INHIBITION OF THE IKK PATHWAY RESTORES PI3-K ACTIVITY IN HIGH-FAT FED RODENT SKELETAL MUSCLES

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ABSTRACT

INHIBITION OF THE IKK PATHWAY RESTORES PI3-K ACTIVITY IN HIGH-FAT FED RODENT SKELETAL MUSCLES

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High-fat feeding impairs insulin-stimulated rates of glucose transport, which appears to be a direct result of insulin being unable to fully activate phosphoinositide (PI)-3 kinase. Activation of the IKK pathway has been reported to contribute to inducing skeletal muscle insulin resistance, and we have recently observed that components of the IKK pathway are activated in high fat-fed rodent skeletal muscle. It is not known if the inhibition of the IKK pathway will rescue insulin-stimulated PI3-K activity in high fat-fed rodent skeletal muscle. Sprague-Dawley rats were fed either a control diet or high fat diet for 4 weeks and then allocated (n=8/group) to normal diet, control (CON), high fat diet, control (HF-CON), high fat diet, IKK inhibited (HF-IKK), high fat diet, JNK inhibited (HF-JNK) or high fat diet, NF-κB inhibited (HF-NF-κB) groups. Inhibitors were provided for 5 days by subcutaneous injection after which all animals underwent hind limb perfusions. Insulin-stimulated rates of 3-MG transport and PI3-K activity were significantly increased in the red quadriceps of HF-IKK and HF-NF-κB compared to HF-CON, IKKβ serine phosphorylation was decreased in HF-IKK and HF-NF-κB, and IRS-1 serine phosphorylation was significantly lower in HF-JNK, HF-NF-κB and HF-IKK compared to HF-CON. Suppressing the activation of the IKK pathway in the skeletal muscle of high fat-fed rats resulted in the rescue of insulin stimulated PI3-K activity.
This suggests that the IKK pathway plays a significant role in the inhibition of insulin-stimulated PI3-K activity in the skeletal muscle of the high fat-fed rat.
INTRODUCTION

The high fat-fed rat is a well-studied rodent model of insulin resistant. The skeletal muscle of this model where it has been repeatedly shown that high fat feeding impairs insulin-stimulated rates of glucose transport (17, 40), which largely is a consequence of an inability to effectively traffic the glucose transporter, GLUT-4, to the plasma membrane (44). Activation of downstream components of the insulin signaling cascade are reduced by high fat feeding (e.g., Akt2 and aPKC λ/ζ activities) leading to decreased GLUT4 translocation to the plasma membrane (17, 33, 40, 44, 53, 55, 56). These downstream impairments are a direct result of insulin being unable to fully activate phosphoinositide (PI)-3 kinase. Based on these observations efforts have been made to identify the potential mechanisms that might account for why the provision of a high fat diet decreases insulin-stimulated PI-3 kinase activity.

Activation of the IKK inflammatory pathway has been reported to contribute to inducing skeletal muscle insulin resistance (3, 5, 20, 22-24, 39). Activated IKKβ has been shown to directly phosphorylate IRS-1 on serine 307 (13). This is of interest as increased IRS-1 serine phosphorylation prevents the insulin-signaling cascade from becoming fully activated (21, 30, 47, 62). Of particular note, we have recently observed that components of the IKK inflammatory pathway are activated in the skeletal muscle collected from high fat-fed animals (54). Specifically, IKK (IkB kinase) serine phosphorylation is significantly increased, which may prevent PI-3 kinase from being fully activated in response to the high fat diet. In addition, high fat feeding increases SOCS (Suppressor of cytokine signaling)-3 expression and co-localization of SOCS-3 with both the insulin receptor (IR)-β subunit and IRS-1 (54), which appears to prevent
IRS-1 from binding with the IR-β subunit thereby also reducing PI-3 kinase activity (11, 37). However, we have found (42) that high fat diet-induced impairments in insulin stimulated skeletal muscle PI3-K activity appear within 7 days of animals being on the diet, and result from increased activation of the IKK inflammatory pathway. Whereas, SOCS-3 expression is not altered until animals have been on the high fat diet for at least 4 weeks (42), suggesting that this is a secondary mechanism of impairment.

Clearly, a high fat diet activates inflammatory responses in skeletal muscle. However, which component or components of the inflammatory pathway, when activated, leads to impaired insulin signaling in skeletal muscle are still unknown. Thus, the objective of this investigation was to determine if the inhibition of a specific component within the IKK inflammatory pathway leads to the rescue of insulin-stimulated activation of PI3-K in response to high fat feeding or if multiple signals within the IKK pathway are activated and collectively contribute impairing insulin signaling in skeletal muscle.
METHODS

Experimental design

Forty-eight male Sprague Dawley rats, approximately 4 wks of age weighing between 155 and 225 g, were obtained from Charles River (Willmington, MA) and placed on rat chow (#112386; Dyets Bethlehem, PA). Animals were housed four per cage in a temperature-controlled environment at 21°C with an artificial 12–12 light-dark cycle. Following a one week acclimation period animals were randomly assigned to either control (CON, n=8) or high-fat fed (HF n=40) dietary groups. The control diet consisted of 20% fat-derived calories (coconut oil) (#112386; Dyets, Bethlehem, PA), and the high-fat diet consisted of 59% fat-derived calories (coconut oil) (#112387; Dyets). The rats were allowed to feed ad libitum for 4 weeks. Following this dietary lead in, the high fat-fed rats were further subdivided into High Fat Diet Control (HF CON, n=8), High Fat Diet-TNF-alpha inhibited (HF-TNFα, n=8), High Fat Diet-JNK inhibited (HF-JNK, n=8), High Fat Diet-NF-κB inhibited (HF-NF-κB, n=8), or High Fat Diet-IKK complex inhibited (HF-IKK, n=8) groups. Animals remained on their respective diets during the entire 5 day experimental period.

The HF TNF-α group received 250mg/kg/d of sodium salicylate (#S396 Fisher Scientific, Waltham, MA) (13, 14). TNF-α increases IKK activity thereby the inclusion of this group provides insight on the contribution of TNF-α to the inhibition of insulin-stimulated PI3-K activation (9).

The HF-JNK group received JNK inhibitor SP600125 (#3-514133h Enzo Life Sciences, Farmingdale, NY) at a dose of 10mg/kg/d dissolved in dimethyl sulfoxide (DMSO) (# D1281 Fisher Scientific) (1). This group was included because TNF-α can
activate JNK, and when JNK is activated it can induce IRS-1 serine 307 phosphorylation (20).

The HF-NF-κB group received the aldehyde proteasome inhibitor MG-132 peptide at a dose of 10mg/kg/d (#10012618 Caymen Chemical, Ann Arbor, MI) dissolved in DMSO (49). The proteasome inhibitor prevents IκBα from degradation thereby preventing NF-κB activity (49). The rationale for this group was NF-κB can autoregulate the inflammatory cascade and thus the possibility exists that TNF-α might be increased as a consequence of rather than the cause of NF-κB activation (2).

The High Fat Diet-IKK Complex Inhibition animals were treated with NF-κB essential modifier-binding domain (NDB) peptide that prevents the regulatory subunit, IKKγ from binding with and activating the two catalytic subunits IKKα and IKKβ of the IKK complex (35) thereby providing insight relative to the importance of the activation of the IKK regulatory subunit. NDB (#61169, Anaspec, San Jose, CA) was given at a dose of 0.75 mg/kg/d.

All compounds were administered for five days as effects from these compounds have been shown to occur within as little as three days of treatment (14, 49). The CON and HF CON group received 10mg/kg/d of DMSO. The experimental compounds, route of administration and doses were based on those used in previously published studies (49). Administration was by subcutaneous injection with a 25 G x 5/8 in needle for the groups CON, HF CON, HF-JNK and HF-NFκB. The HF-TNF-α group received their drug dose through oral gavage. All animals received their drug dose for 5 days every evening.
All experimental procedures were approved by the Institutional Animal Care and Use Committee at California State University, Northridge, and conformed to the guidelines for the use of laboratory animals, published by the U.S. Department of Health and Human Services.

*Surgical Preparation and Hind Limb Perfusions*

Following the dietary lead in and experimental period, all animals were subjected to hind limb perfusion. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (6.5 mg/100 g body wt) and surgically prepared for hind limb perfusion as previously described by Ruderman *et al.* (36) and modified by Ivy *et al.* (26). Following surgical preparation, cannulas were inserted into the abdominal aorta and vena cava, and the animals were killed via an intracardiac injection of pentobarbital sodium, as the right hind limb was washed out with 30 ml of Krebs-Henseleit buffer (KHB) (pH 7.55). The cannulas were placed in line with a nonrecirculating perfusion system, and the hind limb was allowed to stabilize during a 5 min washout period. The perfusate was continuously gassed with a mixture of 95% O₂-5% CO₂ and warmed to 37°C.

Perfusate flow rate was set at 5 ml/min during the stabilization and subsequent perfusion, during which rates of glucose transport were determined. Perfusions were performed in the presence of 500 μU/ml of insulin. The basic perfusate medium consisted of 30% washed time-expired human erythrocytes (Ogden Medical Center, Ogden, UT), KHB, 4% dialyzed BSA (Fisher Scientific, Fair Lawn, NJ) and 0.2 mM pyruvate. The hind limbs were washed out with perfusate containing 1 mM glucose for 5 min in
preparation for the measurement of glucose transport. Glucose transport was measured over an 8 min period using an 8 mM concentration of nonmetabolized glucose analog 3-O-methylglucose (3-MG) (32 μCi 3-[3H] MG/mM; PerkinElmer Life Sciences, Boston, MA) and 2mM mannitol (60 μCi-[1-14C] mannitol/mM; PerkinElmer Life Sciences). Immediately after the transport period portions of the red quadriceps were excised from right hind limb, blotted on gauze dampened with cold Krebs-Henseleit buffer, freeze clamped in liquid N₂ and stored at -80° C for later analysis. Rates of insulin-stimulated skeletal muscle 3-MG transport were calculated as previously described (34, 58, 60).

**Muscle Lysate Preparation.**

Muscle samples were weighed and homogenized 1:10 in homogenization buffer (HB) that contained 50 mM Hepes, 150 mM NaCl, 200 mM sodium pyrophosphate, 20 mM α-glycerolphosphate, 20 mM NaF, 2 mM sodium vanadate, 20 mM EDTA, 1% IGEPAL, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 1 mM MgCl₂, 1 mM CaCl₂, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Muscles were homogenized on ice using a glass pyrex homogenizer and then centrifuged at 18,300 x g for 15 min at 4°C (Micromax RF, International Equipment Co., Needham Heights, MA). The supernatant was collected and quantified for protein content by the Bradford Method using a Benchmark microplate reader (BioRad, Richmond, CA).

**Insulin Signaling Cascade.**

**IRS-1 Associated PI3-K Activity.** Between 100 and 150 mg of insulin-stimulated RQ were weighed frozen and homogenized as previously described (34, 58). The
supernatant was collected, quantified for protein and IRS-1 associated PI 3-kinase activity was determined as we have detailed previously (41).

**Total and Phosphorylated IRS-1.** IRS-1 protein concentration, tyrosine and serine phosphorylation of IRS-1 protein was determined using lysate samples. Samples were combined (1:1) with Laemmli sample buffer and heated at 100°C for 5 minutes. One hundred micrograms of muscle lysate protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run under reducing conditions on 10% resolving gel using a Mini-Protean III dual slab cell (BioRad). Resolved gels were equilibrated in a Bjerrum and Schafer-Nielson Transfer Buffer for 30 min. Resolved proteins were then transferred to Polyvinylidene difluoride (PVDF) membranes using a semidry transfer unit (400 mA, 25 max V for 35 min). The membranes were blocked in 5% non-fat dry milk-Tween 20 tris buffered saline (NFDM-TTBS) blocking solution and then incubated with either affinity purified rabbit polyclonal anti-IRS-1 (Cat# 06-526, Millipore Corporation, Temecula, VA), IRS-1 pY 941 (Cat # 07-848, Millipore) or anti-IRS-1 pSer 307 (Cat # 07-247, Millipore) followed by an incubation with a species specific secondary antibody conjugated to horse radish peroxidase (HRP). Antibody binding was visualized using enhanced chemiluminescence (ECL) in accordance to the manufacturer’s instructions. Images were captured using a ChemiDoc system (BioRad) equipped with a CCD camera and saved to a Dell Optiplex GX620 computer. Bands were quantified using Image Lab analysis software (BioRad). Each gel contained samples from least four experimental groups at one time, the density of the bands was determined, and the data were expressed as a percentage of a standard muscle sample run on each gel such that comparisons could be made across gels. The autoradiographs
presented were selected from samples that best reflected the mean of the experimental group.

**Co-immunoprecipitation of IRS-1, pY with IR-β, IR-β Total Protein**

Concentration. Eighty microliters of Protein-A (PRO-A) bead slurry were incubated with 8 µg of anti-IR-β (Cat# 07-724, Millipore) antibody overnight at 4°C with rotation. Following the overnight incubation PRO-A/antibody complex was washed 3 times with Phosphate Buffered Saline (PBS). The PRO-A/antibody complex was then incubated overnight with rotation at 4°C with 800 µg of lysate protein followed by washing once with PBS, once with 2% Triton X-PBS and a final PBS wash before the addition of 33 µl of Laemmli sample buffer. Samples were heated at 100°C for five minutes. Five microliters of eluted sample were loaded in duplicate and subjected to SDS-PAGE run under reducing conditions on a 10% resolving gel. Resolved proteins were transferred to PVDF membranes and blocked with 5% NFDM-TTBS. The membranes were then incubated either for 2.5 hours at 4°C with anti-IR-β (Cat# 07-724, Millipore) or 12 hours at 4°C with either anti-phospho-tyrosine (Cat# 06-427, Millipore) or anti-IRS-1 (Cat# 06-526, Millipore) primary antibody followed by incubation with species specific secondary antibody conjugated to HRP. Antibody binding was visualized and quantified as described above.

