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Activation of aPKCζ Toward TC10 is Regulated by High Fat Diet and Aerobic Exercise in Skeletal Muscle

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Abstract

We determined whether chronic aerobic exercise reverses high-fat diet-induced impairments in the CAP (c-Cbl associated protein)/c-Cbl (Casitas b-lineage lymphoma) signaling cascade in rodent skeletal muscle. Sprague-Dawley rats were placed into either control (CON, n=16) or high-fat fed (n=32) diet groups for 4 wk. During a subsequent 4 wk experimental period 16 high-fat fed rats remained sedentary (HF), 16 high-fat fed rats completed 4 wk of exercise training (HFX), and CON animals were sedentary and remained on the control diet. Following the intervention period, animals were subjected to hind limb perfusions in the presence (n=8/group) or absence (n=8/group) of insulin. In the plasma membrane fractions, neither highfat feeding nor exercise training altered APS (adaptor protein with PH and SH2 domains), c-Cbl or TC10 protein concentrations. In contrast, CAP protein concentration and insulin-stimulated plasma membrane c-Cbl tyrosine phosphorylation were reduced by high-fat feeding, but exercise training reversed these impairments. Of note was that insulin-stimulated aPKC ζ (atypical protein kinase C) kinase activity toward TC10 was reduced by high-fat feeding but normalized by exercise training. We conclude that chronic 4 wk of exercise training can reverse high-fat diet-induced impairments on the CAP/c-Cbl pathway in high-fat fed rodent skeletal muscle. We also provide the first evidence that the CAP/c-Cbl insulin signaling cascade in skeletal muscle may directly interact with components of the classical (phosphoinositide 3-kinase dependent) insulin signaling cascade.

Keywords

c-Cbl tyrosine phosphorylation; TC10; aPKCζ kinase activity

INTRODUCTION

Insulin-stimulated activation of phosphoinositide 3-kinase (PI3K) is required for GLUT4 translocation and glucose uptake (1–4). In rodent skeletal muscle high-fat feeding impairs insulin-stimulated rates of glucose transport and uptake in part due to decreased PI3K activity and GLUT4 translocation to the plasma membrane (5–11). However, in insulin sensitive tissues activation of the classical PI3K dependent insulin-signaling pathway alone cannot fully account

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for insulin-stimulated glucose transport and GLUT4 translocation (12–15). Rather, a purportedly PI3K independent pathway, the CAP/c-Cbl insulin signaling cascade, is also involved in the regulation of glucose metabolism (16–20). In response to insulin the activation of the CAP/c-Cbl pathway is initiated by the APS (adaptor protein with PH and SH2 domains) recruiting c-Cbl (Casitas b-lineage lymphoma) and a second adaptor protein CAP (c-Cbl associated protein) to the insulin receptor (IR) (20–23). This complex enables the tyrosine residues on c-Cbl to be phosphorylated thereby causing the CAP-Cbl complex to dissociate from the IR and migrate to flotillin. This in turn recruits the CrkII/C3G complex to the lipid raft microdomain of the plasma membrane where the guanine nucleotide exchange factor C3G activates a small GTP-binding protein TC10. Activated GTP bound TC10 causes actin remodeling and enables GLUT4 to dock/fuse to the plasma membrane (16,21–24). Of interest, it has been suggested that the CAP/c-Cbl pathway may not function in an entirely PI3K independent manner (24–27). Specifically, aPKCζ/λ, a component of the PI3K dependent pathway, may interact with TC10 (24,28–30) resulting in increased TC10 threonine phosphorylation (31). We have recently observed that high-fat feeding reduces insulin-stimulated plasma membrane aPKC ζ/λ protein concentration and activation in rodent skeletal muscle (11). Furthermore, Bernard et al. (32) and Prada et al. (33) using skeletal muscle obtained from high fat fed rodents report that CAP protein concentration and insulin-stimulated c-Cbl tyrosine phosphorylation are decreased. Given that high fat feeding modulates components of both the PI3K dependent and CAP/c-Cbl insulin signaling cascades in skeletal muscle we believed the high fat-fed rodent model could provide insight whether interaction exists between these signaling pathways. We hypothesized that in rodent skeletal muscle insulin-stimulated aPKCζ/λ activation results in TC10 threonine phosphorylation and that aPKCζ/λ threonine phosphorylation of TC10 is impaired by high fat feeding.

Aerobic exercise training reverses high fat diet-induced impairments in rodent skeletal muscle (34–36) primarily by enabling the PI3K-dependent insulin signaling cascade to be more fully activated by insulin (11,34–38). Whether exercise training can reverse high fat diet-induced impairments in the CAP/c-Cbl pathway is not known, but we have previously reported aerobic training increases insulin-stimulated c-Cbl tyrosine phosphorylation in normal rodent skeletal muscle (39). We therefore hypothesized that high fat diet-induced impairments on the CAP/c-Cbl signaling cascade and aPKCζ/λ threonine phosphorylation of TC10 are reversible by exercise training.

