistance (50). IκBκB is a catalytic subunit of a trimeric serine kinase that also consists of a second catalytic subunit (IκBKα) and a regulatory subunit (IκBKγ). This enzyme lies upstream of the transcription factor nuclear factor-κB (NF-κB), and together these molecules comprise the principal regulatory pathway for the immune response (5). NF-κB 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-κB dimers are retained in a cytoplasmic location by binding an IκB molecule. When phosphorylated by IκBκB, these inhibitors detach from the complex and are targeted for proteasomal degradation, thus permitting translocation of NF-κB 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 IκBκB-NF-κB 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 IκBκB-NF-κB 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 IκBκB or nuclear translocation of NF-κB is the more significant event in mediating such an
effect. Studies of NF-κB 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 IκBκB, 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 IκBκB-NF-κB pathway is sufficient to cause insulin resistance in muscle and whether this is mediated principally through a direct transcriptional effect of NF-κB or via the kinase activity of IκBκB. 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 vector using the Gateway vector conversion system (Invitrogen, Mount Waverley, Victoria, Australia) by insertion of the cassette into the Eco RV site. The donor vector pDONR201-BSIIMCS was created by recombination of pDONR201 with the multiple cloning site of pBluescriptII KS(+) and ligated into EH114 to make EH114-GW-IκBκB. The excised fragment was subcloned into the corresponding sites of pDONR201-BSIIMCS and then recombined into EH114 to make EH114-p65. pCMV vector containing dIII (GeneWorks, Hindmarsh, South Australia). pcDNA3 expressing the CTCACTATAGGGCGAATTG-3′ PCR using 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTA-11032 with the multiple cloning site of pBluescriptII KS(+) and ligated into EH114 to make EH114-GW-IκBκB. The 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-IκBκB. 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 ± 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, Kempsy, Australia). Approximately 1 wk 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 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-μl 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-μs 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 in 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-[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 IκBκB, 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 was shown to be sufficient to activate total muscle IκBκB (3) but not to induce insulin resistance in muscle, which might disguise the effects of the genetic manipulation (14). At the end of the clamp, rats were euthanized by intravenous injection of pentobarbital 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 (Rd). Endogenous glucose output (EGO) was derived from the difference between Rd and the net glucose infusion rate (GIR). The area under the tracer disappearance curve of 2-deoxy-[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 (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 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-dismembranator II; B. Braun Biotech, Melsungen, Germany) by manual homogenization in RIPA buffer (5 M M 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 μg/ml leupeptin, 10 μg/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, Regensburg Park, NSW, Australia), and aliquots containing 10–60 μg 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 (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 4 μm, deparaffinized, and rehydrated. Tissue slides were retrieved and blocked with hydrogen peroxide followed by serum-protein block (DAKO, Carpenteria, CA) and then incubated in primary antibody (anti NF-κB-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 Axiolab 200M microscope (North Ryde, NSW, Australia) under a ×20, 0.45 LD Achroplan 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.uhmresearch.ca/facilities/wcif/idownload.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 150 corresponding to the level of p65 expression. 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-κB signaling pathway in skeletal muscle is capable of causing local insulin resistance, we aimed to overexpress the transcriptionally active NF-κB 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, and the concurrent 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 ± 17% increase in p65 expression was achieved in test compared with paired control 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-κB 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 71 ± 7%.

RESULTS

Overexpression of the p65 subunit of NF-κB in skeletal muscle. To establish whether specific activation of the NF-κB signaling pathway in skeletal muscle is capable of causing local insulin resistance, we aimed to overexpress the transcriptionally active NF-κB 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, and the concurrent 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 ± 17% increase in p65 expression was achieved in test compared with paired control 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-κB 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 71 ± 7%.
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-κB 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 ± 5g 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, Rd, and EGO values for these animals were 41.7 ± 3.3, 38.9 ± 3.3, and 2.8 ± 2.6 mg·kg\(^{-1}\)·min\(^{-1}\), respectively. There were no differences between paired muscles in metabolic rate 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 IκBKβ overexpression on physiological parameters during hyperinsulinemic-euglycemic clamp**

<table>
<thead>
<tr>
<th>Variable</th>
<th>p65 Overexpression</th>
<th>IκBKβ Overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose disposal into TCM, mg·kg(^{-1})·min(^{-1})</td>
<td>29.7±3.7</td>
<td>26.6±2.5</td>
</tr>
<tr>
<td>Glucose incorporation into TCM, mg·kg(^{-1})·min(^{-1})</td>
<td>10.5±1.7</td>
<td>9.5±1.0</td>
</tr>
<tr>
<td>Stored TCM glycogen, nmol/mg</td>
<td>51±3</td>
<td>61±4</td>
</tr>
</tbody>
</table>

Data are means ± SE for test vs. control muscle groups and demonstrate no effect of either IκBKβ or p65 overexpression in tibialis cranialis muscle (TCM) for 1 wk on glucose disposal or glycogen accumulation.
NF-κB and Muscle Insulin Sensitivity

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 ± 8% (P = 0.011). Thus analysis of glucose disposal and activation of the insulin signaling cascade together provide no evidence that local NF-κB activation is deleterious for muscle insulin sensitivity.

p65 overexpression in muscle has specific negative feedback effects on the classic NF-κB pathway. We were also interested in what effect overexpression of p65 might have on upstream regulators of activation of the classic NF-κB 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 this end, we measured mRNA expression of TLR-2 and TLR-4, both of which have been suggested to play a role in the regulation of the NF-κB pathway in muscle but not to induce insulin resistance when continued for a longer period of time, was also unaffected by the manipulation. (*P < 0.05 vs. control.)