**Pro-inflammatory Signaling and SOCS-3.**

IKKβ, IKK α/β pS 176/180, and IκBα. Skeletal muscle lysate samples were used for western blot analysis of IKK α/β pSer 176/180, IκBα and IκBα pSer 32/36. Protein samples were combined with Laemmli sample buffer (1:1) and heated at 100°C for 5
minutes. One hundred micrograms of muscle lysate protein were subjected to SDS-PAGE run under reducing conditions on a 10% (IKK α/β pSer 176/180, IκBα and IκBα pSer 32/36) resolving gels and transferred to PVDF membranes. The PVDF membranes were blocked using either a 5% BSA-TTBS (IKK α/β pSer 176/180) or a 5% NFDM-TTBS (IκBα and IκBα pSer 32/36) solution followed by incubation with either anti-IKKα/β pSer 176/180 (Cat# 2694S, Cell Signaling, Beverly, MA), anti-IκBα (Cat# 4812, Cell Signaling) or anti-IκBα pSer 32/36 (Cat# 9246L, Cell Signaling) primary antibody solutions followed by incubation with species specific secondary antibody conjugated to HRP. Images were captured and quantified as described above.

**Total IKKβ.** PVDF membranes previously run for IKKα/β pSer 176/180 were stripped with 20 mL of stripping buffer (30 g Glycine, 2 g SDS in 800 mL of TTBS with a pH of 2.2 with 20 mL Tween 20) for 30 minutes twice on a shaker at 50°C. Then the PVDF membranes were washed 3 times for 5 minutes each with 25 mL TTBS. The PVDF membranes were then blocked using a 5% NFDM-TTBS solution followed by incubation with anti-IKKβ (Cat# 2370, Cell Signaling), primary antibody solutions followed by incubation with species specific secondary antibody conjugated to HRP. Images were captured and quantified as described above.

**JNK1 and JNK2/3.** Skeletal muscle lysate samples were used for western blot analysis of JNK1 and JNK2/3. Protein samples were combined with Laemmli sample buffer, subjected to SDS-PAGE run under reducing conditions on a 10% resolving gel and transferred to PVDF membranes. The PVDF membranes were blocked followed by incubation with anti-JNK (#9252, Cell Signaling) primary antibody followed by
incubation with a species specific secondary antibody conjugated to HRP. Images were captured and quantified as described (4, 19).

**SOCS-3 Protein Concentration and Co-immunoprecipitation of SOCS-3 with IR-β and IRS-1.** Eighty microliters of PRO-A bead slurry were incubated with 8 µg of anti-SOCS-3 (Cat# sc-9023, Santa Cruz Biotechnology (SCBT), Santa Cruz, CA) antibody for 2.5 hours at 4°C with rotation. Following the incubation PRO-A/antibody complex was washed 3 times with Phosphate Buffered Saline (PBS). The PRO-A/antibody complex was then incubated overnight with rotation at 4°C with 800 µg of lysate protein followed by washing once with PBS, once with 2% Triton X-PBS and a final PBS wash before the addition of 33 µl of Laemmli sample buffer. Samples were heated at 100°C for 5 minutes. Five microliters of eluted sample were loaded in duplicate and subjected to SDS-PAGE run under reducing conditions on a 10% resolving gel. Resolved proteins were transferred to PVDF membranes and blocked with 5% NFDM-TTBS. Separate gels were run for each protein and transferred to PVDF membrane. The membranes were then incubated with either anti-SOCS-3 primary antibody (Cat# sc-9023, SCBT) for 3 hours at 4°C, anti-IR-β primary antibody (Cat# 07-724, Millipore) for 2.5 hours at 4°C, or anti-IRS-1 primary antibody (Cat# 06-526, Millipore) for 12 hours at 4°C followed by incubation with species specific secondary antibody conjugated to HRP. Antibody binding was visualized and quantified as described above.

*Statistical Analysis.*

An analysis of variance (ANOVA) was used on all variables to determine whether significant differences existed between groups. When a significant $F$-ratio was obtained,
a Tukey HSD post-hoc test was used to identify statistically significant differences (p<0.05) among the means. Statistical analyses were performed using JMP software (SAS Institute Inc., Cary, NC). All values are expressed as means ± SE.
RESULTS

Skeletal Muscle 3-MG Transport

Insulin stimulated rates of 3-MG transport in the RQ of the CON animals (6.63 ± 1.17 µmol/g/h) was greater (p<0.05) than the HF CON animals (3.36 ± 1.2 µmol/g/h). Insulin stimulated rates of 3-MG transport in HF-JNK (6.28 ± 1.2 µmol/g/h), HF-NFκB (7.59 ± 1.2 µmol/g/h) and HF-IKK (7.42 ± 1.0 µmol/g/h) animals were not significantly different (p<0.05) from CON animals.

Insulin Signaling Cascade

IR-β protein concentration and IR-β subunit tyrosine phosphorylation were not significantly different among groups (Fig. 1A). IRS-1 protein concentration (Fig. 1B) was not significantly different among groups. IRS-1 tyrosine phosphorylation (Fig. 1B) was significantly reduced (p<0.05) in HF CON and HF-JNK compared to the CON animals. However, IRS-1 tyrosine phosphorylation in the HF-IKK and HF-NFκB groups

Figure 1. IR-β subunit protein concentration (A) and, IRS-1 concentration, IRS-1 (pγ) and IRS-1 ser307 phosphorylation (B) insulin-stimulated red quadricep obtained from Sprague Dawley rats. *, Significantly different from CON animals (p<0.05). †, significantly different from HF CON animals (p<0.05) Values are expressed as means ± SE.
were not significantly (p<0.05) different from CON animals. IRS-1 serine 307 phosphorylation (Fig. 1B) was significantly lower (p<0.05) in HF-JNK, HF-NFκB and HF-IKK animals compared to HF CON animals. However, IRS-1 serine 307 phosphorylation in HF-JNK, HF-NFκB and HF-IKK animals was not significantly different (p<0.05) from that of the CON animals.

IRS-1 co-immunoprecipitation with IR-β (Fig. 2A) was reduced (p<0.05) in HF CON, HF-JNK, HF-NF-κB and HF-IKK groups compared to CON animals. Although, HF-IKK animals were significantly lower (p<0.05) from CON animals they were significantly higher compared to HF-CON animals. IRS-1 associated PI-3K (Fig. 2B) activity was significantly lower (p<0.05) in HF CON and HF-JNK compared to CON animals. HF-IKK and HF-NFκB animals PI-3K activity was significantly elevated (p<0.05) compared to both CON and HF CON animals.

**Figure 2.** Co-immunoprecipitation of IRS-1 with IR-β subunit (A), insulin-stimulated IRS-1 associated Phosphoinositol 3-kinase (PI3-K) activity (B), insulin-stimulated red quadriceps obtained from Sprague Dawley rats. *, Significantly different from CON animals (p<0.05). †, significantly different from HF CON animals (p<0.05). Values are expressed as means ± SE.

**Pro-inflammatory Signaling Cascades and SOCS-3**

IKKβ protein concentration (Fig. 3A) was similar among groups, except for HF-IKK animals, which was significantly lower (p<0.05) compared to CON and HF CON
animals. IKKα/β serine phosphorylation (Fig. 3A) was significantly elevated (p<0.05) in HF CON and HF-JNK compared to CON animals. HF-IKK and HF-NFκB groups were not significantly different (p<0.05) from the CON animals. IκBα total protein concentration (Fig. 3B) was similar among groups, with the exception of HF-NFκB

![Graphs showing IKKβ, IKK αβ pS, IκBα and JNK1/2/3 protein concentrations](image)

**Figure 3.** IKKβ protein concentration and IKK serine phosphorylation (A), IκBα protein concentration and IκBα serine phosphorylation 32/36 (pS) (B), JNK1 and JNK 2/3 protein concentration (C) in insulin stimulated red quadriceps obtained from Sprague Dawley rats. *, Significantly different from CON animals (p<0.05). †, significantly different from HF CON animals (p<0.05). Values are expressed as means ± SE.

animals which had a significantly higher (p<0.05) protein concentration of IκBα compared to CON animals. IκBα serine phosphorylation in HF CON, HF-JNK and HF-NFκB was significantly higher (p<0.05) from CON animals. IκBα serine
phosphorylation in HF-IKK animals was not significantly different (p<0.05) from CON animals but was significantly reduced (p<0.05) compared to HF CON animals. JNK 1 and JNK 2/3 concentration (Fig. 3C) were significantly increased (p<0.05) among HF CON, HF-IKK and HF-NFκB compared to CON animals. JNK 1 and JNK 2/3 concentration in HF-JNK and HF-NFκB animals were significantly lower (p<0.05) compared to HF CON animals.

SOCS-3 protein concentration (Fig. 4A), SOCS-3 co-immunoprecipitation with IR-β (Fig. 3D) and SOCS-3 co-immunoprecipitation with IRS-1 (Fig. 3D) were all significantly higher (p<0.05) in the HF-JNK, HF-IKK and HF-NFκB animals compared to CON animals, but were significantly lower (p<0.05) than the HF CON animals.

Figure 4. SOCS-3 protein concentration, SOCS-3 co-immunoprecipitation with IR-β, and SOCS-3 co-immunoprecipitation with IRS-1, (A) in insulin stimulated red quadriceps obtained from Sprague Dawley rats. *, Significantly different from CON animals (p<0.05). †, significantly different from HF CON animals (p<0.05). Values are expressed as means ± SE.
DISCUSSION

The high fat-fed rat has been used as a rodent model of insulin resistance in a number of investigations (15, 16, 18, 52, 57). Sprague Dawley rats placed on a high fat diet exhibit hyperinsulemia, hyperglycemia, elevated LDL cholesterol, VLDL triglycerides and marked visceral obesity (10). Of particular interest to us have been the finding that the skeletal muscle of the high fat fed rat is unable to effectively transport glucose as evidenced by decreased rates of insulin stimulated 3-MG transport (18, 45, 52). In agreement with these previous finding we have found in the present investigation that insulin stimulated 3-MG transport rates were significantly reduced in the skeletal muscle of the HF group compared to the CON animals. It has been reported that the decreased rates of insulin stimulated 3-MG transport in the skeletal muscle of high fat-fed rodents appears to be a result of insulin being unable to fully activate PI3-K activity (7, 28). Consistent with these previous reports, we found that insulin was unable to activate PI3-K activity in the HF animals to the same extent that was observed in the CON animals. As it appears that the decrease in insulin stimulated 3-MG transport is primarily a function of the decrease in PI3-K activity (7, 28), it has become of interest to determine why a high fat diet causes impairments in insulin stimulated PI3-K activity.

It has been suggested that the decrease in insulin stimulated PI3-K activity in insulin resistant skeletal muscle is a result of increased activation of the IKK pathway (49, 59, 63, 65). Itani et al. (25) showed that lipid infusion-induced insulin resistance in humans decreased skeletal muscle IκBα protein concentration, which is indicative of increased IKKβ activation. In contrast, the IKKβ knockout mouse does not develop insulin resistance in response to lipid infusion (32, 61). Activated IKKβ has been shown
to directly phosphorylate IRS-1 on serine 307 (43, 59, 64). This is of particular interest as increased IRS-1 serine phosphorylation prevents the insulin signaling cascade from becoming fully activated (60, 64). Additionally, chronic administration of sodium salicylates have been shown to improve insulin sensitivity by impairing the activation of the IKK pathway (42, 46, 49). We performed a preliminary exploration of the IKK pathway in high fat-fed rodent skeletal muscle (42) and found that activation of select components of the IKK pathway were increased in the skeletal muscle of the high fat-fed rat, and that IKKβ serine phosphorylation was elevated. In agreement with these previous findings, we observed in the present investigation that although IKKβ protein concentration was unaltered by the high fat diet, serine phosphorylation of this protein was significantly increased, and likely contributed to the increased IRS-1 serine phosphorylation in the skeletal muscle of the HF animals.

As it appears the insulin stimulated PI3-K activity is decreased in the skeletal muscle of the high fat-fed rat due to increased IKK pathway activation, we were curious if insulin stimulated PI3-K activity could be rescued if activation of the IKK pathway could be attenuated in the skeletal muscle of the high fat-fed rat. This appears to be possible as evidenced by the effect of high dose sodium salicylate treatment suppressing the activation of the IKK pathways (6, 42, 48, 63). However, it was not identified as to what component of the IKK pathway was affected by salicylate treatment. Thus, we attempted to inhibit specific points of the IKK pathway with select compounds known to impair activation of the IKK pathway.

One compound we chose to utilize was the cell permeable NEMO binding domain (NBD) peptide as it has been reported to interrupt the bond between IKKγ and the
IKKα/β complex in rodent skeletal muscle, which subsequently prevents activation of the IKK pathway (8). It has been reported that when IKKβ is activated it directly phosphorylates the IRS-1 on serine 307 (12). This is of interest as increased IRS-1 serine phosphorylation prevents the insulin stimulated signaling cascade from becoming fully activated (20, 31, 61). In the high-fat fed rat we have found that components of the pro-inflammatory pathway are activated (57). In particular, IKK serine phosphorylation was increased, which may have contributed to increased IRS-1 serine phosphorylation, and in turn prevented PI3-K from being fully activated. In the present investigation we found that treating high fat-fed rats with NBD increased insulin-stimulated skeletal muscle PI3-K activity and 3-MG transport compared to high fat control animals. This was likely the result of the NBD treatment decreasing IKKβ phosphoserine concentration in the skeletal muscle of these animals.

We also evaluated the aldehyde proteasome inhibitor MG-132 peptide, as it prevents IκBα from degradation thereby preventing NF-κB activity (49). The rationale for utilizing this inhibitor is that NF-κB can auto-regulate the inflammatory cascade -and thus the possibility exists that TNF-α might be increased as a consequence rather than cause NF-κB activation (29, 35, 49). This is of interest as TNF-α activates c-jun N-terminal kinase (JNK), a member of the MAPK family, and when activated JNK can induce IRS-1 serine 307 phosphorylation (20). We found that the skeletal muscle of the high fat-fed animals that were treated with the MG-132 peptide exhibited a decrease in IKK serine phosphorylation, which appeared to contribute to a decrease in the degree of serine phosphorylation on IRS-1. The decreased IRS-1 serine phosphorylation, in turn, appeared to allow for partial restoration of insulin-stimulated PI3-K activity and 3-MG
transport in the skeletal muscle of these animals. Of note, the positive effect appears to occur upstream of NF-κB activation, thereby suggesting that the increased TNF-α found in the skeletal muscle of high fat-fed animals is not a consequence of NF-κB activation.