MATERIALS AND METHODS

Experimental Design

Forty-eight male Sprague-Dawley rats (Harlan, San Diego, CA) approximately 6 wk of age were placed randomly into either normal diet (n=16) or high-fat diet (n=32) groups. The normal diet (D12328, Research Diets Inc., New Brunswick, NJ) consisted of 73.1% carbohydrates, 10.5% fat, and 16.4% protein. The high-fat diet (D12330, Research Diets Inc.) contained 25.5% carbohydrates, 58% fat, and 16.4% protein. The animals were on their respective diets for 4 weeks and allowed to feed *ad libitum*, which we have previously shown to induce skeletal muscle insulin resistance in male Sprague-Dawley rats (7,8,10). During the subsequent 4 wk experimental period high-fat diet rats continued to eat the high-fat diet and were randomly assigned to either high-fat diet (HF, n=16) or exercise training (HFX, n=16) groups. Exercise training consisted of treadmill running for 1 h/day, 5 day/wk at 32m/min on a 15% incline. The speed was gradually increased during the first week of training such that the animals were running at 32m/min by the 5^{th} day of training and continued to run at this pace for the duration of the exercise training. We have previously shown that when Sprague-Dawley rats are exercised using this speed and grade that red gastrocnemius oxidative capacity is significantly increased (40,41). The third group of rats (CON; n=16) remained on the chow diet for the

duration of the study (8 wk), and acted as a control group. Following the experimental period animals were fasted for 8–12 h prior to undergoing hind limb perfusion. Exercise trained animals undertook their last training bout 36–48 h prior to hind limb perfusion. We have reported serum glucose, insulin, adiponectin, FFA and skeletal muscle lipid content for these animals previously (37).

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 United States Department of Health and Human Resources.

Hind Limb Perfusions

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (6.5 mg 100^{-1} g body wt) and surgically prepared for hind limb perfusion as previously described by Ruderman *et al*. (42) and modified by Ivy *et al*. (43). Following surgical preparation, cannulas were inserted into the abdominal aorta and vena cava, and the animals were killed via an intracardiac injection of pentobarbital as the hind limbs were washed out with 30 ml of Krebs-Henseleit buffer (KHB) (pH 7.55). Immediately, the cannulas were placed in line with a non-recirculating perfusion system, and the hind limbs were allowed to stabilize during a 5 min washout period. The perfusate was continuously gassed with a mixture of 95% O_2-5% CO_2 and warmed to 37° C. Perfusate flow rate was set at 7.5 ml min^{-1} during the stabilization and subsequent perfusion during which rates of glucose transport were determined.

Perfusions were performed in the presence (n = 8/group) or absence (n= 8/group) of 500 μU ml^{-1} insulin. The basic perfusate medium consisted of 30% washed time-expired human erythrocytes (Ogden Medical Center, Ogden, UT), KHB, 4% dialysed bovine serum albumin (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 non-metabolized glucose analogue 3-*O*-methylglucose (3-MG) (32 μCi 3-[^{3}H] MG mM^{-1}, PerkinElmer Life Sciences, Boston, MA) and 2 mM mannitol (60 μCi-[1-^{14}C] mannitol mM^{-1}, PerkinElmer Life Sciences). Immediately after the transport period, portions of the red gastrocnemius (RG) were excised from both hind limbs, blotted on gauze dampened with cold KHB, freeze clamped in liquid N_2 and stored at −80° C for later analysis.

3-MG Transport

Rates of insulin-stimulated skeletal muscle 3-MG transport were calculated as previously described and this data has been reported elsewhere (37).

Muscle Homogenization

Portions were cut from the RG, weighed frozen and homogenized in an ice-cold homogenization buffer (HB) (1:10 wt/vol) containing 50 mM Hepes (pH 7.6), 150 mM NaCl, 20 mM Na-Pyrophosphate, 20 mM β-Glycerophosphate, 10 mM NaF, 2 mM orthovanadate, 2 mM EDTA, 1% IGEPAL, 10% glycerol, 2 mM phenylmethylsufonylfluoride, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μg ml^{-1} leupeptin, and 10 μg ml^{-1} aprotinin. The homogenate was then transferred to a microcentrifuge tube and centrifuged (19,600 × g, 4° C) in a refrigerated microcentrifuge (Micromax RF, International Equipment Co., Needham Heights, MA) for 15 min. The supernatant was collected, labeled as lysate and assayed for protein concentration using the Bradford method (44) adapted for use with a Benchmark microplate reader (BioRad, Richmond, CA).