IkBβ overexpression in skeletal muscle. Although we did not detect an effect of NF-κB activation to attenuate insulin sensitivity in skeletal muscle, the above-described experiment did not rule out the possibility that upstream activation of the IkB-NF-κB pathway could be implicated. Indeed, direct Ser473 phosphorylation of IRS-1 by IkBβ 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-IκBβ 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 IκBβ protein was increased by 655 ± 54% in test compared with paired control muscles (P < 0.001). This resulted in a 470 ± 97% increase in total Ser180/181-phosphorylated IκBα/β (Fig. 4B; P < 0.001) and a consequent 12 ± 5% reduction in IκBβ protein (Fig. 4C; P = 0.033), commensurate with increased NF-κB translocation to the nucleus and transactivation of target genes (5). IκBα expression was unchanged by this manipulation, suggesting that there was no compensatory downregulation of this catalytic subunit, and p65 protein expression was also unchanged (data not shown). Thus we were able to successfully and specifically overexpress IκBβ in rat muscle and demonstrate an appropriate downstream effect of this manipulation.

IkBβ overexpression does not impair muscle glucose disposal. In rats electroporated with IκBβ, we compared glucose disposal and glycogen storage in test and control muscles under hyperinsulinemic-euglycemic clamp conditions. Clamp in this experiment were preceded by an additional 1-h moderate infusion of Intralipid sufficient to activate IκBβ in the muscle but not to induce insulin resistance when continued for a maximum of 3.25 h (3). Rats weighed 234 ± 4g 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 ± 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.
of study and had plasma glucose and insulin concentrations of 8.5 ± 0.2 mM and 301 ± 1006 mU/l, respectively, during the clamp. Clamp GIR, Rd, and EGO values for these animals were 28.0 ± 1.2, 33.3 ± 1.8, and 5.4 ± 1.6 mg·kg⁻¹·min⁻¹, respectively. Similarly to results obtained with p65 overexpression, there was no significant difference in Rg 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 IkBβ 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 IkBβ and p65 rats, respectively).

**AJP-Endocrinol Metab • VOL 294 • FEBRUARY 2008 • www.ajpendo.org**
IkB\(\beta\) overexpression increases phosphorylation of insulin signaling intermediates in muscle. To further investigate whether increased activation of IkB\(\beta\) 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 Tyr\(^{612}\) phosphorylation of IRS-1 (24 ± 11%, \(P = 0.023\); Fig. 5A), Ser\(^{473}\) phosphorylation of Akt (23 ± 3%, \(P < 0.001\); Fig. 5B), and Ser\(^{9}\) phosphorylation of glycogen synthase kinase-3\(\beta\) (12 ± 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 IkB\(\beta\)-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, e-Jun terminal kinase (JNK), or p38 mitogen-activated protein kinase (MAPK) were significantly altered by IkB\(\beta\) 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 IkB\(\beta\)-NF-\(\kappa\)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 IkB\(\beta\) and the p65 subunit of NF-\(\kappa\)B in single muscles of normal adult
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 IkBα 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 we found evidence for a negative feedback effect of p65 overexpression on activation of the IkBβ-NF-κB pathway in muscle, in contrast to the feed-forward effect of NF-κB 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 myocytes. 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 IkBβ-NF-κB 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 IkBβ 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 IkBβ 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 IkBβ 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 IkBβ (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 IkBβ-NF-κB pathway and release of cytokines such as TNF-α, which cause NF-κB-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 IkBβ-NF-κB pathway and preserve muscle insulin sensitivity.

One of the proposed mechanisms whereby increased IkBβ 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 IkBβ 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-κB 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-κB target genes. Furthermore, upregulation of IkBα under the same circumstances would tend to sequester NF-κB in the cytoplasm and also limit the inflammatory response. These findings are in marked contrast to the feed-forward activation of the IkBβ-NF-κB 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-κB 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 IkBβ-NF-κB 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.

ACKNOWLEDGMENTS

We are indebted to Drs. Steven Shoelson (Joslin Diabetes Center, Harvard Medical School) and Shane Grey (Inflammation and Immunity Program, Garvan Institute) for provision of DNA constructs, to Tracie Reinien, Jonathan Davey, and Mercedes Ballesteros for expert technical assistance, and to the staff of the Biological Testing Facility at the Garvan Institute for animal care.

GRANTS

We acknowledge funding from the National Health and Medical Research Council of Australia and the Diabetes Australia Research Trust for this work.

REFERENCES