It has been reported that a high fat diet increases JNK activation in rodent skeletal muscle (38). Thus, it is possible that a portion of the IRS-1 serine phosphorylation may also result from TNF-α induced JNK activation. To inhibit JNK, we utilized the compound SP600125, a competitive MAPK inhibitor that inhibits JNK phosphorylation (3). Although JNK 1 concentration was reduced in the skeletal muscle of the high fat-fed rats treated with SP600125, insulin stimulated PI3-K activity was not different from the high fat diet control animals. This was likely a result of IKKβ serine phosphorylation in the skeletal muscle of these animals being unaffected by SP600125, and thus IRS-1 serine phosphorylation remained elevated, which served to impair activation of PI3-K. However, it is important to note that when a JNK inhibitor is used separately from IKK inhibitors it has been reported that it will not completely restrict IRS-1 serine phosphorylation and IκBα can still be activated (27). Thus, additional work is necessary to ascertain the full contribution of JNK as an inhibitor of insulin-stimulated PI3-K activation.

We have recently evaluated whether suppressor of cytokine signaling 3 (SOCS-3) could account for the reduced insulin-stimulated PI3-K activity in the high fat-fed rodent (50). We observed that SOCS-3 protein concentration was elevated in the skeletal muscle of the high-fat fed animals, and that there was a significantly greater co-immunoprecipitation of SOCS-3 with both the IR-β subunit and IRS-1 in the high-fat fed skeletal muscle. We have hypothesized that this might possibly result in a physical
barrier that impaired IRS-1 from interacting with the IR-β subunit (57). In the present investigation we found that SOCS-3 concentration was elevated in the skeletal muscle of all the high fat-fed animals compared to the normal diet control animals, and SOCS-3 protein concentration was unaffected by any of the experimental treatments. SOCS-3 co-immunoprecipitation with both the IR-β subunit and IRS-1 also remained significantly greater in the skeletal muscle of all the high fat-fed animals compared to the normal diet control animals. However, as noted above, we were able to restore insulin-stimulated PI3-K activity in two of our high fat-fed groups. Thus, it appears that SOCS-3 may not play as significant of a role in impairing insulin-stimulated PI3-K activity in high fat-fed rodent skeletal muscle as we had previously suggested.

**Summary**

We and others (18, 51, 57, 60) have shown that a high fat diet decreases insulin-stimulated PI3-K activity in rodent skeletal muscle. It has been suggested the high fat diet activates the IKK pathway which in turn results in the inhibition of insulin-stimulated PI3-K activation. Thus, in the present investigation we evaluated if activation of the IKK pathway could be attenuated in the skeletal muscle of the high fat-fed rat would result in restoration of insulin-stimulated PI3-K activity. We observed that when the activation of the IKK pathway was reduced in the skeletal muscle of the high fat-fed rat that insulin-stimulated PI3-K activity and rates of 3-MG transport were rescued.
REVIEW OF LITERATURE

Introduction

Approximately one-third of the United States population is obese and 8.3% of the American population is diabetic totaling 25.8 million adults and children. Environmental, as well as genetic factors, contribute to the epidemic of diabetes, which includes, but is not excluded to, lack of physical activity and unhealthy eating habits. According to the Center for Disease Control (CDC), diabetes was the 7th leading cause of death in 2007 (19) and is the leading cause of blindness, stroke, kidney failure, heart disease, and lower limb amputations. Based on current data, the CDC predicts 1 in 3 Americans will be diabetic by 2050. Due to the substantial financial impact that diabetes has had in the United States, estimated at over $174 billion dollars in direct and indirect medical costs annually, alternative means of treating diabetes need to be established. Individuals with diabetes have a 3-4 times higher mortality rate compared to non-diabetic individuals.

There are two different classifications of diabetes, Type I and Type II, the most common form, Type II, diabetes accounts for ninety-five percent of diabetic patients. Type II diabetes, also known as non-insulin dependent diabetes mellitus (NIDDM), is a chronic disease associated with low grade inflammation and improper use of circulating insulin (4, 5, 24, 25, 54). Insulin resistance is the precursor to diabetes and if insulin function is not restored NIDDM is inevitable, but this can take as many as 10-20 years until development (75, 76). In a healthy individual, the binding of insulin to the insulin receptor initiates glucose metabolism in insulin sensitive tissues, more specifically in
skeletal muscle (26, 27). When insulin does not initiate the correct response on its tissues downstream proteins will not activate GLUT 4 transport to the plasma membrane and glucose cannot properly enter the cell. As a result blood glucose levels increase (66, 79, 91, 137). Pancreatic beta cells release greater amounts of insulin to compensate for the increase in circulating glucose, with continued release of such high amounts pancreatic function will be hindered.

This literature review will begin with a description of the insulin signaling cascade and then move to impairments of the insulin signaling cascade to understand insulin resistance. Specific inflammatory proteins will be discussed in detail to further the understanding of the pro-resistant tissue, inflammatory response and it’s contribution to impaired insulin signaling. Finally, there will be a discussion on drug treatment and if it is possible to rescue insulin response in insulin

*Insulin Signaling Cascade*

The insulin receptor plays a crucial role for glucose uptake to the cell and has a heterotetrameric membrane glycoprotein with two alpha (α) subunits found on the outside of the cell, and two beta (β) subunits within the cell that create a link between the extra and intercellular regions to allow for transportation of substrates (22, 34, 54, 64, 65). Insulin binds to the alpha subunit causing it to become activated and bring the two units closer together. Following a conformational change in the β subunits, tyrosine auto-phosphorylation occurs, which is important in order for the receptor’s kinase activity to be towards the intercellular proteins (15, 16, 65, 116). Substrates such as the insulin
receptor substrates (IRS) family contain phosphotyrosine-binding (PTB) and Src homology 2 (SH2) domains which activate downstream proteins.

There are four members of the IRS family that have been identified that are important in the regulation of the insulin signaling cascade. The focus will be on IRS-1 as it pertains most to the subject of this review since IRS-1 and IRS-2 are the two members of the family found in skeletal muscle (15, 16, 46, 122, 123). Characteristics of the IRS proteins consist of an NH$_2$-terminal pleckstrin homology (PH) domain and a PTB domain. The tail, or COOH-terminal, is longest in IRS-1 and IRS-2, and contains 20 tyrosine phosphorylation sites (13, 15, 16, 34, 42, 101, 113, 122, 123) for binding to the SH2 domains on proteins and kinases such as PI3-kinase (17, 47, 52, 99).

Phosphoinositide 3 kinase (PI3-K), due to the tyrosine-phosphorylation of IRS-1, is activated which regulates glucose transport, as well as glycogen and lipid storage and protein synthesis (13, 22, 102). Class IA PI3-K is important for glucose transport, and if PI3-K is inhibited glucose transport will not occur (80, 93, 102, 103). Research has shown that a pharmacological inhibitor of PI3-K known as wortmannin (20, 84, 104), blocks insulin stimulated glucose uptake, proving the importance of PI3-K to the insulin signaling cascade. Two important subunits within PI3-K are the 85 kDa regulatory subunit (p85) and the 110 kDa catalytic subunit (p110), which is stabilized by p85 (17, 30, 79, 80, 84, 90, 91, 107, 137). Tyrosine phosphorylation of IRS-1 creates binding sites for p85. Once bound, regulatory subunit p85 releases its hold on p110 and recruits PI3-K from the cytosol to the plasma membrane (41, 80). Activation of PI3-K causes phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) at the plasma membrane to form tris-phosphorylated inositol (PIP$_3$) (14, 17, 30, 90, 123, 124, 126). Tris-
phosphorylated inositol is important for downstream GLUT-4 trafficking because PIP$_3$ activates 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (37). Protein kinase B (Akt/PKT) and atypical protein kinase C $\zeta$ and $\lambda$ (aPKC $\zeta$/$\lambda$) will then be activated (2, 16, 40, 61, 67, 82, 99, 103) and assist with the translocation of GLUT-4 to the plasma membrane. Following serine/threonine of Akt/PKT, Akt substrate AS160 is dephosphorylated allowing its activation which then removes the brake on GLUT-4. The combination of Akt, AS160, and aPKC $\zeta$/$\lambda$ finish the process of insulin stimulated glucose uptake to the cell by assisting in the translocation of GLUT-4 containing vesicles to the plasma membrane.

*The High Fat-Fed Rat - A Model of Insulin Resistance*

The multi-purpose Sprague Dawley rat was first bred in 1925 and is the most commonly used rodent experimental model in toxicology, reproduction, pharmacology, behavior research, nutrition, diet induced obesity, and oncology (48, 50). Sprague Dawley rats placed on high fat diets exhibit hyperinsulemia, hyperglycemia, elevated LDL cholesterol, VLDL triglycerides and marked visceral obesity (6, 8, 14, 47-50, 72, 93, 106, 125, 129-132, 134, 137). A diet high in polyunsaturated fat (60% fat) compared to a control diet (17% fat) has also shown to replicate the obese model by having a significantly higher body weight over the control diet animals (49, 52, 73, 79, 94, 130, 133, 137).

Research has shown that plasma glucose, plasma insulin, triglycerides, and free fatty acids are higher in high-fat fed rats compared to rats placed on a regular rat chow diet (23, 61). High-fat feeding decreases insulin stimulated glucose transport in as little
as four weeks (48), a key factor in skeletal muscle insulin resistance. The major cause of the reduction in glucose transport is from an impairment of GLUT-4 translocation to the plasma membrane (37, 81, 115, 137). GLUT-4 protein concentration has shown to not be the cause of a decrease in transport function; instead studies have observed that GLUT 4 translocation is the issue (11, 51, 130, 137). The composition of a high fat diet alters the plasma membrane, which presents itself as a possibility for the lack of bonds at the insulin receptor (51).

It has been found that insulin receptor substrate (IRS)-1 tyrosine phosphorylation decreases with high fat feeding (73, 129, 130), but there is no difference in IRS-1 protein concentration. Sprague Dawley rat have also shown a decrease in PI3-K activity in skeletal muscle following a high fat diet. Which, in turn, causes a decrease in GLUT-4 transport to the plasma membrane (11, 52, 73, 129, 130). Findings such as these are the reason why the Sprague Dawley rat is so often used as a model for insulin resistance for further understanding of proteins and pathways. Additionally the stable gene pool of the Sprague Dawley rat minimizes variations between rats.

The Pro-Inflammatory Cascade

As previously mentioned, obesity is linked with chronic low grade inflammation of the white adipose tissue which can lead to type II diabetes (9, 24, 36, 78, 88). Under inflammatory conditions, cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and C-reactive protein (CRP) are secreted resulting in the activation of pro-inflammatory signaling pathways (96). Recent research has confirmed the relationship between inflammatory pathways and insulin resistance. High-fat fed rodents had an
accumulation of free fatty acids (FFAs) in skeletal muscle and hepatic tissue (12), which was thought to cause an alteration in fat metabolism in a study done by Randle et al. over 30 years ago (92). Kim et al.(69) found that the increase in FFAs are responsible for the decrease in glucose transport activity and inhibition of IRS-1 tyrosine phosphorylation (3, 29, 68-70, 97, 98). Intramuscular fat metabolites, acyl Co-A and diacylglycerol, increase in response to the flux in FFA (12, 61, 62, 135). Additionally, protein kinase C-Θ is activated by diacylglycerol and was shown by Itani et al. (62) to directly cause serine phosphorylation on the insulin receptor (61, 69, 70). It was also shown by Itani et al. (62) that PKC inhibitors resulted in a reversal of insulin resistance. An increase in FFAs also increases IKappaB kinase (IKK)-β, another serine/threonine kinase (12), and could have also resulted in serine phosphorylation on the insulin receptor. Regardless of which kinase inhibited IRS-1 tyrosine phosphorylation, FFAs interrupt the insulin signaling cascade with cytokine production and inflammatory pathways.

In a healthy individual, anti-inflammatory cytokines overpower pro-inflammatory cytokines to create equilibrium. However, insulin resistance results in an overproduction of pro-inflammatory cytokines and a deficiency in anti-inflammatory cytokines in skeletal muscle (9, 53, 56-59). The amount of pro-inflammatory cytokines is dependent on fat mass; the higher the body mass the higher the cytokines. Alternatively, with fat loss there is a decrease in pro-inflammatory cytokines (23, 25).

While recent studies have suggested that inflammatory pathways are responsible for the insulin-stimulated impairment of PI3-K activity following a high fat diet there is no answer as to which pro-inflammatory cytokines are activated first.
The IKappaB Kinase (IKK) complex is composed of two catalytic subunits, IKK-α and IKK-β, and the regulator kinase IKK-γ, also known as NEMO. IKK-β is the primary subunit of the IKK/NF-κB pathway and has been of interest to researchers in regards to the inflammatory response. Activation of IKK-β phosphorylates serine residues of IκB allowing NF-κB to disassociate (28, 60, 117) and stimulate pro-inflammatory cytokines. When activated, IKK-γ, will bind to the carboxy terminal of IKK-α or IKK-β on the NEMO binding domain (NBD) which will turn on the IKK complex. With the activation of the IKK complex, downstream regulation of transcription factor NF-κB can occur as well as production of tumor necrosis factor (TNF)-α (23, 117). There exists a positive feedback loop between NF-κB and TNF-α within skeletal muscle and adipose tissue (135). Yang et al. (128) found that the interaction between TNF-α and NF-κB increases the IKK-β response and inhibition of either cytokine resulted in a lesser response from IKK-β. There was no difference in IKK-α activation. This finding is of importance because IKK-β has been demonstrated to cause serine phosphorylation of IRS-1 (12, 61, 62, 70, 100, 135). If IKK-γ is inhibited, the IKK complex will not be turned on, nor will the IKK complex activate NF-κB.

In 2010, Yaspelkis et al. (130) found that high fat feeding did not increase IKKα and IKKβ concentrations but the high fat diet did increase serine phosphorylation of IKKα/β and IκBα phosphorylation. DiDonato previously stated that TNF-α increases IKK activity (28) which was also found true in Yaspelkis’ 2010 study. As stated before activation of IKK β has been reported to directly serine phosphorylate IRS-1 and impair insulin signaling. The findings by Yaspelkis et al. (130) confirm that high fat feeding
results in activation of IKK pathways and impairments of insulin signaling. Cell-permeable peptides have been used to block binding sites on the NBD and have shown lower concentrations of the IKKs and NF-κB (103). Sodium salicylate has been previously used in the treatment for insulin resistance (44, 135) because of its influence on the pro-inflammatory pathways.

*Nuclear Factor Kappa B (NFkB)*

The transcription promoter and member of the Real family, nuclear factor kappa B (NFkB), is found in almost all animal cells. Although NF-κB has been found to be activated by pro-inflammatory cytokines (128), there exists a positive feedback loop between NF-κB and other cytokines, such as TNF-α, (88, 110, 117, 129) to continue the pro-inflammatory response. There are five members of the NF-κB family, the most common one being a p50 and RealA (88) subunit (110, 129). When NF-κB is stable it is bound to its inhibitor IκB (128). Following the inflammatory response IκB is phosphorylated and allows NFκB to disassociate and move freely to the cell nucleus where it can bind to target genes (85, 129). The phosphorylation of IκB occurs from the ubiquitin-proteasome pathway (61, 110) by the action of 26S proteasome. As mentioned previously, NF-κB is required for cytokine production including TNF-α and IL-6. These cytokines are also responsible for activating NF-κB, leading us to believe that they all amplify the response of one another (129).

*C-Jun N-terminal kinases (JNK)*

C-Jun N-terminal kinase (JNK) is activated by free fatty acids and inflammatory cytokines, among those are TNF-α and members of the MAP kinase kinases (MAPKKs),
specifically MKK4 and MKK7 (1, 10, 53, 111, 120, 121). Hirosumi et al. (53) found both genetic (ob/ob) and dietary obese models exhibited high activity of JNK regardless of the tissue, liver, muscle or adipose when compared to lean control. There exist three subgroups of JNK; JNK1 and JNK2 which are found in all cells and tissues, and JNK3 which is found mostly in the heart, testis, and brain (10, 57). In regards to insulin resistance, focus will be on JNK1 and JNK2 because of their prevalence in skeletal muscle. Upon activation of the inflammatory response JNK levels are raised, JNK-interacting proteins (JIPs) are scaffolding molecules that create a link between IRS-1 and JNK for phosphorylation of IRS-1 on Ser\(^{307}\) and inhibiting the signal of the insulin receptor (57, 63, 111, 121). Along the COOH terminus of IRS-1 are JNK binding motifs which indicate a specific relationship between JNK and IRS-1 and explain the increase in phosphorylation of IRS-1 with JNK expression. This knowledge has also lead to the understanding that JNK is a mediator of TNF-\(\alpha\) (10). Davis et al. (96) found that JNK knockout mice had a reduction in insulin resistance following a high fat diet and also a decrease in IRS-1 serine phosphorylation compared to control animals. In a study done by Aguirre et al. (1) mutated Ser\(^{307}\) sites resulted in a decrease in phosphorylation of IRS-1 by JNK and serine phosphorylation on IRS-1 by TNF-\(\alpha\). Although the JNK pathway plays a supporting role in insulin resistance, studies have continued to use JNK inhibitors to determine if there is a rescue of the insulin signaling cascade (96, 120).

**Suppressor of Cytokine Signaling (SOCS)-3**

Suppressor cytokine signaling (SOCS) proteins were discovered in 1997 and comprised of eight family members (SOCS1-7 and CIS) (35, 87, 109). All eight members of this family of proteins (SOCS 1-7 and CIS) contain an N-terminal, Src
homology domain 2 (SH2) and a C-terminal domain, called a SOCS box, varying in length (95, 118). By inhibiting the janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, SOCS proteins can bind to SH2 domains of tyrosine-phosphorylated cytokine receptors and inhibit downstream insulin signaling, a process known as competitive inhibition (39, 45, 104, 118, 119). In addition to competitive binding to the Tyr960 binding site on the IR, SOCS proteins can also disrupt the insulin signal by degradation of IRS proteins (38).

SOCS-3 is prominently found in insulin resistant animal tissue (31, 33, 34, 38, 74, 100, 118) within the liver and skeletal muscle (118, 131). Expression of SOCS-3 is activated by hormones, cytokines and growth factors (31-34, 38, 74, 100, 118) Emanuelli et al. (34) used a yeast two-hybrid system to show the interaction between SOCS-3 and the insulin receptor. In this study by Emmanuelli et al. (34) it was found that a mutation in the insulin receptor Tyr960 stopped the binding of SOCS-3 to the insulin receptor. This data explains that SOCS-3 requires tyrosine 960 in order to bind to the insulin receptor. If SOCS-3 binds to the insulin receptor, IRS-1 is inhibited from binding and phosphorylation cannot occur. To further investigate the role of SOCS-3, Shi et al. (102) found that IRS-1 phosphorylation, PI3-K activity and insulin stimulated glucose transport is enhanced in mice adipose tissue with SOCS-3 deficiencies.

Current research has shown that SOCS-3 may be responsible for impairment of the insulin signaling cascade in the skeletal muscle of the high fat diet. When skeletal muscle tissue is in an insulin resistant state, insulin levels and cytokine concentrations such as TNF-α and NF-κB are elevated, which can also increase the over expression of the SOCS-3 protein as seen in the genetic insulin resistant phenotype in mice (33, 39).
Additionally, recent findings by Solares et al.\(^{(108)}\) have reported that SOCS-3 co-localization with IRS-1 and IR-β occur weeks after being placed on a high fat diet in rodent skeletal muscle tissue. Interestingly, it has also been seen that the activation of a STAT, STAT5B protein, also increases SOCS-3, but the expression of SOCS-3 turns off the STAT protein through negative feedback \((34, 39, 114, 118, 119)\). When stimulated by insulin, STAT5B binds to the insulin receptor causing tyrosine phosphorylation. Due to the increase in SOCS-3, STAT5B can no longer bind to the insulin receptor and will decrease STAT5B \((18, 21, 39, 112)\).

Supporting evidence from Yaspelkis et al.\(^{(130)}\) suggest there is a steric hinderance as between the IR and SOCS-3. When SOCS-3 is present, tyrosine residues are unavailable for IRS-1 phosphorylation and the insulin signal to IRS-1 is hindered. It was found that there was an increase in co-localization of SOCS-3 with IR-β and IRS-1 in high fat fed rodents. Yaspelkis et al.\(^{(130)}\) also reported insulin-stimulated glucose transport was lower in the skeletal muscle in the presence of SOCS-3.

Over expression of SOCS-3 causes IRS-1 proteasomal degradation \((74)\). By definition, proteasomal degradation is the breakdown of the protein, hindering its capabilities, and in this case the binding of IRS-1 to the insulin receptor. Other roles of SOCS-3 and its sites of inhibition are still under investigation. Questions still remain about SOCS-3 and its role in the impairment of insulin action causing a decrease glucose transport and an increase in lipolysis. Investigations on SOCS-3 expression still continue to determine if its activation is independent of the other pro-inflammatory pathways.

*Inhibition of Pro-Inflammatory Pathways*
To date there are few studies that focus on using drug treatment to reverse the pro-inflammatory pathways in skeletal muscle. However, there has been research done in the field of cardiac and neurological tissues (7, 44), and while they do have limited applicability to skeletal muscle insulin signaling, the inflammatory response is similar within cardiac and skeletal tissue. Diseases such as Parkinson’s, irritable bowel syndrome, sepsis, diabetes and other chronic inflammatory diseases all place tissues and organs within the body under extreme amount of stress which results in the activation of the pro-inflammatory pathways. The activity of cytokines such as IKK, NF-κB and JNK are all increased in these diseases and research is in the early stages of determining if inhibition of these pathways decrease the expression of these cytokines (84, 107, 128). Although research has been done on rescuing of PI3-K activity through drug treatment, questions still remain because PI3-K is so important for GLUT-4 translocation. There is reason to believe, based on the published data, that inhibitory drugs such as MG-132, SP600125 and NEMO binding domain peptide inhibitors can rescue PI3-K activity in skeletal muscle.

The IKK complex regulator, IKKγ is responsible for the phosphorylation of IκB which then allows for the disassociation of NF-κB. The NF-κB, inhibitor MG-132, has shown inhibition of both NF-κB and the IKK complex in vivo as well as in vitro (128). MG-132 is a peptide aldehyde proteasome inhibitor (107, 110, 128, 129) that limits NF-κB expression and movement by inhibiting IκB phosphorylation. The few studies that have used this potent inhibitor have shown a decrease in NF-κB activation as well as IKK-β expression (107). Snyder et al. (107) examined rat aortic vessels following treatment with MG-132 for septic shock. It was suspected that NF-κB was a possible
treatment site for septic shock due to a lack of success with TNF-α inhibitors (77, 83, 136). From their findings MG-132 is a possible treatment to inhibit the inflammatory response by NF-κB. In another study by Yang et al. (128) NF-κB and IκB expression were both lowered following MG132 treatment in coronary microvessel tissues.

NEMO-binding domain (NBD) peptide inhibitors have been evaluated to help design anti-inflammatory drugs for inflammatory diseases such as rheumatoid arthritis, bone erosion, and insulin resistance (21). NBD peptide inhibitors block the phosphorylation site on IKK complex, more specifically IKK-β is not activated to phosphorylate serine residues on IκBα. This means NF-κB cannot disassociate with IκBα and stimulate inflammatory cytokines production (105). It is important to note that although NF-κB is not activated, there is still a basal response of NF-κB that is continuous and does not require a pathway to be turned on. This is mentioned because when NF-κB concentrations are measured following use of NBD inhibitors there will be NF-κB circulating within the cytoplasm (45, 85).

JNK is believed to be a key regulator in cytokine expression and its influence on insulin signaling has opened the doors for potential drug therapies as well (1, 55, 117, 128). The JNK inhibitor SP600125 reduces JNK phosphorylation (43). Bennett et al. (9) observed that JNK inhibitors such as CC105 and SP600125 protect pancreatic islet cells preventing insulin resistance. In a study done by Yang et al. (128), SP600125 decreased the serine phosphorylation of IRS-1 and increased IRS-1 tyrosine phosphorylation. This observation verified that JNK and IKK-β interfere with proper insulin signaling. Thus, the insulin signal response is increased. It was also found that TNF-α was reduced following treatment of SP600125 when looking at inflammatory bowel disease (86).
When used separately from IKK inhibitors, JNK inhibitors do not completely restrict serine phosphorylation because there is no blockage on IκBα activation (43).

Previous studies have also used sodium salicylate for inhibition of IKK-β, and have shown a decrease blood glucose levels in rodent models (71, 89, 127, 135, 138). However, new inhibitors have been suspected to play a larger role in inhibition of pro-inflammatory pathways and could lead to a better understanding of pro-inflammatory pathways. As previously mentioned, the few studies that exist on JNK, NF-κB and IKK inhibitors have shown potential for limiting the pro-inflammatory response in chronic inflammatory diseases and create potential for rescuing PI3-K activity in skeletal muscle insulin resistance.

**Summary**

Skeletal muscle insulin resistance can develop as a result of the consumption of a high-fat diet and can eventually lead to type II diabetes. In normal functioning skeletal muscle insulin binds to the insulin receptor and tyrosine phosphorylation of IRS-1 activates PI3-K. The activation of PI3-K is important for activation of PKC ζ/λ and Akt2. As a result of PKC ζ/λ and Akt2 activation, GLUT-4 containing vesicles can freely move to the plasma membrane for glucose transport. Literature has shown that a decrease in insulin response, PI3-K activity decreases GLUT-4 migration to the plasma membrane and leads to insulin resistance. Normal GLUT-4 translocation is critical for proper cellular function.

Research on insulin resistance has focused on cytokine production and SOCS-3 activation. It has been shown that SOCS-3 interferes with the interaction between IR-β
and IRS-1 resulting in proteasomal degradation and a reduction in PI3-K activity. Activation of cytokines, IKK, JNK and NF-κB are responsible for an increase in serine phosphorylation of IRS-1. Thus far little research has been done in regards to rescuing PI3-K activity via inhibition of the pro-inflammatory pathways. Inhibition of the pro-inflammatory pathways, such as IKK, JNK and/or NF-κB will decrease the pro-inflammatory response in cardiac and neurological tissue. With further research of these inhibitors, there is a potential that insulin signaling cascade and GLUT-4 transport to the plasma membrane might be rescued in insulin resistant tissue.
REFERENCES

ABSTRACT, INTRODUCTION, METHODS, RESULTS AND DISCUSSION


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REFERENCES

REVIEW OF LITERATURE


APPENDIX A

Protocol Solutions

Solutions

Homogenization Buffer (HB) (20 mL) or (100 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 M HEPES (pH 7.6)</td>
<td>1000 μL</td>
<td>5000 μL</td>
</tr>
<tr>
<td>5.0 M NaCl</td>
<td>600 μL</td>
<td>3000 μL</td>
</tr>
<tr>
<td>100 mM Na-Pyrophosphate</td>
<td>4000 μL</td>
<td>20000 μL</td>
</tr>
<tr>
<td>1.0 M β-Glycerophosphate</td>
<td>400 μL</td>
<td>2000 μL</td>
</tr>
<tr>
<td>0.5 M NaF</td>
<td>400 μL</td>
<td>2000 μL</td>
</tr>
<tr>
<td>100 mM Sodium Othrovanadate</td>
<td>400 μL</td>
<td>2000 μL</td>
</tr>
<tr>
<td>80 mM EDTA (pH 8.0)</td>
<td>500 μL</td>
<td>2500 μL</td>
</tr>
<tr>
<td>10% IGEPAL</td>
<td>2000 μL</td>
<td>10000 μL</td>
</tr>
<tr>
<td>100 % Glycerol</td>
<td>2000 μL</td>
<td>10000 μL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>8010 μL</td>
<td>40050 μL</td>
</tr>
<tr>
<td>100 mM Phenylmethylsulfonyl</td>
<td>400 μL</td>
<td>2000 μL</td>
</tr>
<tr>
<td>0.4 M MgCl₂</td>
<td>50 μL</td>
<td>250 μL</td>
</tr>
<tr>
<td>0.5 M CaCl₂</td>
<td>40 μL</td>
<td>200 μL</td>
</tr>
<tr>
<td>Leupeptin (5 mg/mL)</td>
<td>100 μL</td>
<td>500 μL</td>
</tr>
</tbody>
</table>

Add reagents to a conical tube, vortex and store at -20° C.
2X Treatment Buffer Also Known As Lammaelli Buffer (2X TB) (10 mL)

Add all reagents except Bromophenol Blue to a conical tube, pH to 6.8, add Bromophenol Blue and bring to volume with ddH₂O in a graduated cylinder. Store at -20° C.

Phosphate Triton Azide (PTA) Buffer (200 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 20</td>
<td>1 mL</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>0.04 g</td>
</tr>
<tr>
<td>BSA</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

Add reagents to ~ 100 mL of ddH₂O in a beaker with a magnetic stir bar, pH to 7.2 and bring to volume with ddH₂O in a graduated cylinder.

Phosphate Buffered Saline (PBS) (500 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>4 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.7 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.7 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1 g</td>
</tr>
</tbody>
</table>
Add reagents to ~ 400 mL of ddH₂O in a beaker with a magnetic stir bar, pH to 7.4 and bring to volume with ddH₂O in a graduate cylinder.

“**A**” Wash (2500 µL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% IGEPAL</td>
<td>125 µL</td>
</tr>
<tr>
<td>100 mM Na₃VO₄</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>PBS</td>
<td>2370 µL</td>
</tr>
</tbody>
</table>

Add reagents to 12 x 75 mm glass test tube and vortex.

“**B**” Wash (2500 µL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-HCl</td>
<td>250 µL</td>
</tr>
<tr>
<td>2 M LiCl₂</td>
<td>625 µL</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>100 mM Na₃VO₄</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>1620 µL</td>
</tr>
</tbody>
</table>

Add reagents to 12 x 75 mm glass test tube and vortex.

“**C**” Wash (5000 µL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-HCl</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

53
Add reagents to a 13 x 100 mm glass test tube and vortex.

### Preparation of Protein-A (PRO-A) Sepharose Beads

1. Add 0.5 g of PRO-A sepharose (Amersham) to 10 mL of ddH$_2$O in a 15 mL test tube to swell the beads. Gently mix beads.
2. Place on rotator for 20 min at 4° C.
3. Centrifuge at 1,010 x g for 10 min at 4° C.
4. Discard supernatant and resuspend beads in an equal volume of ddH$_2$O. Gently mix beads.
5. Place on rotator for 20 min at 4° C.
6. Centrifuge at 1,010 x g for 10 min at 4° C.
8. Centrifuge at 1,010 x g for 10 min at 4° C.
10. Using large orifice pipette tips, aliquot 1 mL of PRO-A beads into 1.5 mL screw top tubes. Gently vortex between each aliquot and store at -20°C.

APPENDIX B

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

Solutions

4X Resolving Gel Buffer (200 mL):

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Base</td>
<td>36.3 g</td>
</tr>
</tbody>
</table>

Add reagents to ~ 150 mL of ddH2O in a beaker with magnetic stir bar, adjust pH to 8.8 and bring to volume with ddH2O in a graduated cylinder.

4X Stacking Gel Buffer (50 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Base</td>
<td>3 g</td>
</tr>
</tbody>
</table>

Add reagents to ~ 40 mL of ddH2O in a beaker with magnetic stir bar, adjust pH to 6.8 and bring to volume with ddH2O in graduated cylinder.

10% SDS (100 mL)
Add reagents to ~ 80 mL of ddH₂O in a beaker with magnetic stir bar and bring to volume with ddH₂O in a graduated cylinder.

10X Running Buffer (10X RB) (1 L)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M Tris-Base</td>
<td>30.28 g</td>
</tr>
<tr>
<td>1.9 M Glycine</td>
<td>144.2 g</td>
</tr>
</tbody>
</table>

Add reagents to ~ 800 mL ddH₂O in a beaker with magnetic stir bar and bring to volume with ddH₂O in a graduated cylinder.

1X Running Buffer (1X RB) (1 L)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Running Buffer</td>
<td>100 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10 mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>890 mL</td>
</tr>
</tbody>
</table>

Add reagents in a beaker with magnetic stir bar.

10% Ammonium Persulfate (10% APS) (1 mL)
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>0.1 g</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

Add reagents to a 1.5 mL microcentrifuge tube and vortex.

**Water Saturated N-Butanol (55 mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Butanol</td>
<td>50 mL</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>5 mL</td>
</tr>
</tbody>
</table>

Add reagents to a beaker and mix with a magnetic stir bar.

**Polyacrylamide Resolving Gel Solution (4 Gels, 0.75 mm Thickness)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>7.5% Gel</th>
<th>10% Gel</th>
<th>12% Gel</th>
<th>15% Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer Solution</td>
<td>5 mL</td>
<td>6.6 mL</td>
<td>8.3 mL</td>
<td>10.0 mL</td>
</tr>
<tr>
<td>4X Running Gel Buffer</td>
<td>5 mL</td>
<td>5 mL</td>
<td>5.0 mL</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>200 µL</td>
<td>200 µL</td>
<td>0.2 mL</td>
<td>0.2 mL</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>9.8 mL</td>
<td>8 mL</td>
<td>3.2 mL</td>
<td>2.4 mL</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µl</td>
<td>100 µL</td>
<td>115µl</td>
<td>115µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>6.6 µL</td>
<td>6.6 µL</td>
<td>14µL</td>
<td>14µL</td>
</tr>
</tbody>
</table>

Add reagents to a conical tube and invert several times with a Pasteur pipette to mix.
Stacking Gel Solution (4 Gels, 0.75 mm Thickness)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer Solution</td>
<td>880 µL</td>
</tr>
<tr>
<td>4X Running Gel Buffer</td>
<td>1.67 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>66 µL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>4.06 mL</td>
</tr>
<tr>
<td>10% APS</td>
<td>33.4 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>3.4 µL</td>
</tr>
</tbody>
</table>

Add reagents to a conical tube and invert several times with a Pasteur pipette to mix.

Procedure

1. Assemble gel apparatus according to the manufacturer’s directions.
2. Prepare resolving gels.
3. Invert several times with a Pasteur pipette, avoiding bubbles. Fill caster approximately 3/4 full.
4. Overlay resolving gel with 500 µL of water saturated N-Butanol and allow gel to polymerize for approximately 1 h.
5. Pour N-Butanol off the resolving gel, rinse with ddH₂O and dry with Kimwipe.
6. Prepare stacking gel.
7. Invert several times with a Pasteur pipette, avoiding bubbles. Overlay resolving gel with stacking gel and insert combs. Allow approximately 45 min for polymerization.
8. Assemble gel apparatus chamber for electrophoresis according to manufacturer’s directions.

9. Fill inner chamber with 1X RB and fill outer chamber approximately 1/4 full.
APPENDIX C

Semi-Dry Transfer

Solutions

Transfer Buffer (1 L)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 mM Tris-Base</td>
<td>5.82 g</td>
</tr>
<tr>
<td>39 mM Glycine</td>
<td>2.93 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>3.75 mL</td>
</tr>
<tr>
<td>20% MeOH</td>
<td>200 mL</td>
</tr>
</tbody>
</table>

Add reagents to ~ 700 mL of ddH$_2$O in a beaker with magnetic stir bar and bring to volume with ddH$_2$O in a graduated cylinder. pH should range between 9.0 and 9.4. Do not adjust pH with acid or base.

Non-SDS Transfer Buffer (1 L)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.02 mM Tris-Base</td>
<td>3.03 g</td>
</tr>
<tr>
<td>191 mM Glycine</td>
<td>14.41 g</td>
</tr>
<tr>
<td>20% MeOH</td>
<td>200 mL</td>
</tr>
</tbody>
</table>

Add reagents to ~ 800 mL of ddH$_2$O in a beaker with magnetic stir bar and bring to volume with ddH$_2$O in a graduated cylinder. pH should range between 9.0 and 9.4. Do not adjust pH with acid or base.
### 10X Tris Buffered Saline (TBS) (1 L)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mM Tris-Base</td>
<td>24.22 g</td>
</tr>
<tr>
<td>5 mM NaCl</td>
<td>292.2 g</td>
</tr>
</tbody>
</table>

Add reagents to ~ 800 mL of ddH₂O in a beaker with magnetic stir bar, pH to 7.5 and bring to volume with ddH₂O in a graduated cylinder.

### Tween Tris Buffered Saline (TTBS) (1 L)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X TBS</td>
<td>100 mL</td>
</tr>
<tr>
<td>Tween 20</td>
<td>600 µL</td>
</tr>
</tbody>
</table>

Add reagents to ~ 800 mL of ddH₂O in a beaker with magnetic stir bar, pH to 7.5 and bring to volume with ddH₂O in a graduated cylinder.

### Blocking Solution (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Non-Fat Dry Milk (NFDM) or Bovine</td>
<td>1 g</td>
</tr>
</tbody>
</table>

Add NFDM or BSA to TTBS and mix in beaker with magnetic stir bar. Need ~ 50 mL of blocking solution for each PVDF membrane.
Procedure

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 min.

2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 30 seconds then soak in transfer buffer for 30 min.

3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. Secure to electrode and roll out any air bubbles with a wet glass test tube.

4. Place PVDF membrane on top of blot paper and roll out any air bubbles with wet glass test tube. Pour a small amount of transfer buffer over PVDF membrane to avoid membrane from drying out.

5. Place resolving gel on top of PVDF membrane. Roll out any air bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Roll out any air bubbles with a wet glass test tube.

7. Run protein transfer.

8. Following protein transfer, place PVDF membrane in approximately 30 mL of blocking solution. Block overnight between 2 hours to overnight at 4° C.
**APPENDIX D**

**Immunoblotting**

**Solutions**

**IR-β Primary Antibody (1:1,000) (30 mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>30 mL</td>
</tr>
<tr>
<td>Rabbit Polyclonal IR-β Antibody</td>
<td>30 μL</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.0125 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.3 g</td>
</tr>
</tbody>
</table>

**IRS-1 Primary Antibody (1:1,000) (40 mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>40 mL</td>
</tr>
<tr>
<td>Rabbit Polyclonal IRS-1 Antibody</td>
<td>40 μL</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.02 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.40 g</td>
</tr>
</tbody>
</table>
### IRS-1 pY Primary Antibody (1:250) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Rabbit Polyclonal IRS-1 pY Antibody</td>
<td>80 µL</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.01 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.20 g</td>
</tr>
</tbody>
</table>

### IRS-1 pSer 307 Primary Antibody (1:500) (40 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>40 mL</td>
</tr>
<tr>
<td>Rabbit Polyclonal IRS-1 pSer 307 Antisera</td>
<td>80 µL</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.02 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.40 g</td>
</tr>
</tbody>
</table>

### IKK α Primary Antibody (1:250) (10 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>10 mL</td>
</tr>
<tr>
<td>Rabbit Monoclonal IKK α Antibody</td>
<td>40 µL</td>
</tr>
<tr>
<td>(cat # 2682, cell signal)</td>
<td></td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.005 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>
### IKK β Primary Antibody (1:500) (20mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Rabbit Monoclonal IKKβ Antibody (Cat# 2370, Cell Signaling)</td>
<td>40 μl</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.01 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.20 g</td>
</tr>
</tbody>
</table>

### Phospho IKK α/β (Ser 176/180) Primary Antibody (1:500) (40mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>40 mL</td>
</tr>
<tr>
<td>Rabbit Polyclonal anti-IKK α/β Ser 176/180 Antibody</td>
<td>80 μl</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.02 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.40 g</td>
</tr>
</tbody>
</table>

### IκBα Primary Antibody (1:500) (40 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>40 mL</td>
</tr>
<tr>
<td>Rabbit Monoclonal IκBα Antibody</td>
<td>60 μl</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.02 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.40 g</td>
</tr>
</tbody>
</table>
**IKBα pSer 32/36 Primary Antibody (1:500) (20 mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Mouse Monoclonal IκBα pSer 32/36 Antibody</td>
<td>40 μl</td>
</tr>
<tr>
<td>NaN3</td>
<td>0.01 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.20 g</td>
</tr>
</tbody>
</table>

**Anti-Phosphotyrosine Primary Antibody (1:500) (25 mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>25 mL</td>
</tr>
<tr>
<td>Rabbit Polyclonal Anti-Phosphotyrosine Antibody (Cat# 06-427, Millipore)</td>
<td>200 μl</td>
</tr>
<tr>
<td>NaN3</td>
<td>0.0125 g</td>
</tr>
<tr>
<td>BSA</td>
<td>0.25 g</td>
</tr>
</tbody>
</table>

**Suppressor of Cytokine Signaling 3 (SOCS-3) Primary Antibody (1:500) (30 mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>30 mL</td>
</tr>
<tr>
<td>Rabbit Polyclonal SOCS-3 Antibody</td>
<td>60 μl</td>
</tr>
<tr>
<td>NaN3</td>
<td>0.015 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.30 g</td>
</tr>
</tbody>
</table>
**JNK 1 and JNK 2/3 Primary Antibody (1:7500) (20mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Rabbit Monoclonal JNK Antibody</td>
<td>25 µl</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.01 g</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.20 g</td>
</tr>
</tbody>
</table>

**IR-β subunit Secondary Antibody (1:10,000) (20 mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Rabbit (sc-2004, SCBT)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

**Phosphotyrosine Secondary Antibody (1:10,000) (20 mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Rabbit (sc-2004, SCBT)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>
### IRS-1 Secondary Antibody (1:10,000) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Rabbit (sc-2004, SCBT)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

### IRS-1 pSer 307 Secondary Antibody (1:10,000) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Rabbit (sc-2004, SCBT)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

### IKKα Secondary Antibody (1:10,000) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Rabbit (sc-2004, SCBT)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>
### IKKβ Secondary Antibody (1:10,000) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Rabbit (sc-2004, SCBT)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

### Phospho-Serine IKK α/β Secondary Antibody (1:10,000) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Rabbit (sc-2004, SCBT)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

### IκBα Secondary Antibody (1:10,000) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Donkey Anti-Goat IgG (sc-2033, SCBT)</td>
<td>2 µL</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>
IκBα pSer 32/36 Secondary Antibody (1:10,000) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Mouse (sc-2055, SCBT)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

SOCS-3 Secondary Antibody (1:20,000) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Rabbit (sc-2004, SCBT)</td>
<td>2 µL</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

JNK 1 and JNK 2/3 Primary Antibody (1:500) (20 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTBS</td>
<td>20 mL</td>
</tr>
<tr>
<td>Goat Anti-Rabbit (sc-2004, SCBT)</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFDM</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>
**Procedure**

1. Following the overnight incubation with blocking solution, rinse membrane 3X in TTBS for 15 min.

2. Incubate membrane on primary antibody solution per protocol on Lab Quake.

3. Recycle primary antibody and rinse membrane 3X in TTBS for 5 min.

4. Incubate membrane in secondary antibody solution per protocol on Lab Quake.

5. Discard secondary antibody and rinse membrane 2X in TTBS for 5 min.
ADDENDIX E

Enhanced Chemiluminescence

Enhanced Chemiluminescence (ECL) (1.5 mL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal/Enhancer Solution</td>
<td>1000 µL</td>
</tr>
<tr>
<td>Peroxide Buffer</td>
<td>500 µL</td>
</tr>
</tbody>
</table>

Add solutions to 12 x 75 mm test tube and vortex.

Procedure

1. Prepare Visualizer Enhanced Chemiluminescence Substrate (Millipore).
2. Pour ECL solution directly on PVDF membrane and shake for 5 min.
3. Place PVDF membrane between two cut transparency and remove any air bubbles.
4. Turn on Chemi Doc system (BioRad, Richmond, CA) and select Image Lab Software on Dell computer Chemi Doc system (BioRad).
5. Place PVDF membrane on Chemi Doc tray.
6. Select “auto expose” to acquire image.
7. Capture and save image.
8. Using the Image Lab Software, select “free hand contour tool” to quantify bands.
9. Select “volume analysis” to view data report.
10. Express samples as a percentage of the standard.

**APPENDIX F**

**PI3 Kinase Assay**

**Solutions**

**Homogenization Buffer**

<table>
<thead>
<tr>
<th>FW</th>
<th>Cat #</th>
<th>Stock Solution</th>
<th>°C</th>
<th>Final Vol.</th>
<th>Final Vol. 200 mL</th>
<th>Con.</th>
</tr>
</thead>
<tbody>
<tr>
<td>238.30</td>
<td>BO310-100</td>
<td>1.0 M HEPES (pH 7.6)</td>
<td>4</td>
<td>1000 µL</td>
<td>10000 µL</td>
<td>50 mM</td>
</tr>
<tr>
<td>58.4</td>
<td>S390-500</td>
<td>5.0 M NaCl</td>
<td>RT</td>
<td>600 µL</td>
<td>6000 µL</td>
<td>150 mM</td>
</tr>
<tr>
<td>446.06</td>
<td>02793-500</td>
<td>100 mM Na-Pyrophosphate</td>
<td>RT</td>
<td>4000 µL</td>
<td>40000 µL</td>
<td>20 mM</td>
</tr>
<tr>
<td>216.00</td>
<td>S299-100</td>
<td>1.0 M β-Glycerophosphate</td>
<td>RT</td>
<td>400 µL</td>
<td>4000 µL</td>
<td>20 mM</td>
</tr>
<tr>
<td>41.9</td>
<td>S299-100</td>
<td>0.5 M NaF</td>
<td>RT</td>
<td>400 µL</td>
<td>4000 µL</td>
<td>10 mM</td>
</tr>
<tr>
<td>183.9</td>
<td>02793-500</td>
<td>100 mM Sodium Othovranadate</td>
<td>RT</td>
<td>400 µL</td>
<td>4000 µL</td>
<td>2 mM</td>
</tr>
<tr>
<td>372.24</td>
<td>CA-630</td>
<td>10% IGEPAL</td>
<td>RT</td>
<td>2000 µL</td>
<td>20000 µL</td>
<td>1%</td>
</tr>
<tr>
<td>92.10</td>
<td>G33-500</td>
<td>100 % Glycerol</td>
<td>RT</td>
<td>2000 µL</td>
<td>20000 µL</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>ddH2O</td>
<td></td>
<td>RT</td>
<td>8010 µL</td>
<td>80100 µL</td>
<td></td>
</tr>
<tr>
<td>174.20</td>
<td>195381</td>
<td>100 mM Phenylmethyisulfonyl</td>
<td>-20</td>
<td>400 µL</td>
<td>4000 µL</td>
<td>2 mM</td>
</tr>
<tr>
<td>95.21</td>
<td>M-8266</td>
<td>0.4 M MgCl2</td>
<td>RT</td>
<td>50 µL</td>
<td>500 µL</td>
<td>1 mM</td>
</tr>
<tr>
<td>147.02</td>
<td>C79-3</td>
<td>0.5 M CaCl2</td>
<td>RT</td>
<td>40 µL</td>
<td>400 µL</td>
<td>1 mM</td>
</tr>
<tr>
<td>475.60</td>
<td>BP2662-25</td>
<td>Leupeptin (5 mg/mL)</td>
<td>-20</td>
<td>100 µL</td>
<td>1000 µL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aprotinin (1 mg/mL)</td>
<td>-20</td>
<td>100 µL</td>
<td>1000 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **1.0 M HEPES** – add 10.724 g HEPES to 35 mL ddH2O, pH to 7.5 with NaOH, bring to 45 mL
• **5.0 M NaCl** – add 11.677 g NaCl to 30 mL ddH₂O, apply gentle heat until dissolved, bring to 40 mL.

• **100 mM Na-Pyrophosphate** – add 2.0075 g to 35 mL ddH₂O, bring to 45 mL.

• **1.0 β-Glycerophosphate** – add 9.72 g to 30 mL ddH₂O, bring to 45 mL, filter Whatman #1.

• **0.5 NaF** – add 0.9448 g to 35 mL ddH₂O, bring to 45 mL.

• **100 mM Sodium Orthovanadate** – add 0.5517 g to 20 mL, gently heat 5 minutes, bring to 30 mL, aliquot into 1.0 mL micro-centrifuge tubes.

• **80 mM EDTA** – add 1.191 g EDTA to 30 mL ddH₂O. pH to 8.0 with NaOH, bring to 30 mL.

• **10% IGEPAL** – add 10 mL IGEPAL to 90 mL ddH₂O (difficult to dissolve).

• **100% Glycerol**

• **100 mM Phenolmethylsulfonyfluoride** – add 0.6968 g to 30 mL 100% ethanol, bring to 40 mL with EtOH.

• **0.4 M MgCl₂** – add 3.659 g to 35 mL ddH₂O, bring to 45 mL.

• **0.5 M CaCl₂** – add 2.498 g to 35 mL ddH₂O, bring to 45 mL.

**PTA Buffer (To Make 200 mL):**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 20</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 g</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.04 g</td>
</tr>
<tr>
<td>BSA</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>
- Add reagent to ~100 mL of ddH₂O. pH to 7.2 and bring up to 200 mL with ddH₂O.

**PBS (To Make 500 mL)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>4.0 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.7 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.7 g</td>
</tr>
<tr>
<td>KCL</td>
<td>0.1 g</td>
</tr>
</tbody>
</table>

**Dilute ATP:**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>40 μL</td>
</tr>
<tr>
<td>50 mM stock ATP</td>
<td>10 μL</td>
</tr>
</tbody>
</table>

**ATP Solution (For 5 Samples or 4 Samples and 1 Standard)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Vol</th>
<th>Final Vol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 μL</td>
<td>70 μL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>22.25 μL</td>
<td>31.15 μL</td>
</tr>
<tr>
<td>0.4 M MgCl₂</td>
<td>6.25 μL</td>
<td>8.75 μL</td>
</tr>
<tr>
<td>4x HEPES</td>
<td>12.5 μL</td>
<td>17.5 μL</td>
</tr>
<tr>
<td>Dilute ATP</td>
<td>1 μL</td>
<td>1.4 μL</td>
</tr>
</tbody>
</table>

- Add [γ⁻³²P] ATP only when directed in assay. Section 3, step 7. The amounts below are what are required.
- 16 samples plus a standard, requires two 50 μL ATP Solutions and one 70 μL ATP Solution.

- Sixteen samples require 10 μL each, the total amount is 160 μL plus 10 μL for the standard (half of one sample per TLC Plate, basically the Standard is not ran in duplicate).

### “A” Wash

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Vol 2500 μL</th>
<th>Final Vol 25 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% IGEPAL</td>
<td>125 μL</td>
<td>1.250 mL</td>
</tr>
<tr>
<td>100 mM NaVO₄</td>
<td>2.5 μL</td>
<td>.25 mL</td>
</tr>
<tr>
<td>1 M Dithiothreitol (DTT)</td>
<td>2.5 μL</td>
<td>.25 mL</td>
</tr>
<tr>
<td>PBS</td>
<td>2370 μL</td>
<td>23.7 mL</td>
</tr>
</tbody>
</table>

### “B” Wash

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Vol 2500 μL</th>
<th>Final Vol 25 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-HCL (pH 7.5)</td>
<td>250 μL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>2 M LiCl₂</td>
<td>625 μL</td>
<td>6.25 mL</td>
</tr>
<tr>
<td>1 M DTT</td>
<td>2.5 μL</td>
<td>.25 mL</td>
</tr>
<tr>
<td>100 mM NaVO₄</td>
<td>2.5 μL</td>
<td>.25 mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>1620 μL</td>
<td>16.20 mL</td>
</tr>
</tbody>
</table>

### “C” Wash

---

76
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Vol</th>
<th>Final Vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-HCl (pH 7.5)</td>
<td>2500 μL</td>
<td>25 mL</td>
</tr>
<tr>
<td>NaCl</td>
<td>50 μL</td>
<td>.5 mL</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>250 μL</td>
<td>2.50 mL</td>
</tr>
<tr>
<td>1 M DTT</td>
<td>5 μL</td>
<td>50 μL</td>
</tr>
<tr>
<td>100 mM Na$_3$VO$_4$</td>
<td>5 μL</td>
<td>50 μL</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>4590 μL</td>
<td>45.90 mL</td>
</tr>
</tbody>
</table>

**TLC Running Buffer**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Vol</th>
<th>Final Vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>74.81 mL</td>
<td>448.86 mL</td>
</tr>
<tr>
<td>Methanol</td>
<td>58.60 mL</td>
<td>351.6 mL</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>14.09 mL</td>
<td>84.54 mL</td>
</tr>
<tr>
<td>Ammonium Hydroxide</td>
<td>2.49 mL</td>
<td>14.94 mL</td>
</tr>
</tbody>
</table>

- 150 mL is for one TLC Plate. 8 samples per TLC Plate allows for 6 TLC Plates to account for a completed assay when there are six groups of eight animals.

**Procedure:**

**MUSCLE HOMOGENIZATION**

1. Chip off approximately 80-110 mg pieces of tissue in liquid N$_2$.
2. Thaw aliquots of homogenization buffer (HB)
3. Pipette 10x dilution (muscle: HB) into a grinding tube and homogenize with a glass homogenizer at 0° C until no solid mass remains (with the exception of connective tissue).

4. Transfer samples to 15mL Nalgene centrifuge tubes with pasteur pipettes.

5. Spin samples in large **RC5B** centrifuge using **SS34 rotor** at **14,500 RPM at 4°C** for **15 minutes**.

6. After spin aliquot liquid (muscle lysate) into two separate micro-centrifuge tubes labeled the same. This is done so that the same sample will not be constantly frozen and thawed. Store samples in -80°C freezer.

**PREPARATION OF PROTEIN-A SEPHAROSE BEADS**

1. Turn on the **RC5B** centrifuge at 4°C. Get the **SS34 rotor** from the refrigerator.

2. Fill one or two 15 mL Nalgene centrifuge tubes with 10 mL of H₂O to balance the rotor.

3. Weigh out 0.5 grams of beads into a 15 mL Nalgene centrifuge tube. You can do this in multiple Nalgene tubes at the same time to save time.

4. Add 10 mL of ddH₂O and seal with several layers of Parafilm.

5. Rock gently to mix beads evenly. Rock to hard and the beads can crack.

6. Place tubes on the rotator in the refrigerator for 30 minutes.

7. Spin in **RC5B** at 2800 rpm for 10 minutes.

8. Remove the supernatant and add 10 mL of ddH₂O.

9. Place tubes back on the rotator in the refrigerator for 15 minutes.

10. Remove the tubes and spin in the **RC5B** at 2800 rpm for 10 minutes.
11. Remove the supernatant and add a volume of PTA equal to the spun down beads, rock to re-suspend beads.

12. Spin in RC5B at 2800 rpm for 10 minutes.

13. Remove the supernatant and add a volume of PTA equal to the spun down beads rock to re-suspend beads.

14. Aliquot re-suspended beads into separate 1 mL screwtop Nalgene centrifuge tubes.

15. Store in the -20°C freezer.

**Day Before Running the Assay**

1. Verify the amount of HB made is sufficient for washing the PRO-A BEADS.

2. Verify the amount of solutions prepared so that they last throughout the entire assay.

**Day of the Assay**

**IMMUNOPRECIPITATION (IP) OF IRS-1 [SECTION ONE]**

1. Get ice, samples (stored in -80°C), antibody (anti-rabbit α-IRS-1, Cat #06248 UBT, or anti-rabbit α-IRS-1 Pre-CT, Cat # 06-526, Millipore), Protein-A Sepharose and HB (-20°C freezer) and thaw on ice. Run 9 samples plus a standard on every TLC plate.

   a. The volumes of HB, α-IRS-1, and sample are as follows: for each sample that corresponds to 250 μg of protein (based on Bradford Assay), volume that corresponds to 4 μg of α-IRS-1, and bring to 125 μL with HB.
b. set out 100 mM sodium vanadate, 50 mM ATP solution and 1 M DTT (-20°C freezer) on ice, to be used later.

2. Quickly vortex (below setting 3 so as to not crack the beads), then pulse in a mini-microcentrifuge for 10s each (to pull solution to bottom of micro-centrifuge tube). Incubate samples on ice for **2 hours**.

3. During the last 40 minutes of the 2 hour incubation, set out solutions for the A, B, and C washes, turn on the sonicator and refrigerated micro-centrifuge and let cool to 4°C. Wash the Protein-A Sepharose beads by spinning thawed mixture for 3 mins (4°C) at 14,000 rpm.

4. Discard supernatant and add equal volume of HB to beads. Flick tubes to mix, and spin in the refrigerated micro-centrifuge (14,000 rpm, 4°C) for 3 minutes.

5. Discard supernatant and add equal volume of HB to beads. Flick tubes to mix, and spin in the refrigerated micro-centrifuge (14,000 rpm, 4°C) for 3 minutes.

6. After the final spin (of three washes) discard the supernatant and add an equal volume of HB to beads.

7. Using a cut pipette tip, add 80 μL of Protein-A Sepharose beads to each sample. Vortex beads (below setting 3 so as to not crack the beads), between each addition to another sample. Rotate samples for **1.5 hours** in refrigerator.

**WASHING BEADS AFTER IP IS COMPLETE**

8. Prepare washes and future solutions during the IP rotating/sitting times outlined above.

9. Make the dilute ATP solution in a separate micro-centrifuge tube labeled “DATP”.

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10. Make the ATP solution in another micro-centrifuge tube labeled “ATP”. Do not add the radioactive $^{32}$P ATP yet!!!!

11. Get 10 mL ddH$_2$O and place on ice. Dilute 80 mM EDTA (used in making the HB) (pH 8.0) (1:8) in a 1.5 mL micro-centrifuge tube and label it “EDTA”.

12. Label two 12X75 mm glass test tubes. One “A” and once “B”. Label a 13x100 mm glass tube “C”.

13. After the 1.5 hour incubation is at its end, spin in the refrigerated micro-centrifuge (14,000 °C) for 10 minutes.

14. When the 10 minutes centrifugation is complete, take samples out of the refrigerated micro-centrifuge and discard the supernatant of each sample. Add 200 μL of wash buffer “A” and flick tube to mix. Centrifuge for 10 minutes.

15. At the end of the “A” wash, take samples out of centrifuge and discard the supernatant. Add 200 μL of wash buffer “B” and flick tube to mix. Centrifuge for 10 minutes.

16. At the end of the “B” wash, take samples out of centrifuge and discard the supernatant. Add 200 μL of wash buffer “C” and flick tube to mix. Centrifuge for 10 minutes.

17. At the end of the “C” wash, take samples out of centrifuge and discard the supernatant. Add 200 μL of wash buffer “C” once more and flick tube to mix. Centrifuge for 10 minutes.

18. At the end of the “C” wash, take samples out of centrifuge and discard the supernatant.

**PREPARATION AND SONICATION OF PHOSPHATIDYLINOSITOL (PI)**
[SECTION TWO]

1. Prior to making the wash buffers, get a tube of PI from the -20°C freezer and place it under a light stream of air to evaporate the solution. The evaporation should take approximately 7 minutes under the light stream of air. After evaporating the PI, there should be a small white pellet at the bottom of the tube. Add 125 μL of ice cold 4x HEPES and 375 μL of ice cold ddH₂O. Leave the PI out at room temperature and get a 40 mL beaker filled with ice water for use during sonication.

2. The easiest way to maintain an ice cold liquid for the PI is to add some NaCl to the water to drop the freezing point. The 40 mL beaker has the NaCl in it, with some ice in it. Then you have to cover the beaker with Parafilm. Once covered you slice a small opening to allow the micro-centrifuge tube that possess the PI solution in it, to be half to three-quarters in the ice water. Next you get a larger 200 mL beaker fill it half with ice and then place the 40 mL beaker on top of the ice and pack the remaining open sections of the beaker with ice to hold it in position. The purpose of this is to maintain the coldest possible temperature for the PI to be in without freezing.

3. Sonication of PI:
   a. This is done during each A, B, and C washes spins. Remove the lid of the micro-centrifuge tube, cover with Parafilm and vortex PI for one minute. Sonicate PI in the ice water described above for one minute with bursts every three seconds. If the PI becomes warm after each minute of sonication, increase the amount of time between each burst and make sure
the ice water is as cold as possible. If the temperature of the PI becomes too warm, a conformational change of the lipid will occur rendering it useless. Perform sonication approximately 15 times, vortexing PI for one minute every third minute of sonication.

b. The sonicator has a distinct pitch that is extremely high and will make the ENTIRE solution go MILKY WHITE. When this is achieved after removing the supernatant of the final “C” wash add 20 μL to each sample only when it is MILKY WHITE!! Once the PI has been added to each sample place on vortex (BELOW SETTING #3!) for 7 minutes. You may have to achieve the MILKY WHITE coloring between each sample which is okay as long as the PI remains as cold as possible.

SETTING UP FOR AND CONDUCTING THE REACTION – THE MOST IMPORTANT SEGMENT [SECTION THREE]

1. Turn on the hot plate so that it can reach 100°C while having H₂O in each opening. It takes time to reach operating temperature so plan accordingly.

2. Get timers and set to go off in 8 minutes, for as many samples that are being run.

3. Add 600 μL of chloroform and 600 μL of MeOH to a 12x75 mm test tube, vortex thoroughly and set behind the Nalgene shield. A one to one solution of the two is all that is needed so volume can be changed depending on number of samples being run.

4. Now get three pipettes and place behind the shield.
   a. Set one at 10 μL for the addition of [Y-³²P] ATP solution to each sample.
   b. Set one at 15 μL to stop the kinase reaction with 4 M HCL.
c. Set one at 130 μL for the chloroform/MeOH.

5. You will also need a box of 200 μL pipette tips and 10 μL pipette tips for use with each sample.

6. Add 8 μL of [γ-32P] ATP to the “ATP” solution, vortex thoroughly and place behind shield. This is the amount necessary for a **TOTAL OF 5 SAMPLES OR 4 SAMPLES and 1 STANDARD**.

7. Add **10 μL of [γ-32P] solution** (tube labeled “ATP”) to the first sample tube and vortex (at 3 or 4) for 20 seconds to ensure a thorough mix. Discard tips in **500 mL beaker** with plastic bag inside as a barrier for the [γ-32P]. Place sample in hot plate to incubate and break the PRO-A beads releasing the IRS-1 into the solution. During the time between each addition of [γ-32P] ATP solution, vortex each sample.

8. At 7.5 minutes, place the first sample in a small micro-centrifuge and pulse for 20 seconds.

9. At **8 minutes EXACTLY** stop the reaction in the first tube by adding **15 μL of 4 M HCL**, quickly vortex, and add 130 μL of the chloroform/MeOH solution. Vortex for one minute. Continue for this process for all samples. Stagger the samples so that there is enough time to complete this step **without haste or error**.

10. Once completed with all samples place samples in refrigerated micro-centrifuge and spin (14,000 rpm, 4°C) for 3 minutes.

11. During the spin of the samples make the **TLC RUNNING BUFFER** should be made. After all samples have spun they should have three layers: a pink layer on
top, the beads in the middle, and your desired sample at the bottom of the micro-
centrifuge tube.

PLACING SAMPLE ON THE TLC PLATE FOR THIN LAYER

CHROMATOGRAPHY SEPERATION. [SECTION FOUR]

12. Take a TLC plate and with the white side facing up, apply nine small pencil dots
   approximately 1 cm apart and 1 inch above the bottom of the TLC plate.

13. Set the Nalgene shield, TLC Plate, a pipette set at 20 μL, and the remaining
   chloroform/MeOH solution inside of the hood.

14. The shield should lay across the TLC Plate so as to provide a barrier to stop the
   radiation from contaminating the upper portions of the TLC plate.

15. Take samples from the refrigerated micro-centrifuge and place them behind the
   shield as well.

16. Pre-wet the pipette tip in the chloroform/MeOH solution. Gently penetrate the top
   two layers of your sample tube and extract 20 μL from the bottom sample layer.

17. Spot the sample onto the TLC Plate, do not touch the TLC Plate, inside use
   capillary action to draw out the sample. Blow dry between the addition of another
   drop to ensure the smallest drop.

18. MAKE THE SPOTS DROPPED THE SMALLEST POSSIBLE TO
   ACHIEVE THE GREATEST RESULTS. THE SUCCESS OF THE IMAGE
   DEPENDS ON HOW SMALL YOU MAKE THE SPOTS.

19. Repeat steps 16 and 17 for each pencil marking, remember the TLC Plate has
   samples in duplicate not the entire sample on one dot.
20. Once complete, place TLC Running Buffer in the TLC tank and let settle (so there are no waves or ripples in liquid), then place the TLC Plate so that it rests against the mid line ridge at the bottom of the TLC tank.

21. Allow the TLC Running Buffer to run up the plate for **1 hour**.

22. Go blank the Phospho-screens using the full **15 minutes** to achieve an entire blank.

23. After the 1 hour has elapsed, remove the TLC Plate from the tank and let stand to dry for **15 minutes**.

24. Once dry, cover the plate in saran wrap and place inside the Phospho-Imaging Cassette, with the white side facing the door of the cassette. Place the Phospho-Screen on top of the TLC Plate with the white side facing the TLC Plate. Leave the cassette behind a Nalgene shield and leave it to stand for 8-16 hours depending on the freshness of the [Y-\(^{32}\)P] ATP.

25. Following the exposure, turn the Phospho-Imager on and place the Phospho-Screen into the Phospho-Imager with the white side up. Open Quantity One software, and select PMI, select the target range for the imager to examine based off of the squares within the cassette and hit “go”. For each sample you should be able to visualize a dark black spot on the bottom of the image, with a straight line pointing up and ending with a distinct “V” shaped pattern. The “V” pattern is your area of interest and is what is quantified. Calculate each sample as a percentage of the standard that was applied to the TLC Plate.

26. Discard the TLC Plate in two plastic ziplock bags, and store in freezer until Health and Safety removes them.
APPENDIX G

New Procedure: \( \text{IκB}\alpha \) Total Protein Concentration

Preparation of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

Solutions - Refer to Appendix

Procedure:

1. Cast 10% SDS-PAGE gel.
2. Load 100 \( \mu \)g muscle lystate.
3. Set the power supply to 140 volts for 120 minutes.

Semi-Dry Transfer

Solutions – Refer to Appendix

Procedure:

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.
2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.
3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.
4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entre membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 30 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in NFDM blocking solution for 2 hour minimum to overnight at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml primary antibody solution for 12 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 4 X with 15 ml TTBS for 5 minutes.

5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

7. Follow Appendix
APPENDIX H

New Procedure: Phospho IκBα Ser 32/36

Preparation of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

**Solutions**- Refer to Appendix

**Procedure:**

1. Cast 10% SDS-PAGE gel.
2. Load 100 μg muscle lysate.
3. Set the power supply to 140 volts for 120 minutes.

**Semi-Dry Transfer**

**Solutions** – Refer to Appendix

**Procedure:**

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.
2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.
3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.
4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entire membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 30 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in BSA blocking solution for 2 hour minimum to overnight at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml primary antibody solution for 14 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

5. Incubate membrane in secondary **MOUSE** antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

7. Follow Appendix
APPENDIX I

New Procedure: JNK 1 Total Protein Concentration

Preparation of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

**Solutions** - Refer to Appendix

**Procedure:**

1. Cast 10% SDS-PAGE gel.
2. Load 100 μg muscle lysate.
3. Set the power supply to 200 volts for 90 minutes.

**Semi-Dry Transfer**

**Solutions** – Refer to Appendix

**Procedure:**

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer 300 minutes.
2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.
3. Wet a sheet of blot paper in transfer buffer NOT and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.
4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entire membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 35 minutes.

Western Blotting

Solutions: Refer to Appendix

1. Following protein transfer, place PVDF membrane in NFDM blocking solution for 1 hour minimum to overnight at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml primary antibody solution for 12 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

7. Follow Appendix
APPENDIX J

New Procedure: JNK 2/3 Total Protein Concentration

Preparation of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

Solutions- Refer to Appendix

Procedure:

4. Cast 10% SDS-PAGE gel.
5. Load 100 μg muscle lysate.
6. Set the power supply to 200 volts for 90 minutes.

Semi-Dry Transfer

Solutions – Refer to Appendix

Procedure:

8. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer 300 minutes.
9. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.

10. Wet a sheet of blot paper in transfer buffer NOT and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.

11. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entire membrane has a film of transfer buffer.

12. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

13. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

14. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 35 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

8. Following protein transfer, place PVDF membrane in NFDM blocking solution for 1 hour minimum to overnight at 4°C on Lab Quake.

9. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

10. Place on 5 ml primary antibody solution for 12 hours at 4°C on Lab Quake.

11. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

12. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.
13. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

14. Follow Appendix
APPENDIX K

New Procedure: Phospho IKK α/β Ser 176/180

Preparation of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

**Solutions** - Refer to Appendix

Procedure:

1. Cast 10% SDS-PAGE gel.
2. Load 100 μg muscle lysate.
3. Set the power supply to 200 volts for 90 minutes in ice bath.

Semi-Dry Transfer

**Solutions** – Refer to Appendix

Procedure:

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 10 minutes.
2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 10 minutes.
3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.
4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entire membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 150 mA or .15 Amps depending on machine with a max voltage of 25 for 27 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in BSA blocking solution for 1 hour and 15 minutes minimum to overnight at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml primary antibody solution for 13 – 20 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

7. Follow Appendix
APPENDIX L

New Procedure: IKK α Total Protein Concentration

Strip and re-prob.

Solutions: Refer to Appendix

Procedure:

1. Using previously ran Phospho IKK α/β Ser 176/180 membranes.

2. Place 20 ml stripping buffer over membrane and place on Lab Quake for 30 minutes.

3. Discard stripping buffer and place on fresh 20 ml stripping buffer and place on Lab Quake for 30 minutes.

4. Discard stripping buffer and rinse membrane 3 X with 25 ml TTBS for 5 minutes.

5. After rinsing membrane rinse 1 X with 25 ml ddH$_2$O for 6 minutes.

Western Blotting

Solutions: Refer to Appendix

1. Following stripping, place PVDF membrane in NFDM blocking solution for 16 – 24 hours at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml primary antibody solution for 14 – 18 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.
5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

7. Follow Appendix
New Procedure: IKK β Total Protein Concentration

Strip and re-prob.

**Solutions**- Refer to Appendix

**Procedure:**

1. Using previously ran Phospho IKK α/β Ser 176/180 or IKK α membranes.
2. Place 20 ml stripping buffer over membrane and place on Lab Quake for 30 minutes.
3. Discard stripping buffer and place on fresh 20 ml stripping buffer and place on Lab Quake for 30 minutes.
4. Discard stripping buffer and rinse membrane 3 X with 25 ml TTBS for 5 minutes.
5. After rinsing membrane rinse 1 X with 25 ml ddH₂O for 6 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following stripping, place PVDF membrane in NFDM blocking solution for 1 hour and 15 minutes at 4°C on Lab Quake.
2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.
3. Place on 5 ml primary antibody solution for 15 – 24 hours at 4°C on Lab Quake.
4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.
5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

7. Follow Appendix
APPENDIX N

New Procedure: IRS-1 Total Protein Concentration

Preparation of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

Solutions - Refer to Appendix

Procedure:

1. Cast 7.5% SDS-PAGE gel.
2. Load 100 μg muscle lysate.
3. Set the power supply to 200 volts for 120 minutes.

Semi-Dry Transfer

Solutions – Refer to Appendix

Procedure:

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.
2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.
3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode.
   After placement roll out any air bubbles with a wet glass fleaker.
4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entre membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 60 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in NFDM blocking solution for 4 hours minimum at 4°C or room temperature on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml primary antibody solution for 12-15 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

7. Follow Appendix
APPENDIX O

New Procedure: Phospho IRS-1 Tyrosine 941

Preparation of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

Solutions - Refer to Appendix

Procedure:

1. Cast 7.5% SDS-PAGE gel.
2. Load 100 μg muscle lysate.
3. Set the power supply to 200 volts for 120 minutes.

Semi-Dry Transfer

Solutions – Refer to Appendix

Procedure:

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.
2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.
3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.
4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entre membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 60 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in NFDM blocking solution for 4 hours minimum to overnight at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml primary antibody solution for 12-15 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

Follow Appendix
APPENDIX P

New Procedure: Phospho IRS-1 Serine 307

Preparation of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

Solutions - Refer to Appendix

Procedure:

1. Cast 7.5% SDS-PAGE gel.
2. Load 100 μg muscle lysate.
3. Set the power supply to 200 volts for 120 minutes.

Semi-Dry Transfer

Solutions – Refer to Appendix

Procedure:

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.
2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.
3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.
4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entire membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 60 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in NFDM blocking solution for 4 hours minimum to overnight at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml primary antibody solution for 12 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

Follow Appendix
APPENDIX Q

New Procedure: SOCS-3 Total Protein Concentration,

SOCS-3 & IR-β, SOC-3 & IRS-1 Co-Immunoprecipitation

Preparation of Sample and Sodium Dodecyl Sulfate-Polyacrylamide Gel

Electrophoreses (SDS-PAGE)

Solutions- Refer to Appendix

Procedure:

**DAY ONE:** Get Ice (all samples, washes and buffers should be kept on ICE)

Prepare Protein-A Sepharose beads, following protocol of Protein-A Sepharose beads.

1. Vortex Protein-A Sepharose beads before using at a speed of 2 NEVER GO ABOVE 3, this will crack the beads and ruin them.
2. (use cut pipette tips) into 0.6ml tubes; vortex by hand every time you aliquot beads. *(you will be washing each individual 0.6 ml tube with PBS)*
3. Spin the tubes for 2 minutes at 13,000 rpm, at 4°C
4. Get rid of the supernatant (be gentle and slow so you won’t lose beads).
5. Wash with **180 µl of PBS** (vortex by hand, then spin). Repeat for a total of three washes.
6. Bring the volume to **150 µl per tube with PBS** (add 150 µl of PBS).
7. Add 8 µg of **SOCS-3** antibody; check the label of the antibody for the concentration of the antibody. (i.e. 100 µg/100 µl, you add 8 µl of antibody. 100 µg/200 µl then you add 16 µl of antibody).

8. Vortex gently by hand and incubate in the rotator for 2 hours at 4°C.

9. Spin for 2 min. at 13,000 rpm and get rid of the SOCS-3 antibody / PBS supernatant.

10. Wash with **180 µl of PBS** (vortex by hand, then spin) Repeat for a total of **three** washes. Make sure you take out as much as possible without taking out beads on final spin.

11. Add the sample based on **800 µg** concentration.

12. Vortex gently by hand and incubate in the rotator for overnight at 4°C.

**DAY TWO:** Get Ice (all samples, washes and buffers should be kept on ICE)

1. Prepare the 2% triton-x/PBS solution:
   a. You need 200 µl of this solution so the formula is
      i. # of samples  X  (4 µl triton-x + 200 µl PBS)
      ii. You can mass produce this and store in fridge so that it can be used for every sample.
      iii. Use this time to write out the additional 0.6 tubes “IP for SOCS-3”

2. Spin the sample after the incubation is over (13,000 rpm for 2 minutes)

3. Take out the lysate and store in *previously* labeled tubes.

4. Wash beads as follows:
a. Add 180 µl per tube with PBS, spin it and take out the supernatant and discard.

b. Add 180 µl per tube with 2% triton-X/PBS, spin it and take out the supernatant and discard.

c. Add 180 µl per tube with PBS, spin it and take out the supernatant and discard. Make sure to remove the most supernatant as possible during this last step.

5. Add 33 µl of 2xTB (aka Lamaelli Buffer).

6. Heat samples in heat block at 100°C for 10 minutes

7. Store samples overnight or continue to day 3.

**Probing for IR-β.**

**DAY THREE:**

1. Cast 15% SDS-PAGE gels

2. Thaw samples, spin samples down 13,000 rpm for 2 minutes at 4°C. You will have a pellet and a supernatant.

3. Load between 5 µl of the supernatant to the stacking gel.

4. Set the power supply to 200 volts for 100 minutes.

**Semi-Dry Transfer**

**Solutions** – Refer to Appendix

**Procedure:**
1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.

2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.

3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.

4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entire membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 18 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in NFDM blocking solution for 1 hour and 15 minutes minimum to overnight at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml **IR-β primary antibody solution** for 2.5 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.
5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

7. Follow Appendix

**Probing for IRS-1.**

**DAY THREE:**

1. Cast 10% SDS-PAGE gels

2. Thaw samples, spin samples down 13,000 rpm for 2 minutes at 4°C. You will have a pellet and a supernatant.

3. Load between 5 µl of the supernatant to the stacking gel.

4. Set the power supply to 200 volts for 120 minutes.

**Semi-Dry Transfer**

**Solutions** – Refer to Appendix

**Procedure:**

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.

2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.
3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.

4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entire membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 45 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix Following protein transfer, place PVDF membrane in NFDM blocking solution for 1 hour and 15 minutes minimum to overnight at 4°C on Lab Quake.

1. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

2. Place on 5 ml **IRS-1 primary antibody solution** for 12 hours at 4°C on Lab Quake.

3. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

4. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

5. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.
Follow Appendix

**Probing for SOCS-3.**

**DAY THREE:**

1. Cast 10% SDS-PAGE gels
2. Thaw samples, spin samples down 13,000 rpm for 2 minutes at 4°C. You will have a pellet and a supernatant.
3. Load between 5 µl of the supernatant to the stacking gel.
4. Set the power supply to 150 volts for 100 minutes.

**Semi-Dry Transfer**

**Solutions** – Refer to Appendix

**Procedure:**

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.
2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.
3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.
4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entre membrane has a film of transfer buffer.
5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.
6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 35 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in NFDM blocking solution for 1 hour and 15 minutes minimum to overnight at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml **SOCS-3 primary antibody solution** for 3 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.

6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

Follow Appendix
APPENDIX Q

New Procedure: IR-β Total Protein Concentration,

IR-β & Phospho Tyrosine IR-β and IR-β & IRS-1 Co-Immunoprecipitation

Preparation of Sample and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE)

**Solutions**- Refer to Appendix

Procedure:

**DAY ONE:** Get Ice (all samples, washes and buffers should be kept on ICE)

Prepare Protein-A Sepharose beads, following protocol of Protein-A Sepharose beads.

1. Vortex Protein-A Sepharose beads before using at a speed of 2 Never Go Above 3, this will crack the beads and ruin them.

2. (use cut pipette tips) into 0.6ml tubes; vortex by hand every time you aliquot beads. (you will be washing each individual 0.6 ml tube with PBS)

3. Spin the tubes for 2 minutes at 13,000 rpm, at 4°C

4. Get rid of the supernatant (be gentle and slow so you won’t lose beads).

5. Wash with 180 µl of PBS (vortex by hand, then spin). Repeat for a total of three washes.

6. Bring the volume to 150 µl per tube with PBS (add 150 µl of PBS).
7. Add 8 µg of IR-β antibody; check the label of the antibody for the concentration of the antibody. (i.e. 100 µg/100 µl, you add 8 µl of antibody. 100 µg/200 µl then you add 16 µl of antibody).

8. Vortex gently by hand and incubate in the rotator for 2 hours at 4°C.

9. Spin for 2 min. at 13,000 rpm and get rid of the IR-β antibody / PBS supernatant.

10. Wash with 180 µl of PBS (vortex by hand, then spin) Repeat for a total of three washes. Make sure you take out as much as possible without taking out beads on final spin.

11. Add the sample based on 800 µg concentration.

12. Vortex gently by hand and incubate in the rotator for overnight at 4°C.

**DAY TWO:** Get Ice (all samples, washes and buffers should be kept on ICE)

1. Prepare the 2% triton-x/PBS solution:
   a. You need 200 µl of this solution so the formula is
      i. # of samples X (4 µl triton-x + 200 µl PBS)
      ii. You can mass produce this and store in fridge so that it can be used for every sample.
      iii. Use this time to write out the additional 0.6 tubes “IP for IR-β”

2. Spin the sample after the incubation is over (13,000 rpm for 2 minutes)

3. Take out the lysate and store in previously labeled tubes.

4. Wash beads as follows:
   a. Add 180 µl per tube with PBS, spin it and take out the supernatant and discard.
b. Add 180 µl per tube with 2% triton-X/PBS, spin it and take out the supernatant and discard.

c. Add 180 µl per tube with PBS, spin it and take out the supernatant and discard. Make sure to remove the most supernatant as possible during this last step.

5. Add 33 µl of 2xTB (aka Lamaelli Buffer).

6. Heat samples in heat block at 100°C for 10 minutes

7. Store samples overnight or continue to day 3.

**Probing for IR-β Total Protein Concentration.**

**DAY THREE:**

1. Cast 10% SDS-PAGE gels

2. Thaw samples, spin samples down 13,000 rpm for 2 minutes at 4°C. You will have a pellet and a supernatant.

3. Load between 5 µl of the supernatant to the stacking gel.

4. Set the power supply to 200 volts for 100 minutes.

**Semi-Dry Transfer**

**Solutions** – Refer to Appendix

**Procedure:**

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.
2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.

3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.

4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entire membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 18 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in NFDM blocking solution for 1 hour and 15 minutes minimum to overnight at 4°C on Lab Quake.

2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.

3. Place on 5 ml IR-β primary antibody solution for 2.5 hours at 4°C on Lab Quake.

4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.
6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

7. Follow Appendix

**Probing for IRS-1 Co-immunoprecipitation.**

**DAY THREE:**

1. Cast 10% SDS-PAGE gels

2. Thaw samples, spin samples down 13,000 rpm for 2 minutes at 4°C. You will have a pellet and a supernatant.

3. Load between 5 µl of the supernatant to the stacking gel.

4. Set the power supply to 200 volts for 120 minutes.

**Semi-Dry Transfer**

**Solutions** – Refer to Appendix

**Procedure:**

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.

2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.

3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.

4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entre membrane has a film of transfer buffer.
5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.

7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 35 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix Following protein transfer, place PVDF membrane in NFDM blocking solution for 1 hour and 15 minutes minimum to overnight at 4°C on Lab Quake.

1. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.
2. Place on 5 ml IRS-1 primary antibody solution for 12 hours at 4°C on Lab Quake.
3. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.
4. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.
5. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

Follow Appendix

**Probing for pY Co-immunoprecipitation.**
DAY THREE:

1. Cast 10% SDS-PAGE gels

2. Thaw samples, spin samples down 13,000 rpm for 2 minutes at 4°C. You will have a pellet and a supernatant.

3. Load between 5-10 µl (~5 µl) of the supernatant to the stacking gel.

4. Set the power supply to 200 volts for 120 minutes.

Semi-Dry Transfer

Solutions – Refer to Appendix

Procedure:

1. Following the completion of SDS-PAGE, separate resolving gel from gel plates and soak in transfer buffer for 30 minutes.

2. Soak polyvinylidene fluoride (PVDF) membrane in methanol for 10 seconds then soak in transfer buffer for 30 minutes.

3. Wet a sheet of blot paper in transfer buffer and place on the Trans-Blot electrode. After placement roll out any air bubbles with a wet glass fleaker.

4. Place PVDF membrane on top of blot paper and roll out any air bubbles with a wet fleaker. Ensure that the entre membrane has a film of transfer buffer.

5. Place resolving gel on top of PVDF membrane. Use a gloved finger or wet wedgie to remove all bubbles.

6. Wet another sheet of blot paper in transfer buffer and place on top of resolving gel. Apply small amount of pressure to remove all air bubbles with a wet fleaker.
7. Set the power supply to constant amperage then set to 400 mA or .4 Amps depending on machine with a max voltage of 25 for 18 minutes.

**Western Blotting**

**Solutions:** Refer to Appendix

1. Following protein transfer, place PVDF membrane in NFDM blocking solution for 1 hour and 15 minutes minimum to overnight at 4°C on Lab Quake.
2. After blocking has elapsed, rinse membranes 3 X with 15 ml TTBS for 5 minutes.
3. Place on 5 ml py primary antibody solution for 12 hours at 4°C on Lab Quake.
4. Recycle primary antibody and rinse membrane 3 X with 15 ml TTBS for 5 minutes.
5. Incubate membrane in secondary antibody solution for 1 hour at 4°C on Lab Quake.
6. Discard secondary antibody solution and rinse membrane 3 X with 15 ml TTBS for 5 minutes.

Follow Appendix