Plasma Membrane Fractionation

Plasma membrane fractions were prepared as described previously (45). This procedure provides an enriched plasma membrane fraction and a cytosolic fraction that is devoid of plasma membranes (46). Briefly, a portion of the RG was homogenized in 8x (wt/vol) ice-cold buffer containing 20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, 10% glycerol, 3 mM benzamidine, 10 μM leupeptin, 5 μM pepstatin A, and 1 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged at 100,000 × *g* for 30 min at 4° C, and the supernatant was collected as the cytosolic fraction. The pellet was resuspended by agitation in 4× (wt/vol) ice-cold homogenization buffer to which 1% Triton X was added. The resuspended pellet was then centrifuged at 15,000 × *g* for 10 min at 4° C. The supernatant, representing the plasma membrane fraction, was collected.

Western Blotting

Plasma membrane samples from the RG (100 μg of protein for CAP, APS, c-Cbl, and Flotillin) were added to Laemmli buffer (47). Sample proteins were subjected to SDS-PAGE run under reducing conditions on a 7.5% (CAP and c-Cbl) or 10% (APS and Flotillin) resolving gel in a MiniProtean 3 dual-slab cell (BioRad). Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a semidry transfer unit (10 V for 55 min). Membranes were then blocked in 5% nonfat dry milk/Tris-Tween-buffered saline and incubated with anti-CAP [Cat# 06-994, UBT], anti-APS [sc-8894, Santa Cruz Biotechnology (SCBT) Santa Cruz, CA], anti-c-Cbl [Cat# 05-440, UBT], or mouse polyclonal anti-Flotillin-1 [Cat# 610820, BD Transduction Laboratories, BD Biosciences Pharmingen, San Diego, CA] followed by the species-specific immunoglobulin (Ig) G secondary antibody conjugated to horseradish peroxidase (HRP). Antibody binding was visualized by enhanced chemiluminescence in accordance to manufacturer's instructions. Images were captured using a ChemiDoc system (BioRad) equipped with a CCD camera and saved to a Macintosh G4 computer. Protein bands were quantified using Quantity One analysis software (BioRad). The data is expressed as a percentage (arbitrary units) of a muscle sample standard that was run on each gel.

Immunoprecipitation

c-Cbl tyrosine phosphorylation and TC10 protein concentration were determined using immunoprecipitation followed by western blotting. Sixty microliters of Pro-A slurry were incubated with 4 μg of anti-c-Cbl [Cat# 05-440, UBT] or anti-TC10 [Cat# sc-12637, SCBT] overnight at 4° C with rotation. Following an overnight incubation, plasma membrane samples (1000ug of protein for c-Cbl tyrosine phosphorylation and TC10 protein concentration) were added to the immunocomplexes and placed on rotation for 2 h at 4°C. The Pro-A beads were resuspended in 25 μl of Laemmli buffer, subjected to SDS-PAGE on a 7.5% (c-Cbl tyrosine phosphorylation) or 12.5% (TC10 protein concentration) resolving gel and transferred to PVDF membranes as described above. Membranes were then subjected to western blotting, the proteins were visualized and quantified as described above using either antiphosphotyrosine [Cat# 02-247, UBT] or anti-TC10 [Cat# sc-12637, SCBT] as the primary antibody.

aPKCζ Kinase Activity Toward TC10

We determined whether insulin-stimulated activation of aPKCζ resulted in increased TC10 threonine phosphorylation in mammalian skeletal muscle. The rationale for this assay is that TC10 has a consensus aPKCζ threonine phosphorylation site (31). One hundred microliters of Pro-A slurry were incubated with 4 μg of anti-PKCζ [Cat# sc-216, SCBT] overnight at 4°C with rotation. Following an overnight incubation, 1000 μg of sample protein was added to the immunocomplexes and placed on rotation for 2 h at 4°C. The immunocomplexes were then washed 3 times with PBS. After the final wash, the supernatant was completely removed and the remaining bead/immunocomplex was subjected to a kinase assay using a TC10 substrate.

The TC10 substrate, amino acid sequence ILTPKKHT(179)VKKIGS of TC10 containing threonine 179 that is phosphorylated in response to insulin stimulation, was synthesized by American Peptide Company, Inc. (Sunnyvale, CA). Twenty microliters of assay dilution buffer was added to the bead/immunocomplex in addition to 10 μl of the custom TC10 substrate. The kinase reaction was initiated by the addition of 500 μl assay dilution buffer, 75 mM $MgCl_2$, 1 M adenosine triphosphate (ATP), and [γ-^{32}P] ATP (PerkinElmer Life Sciences) and warmed to 37° C with constant mixing for 10 min. The reaction was terminated by the addition of 80 μl of Tris-tricine sample buffer and heated at 95° C for 5 min. Fifteen microliters of samples were loaded onto a 20% Tris-tricine polyacrylamide gel in duplicate and electrophoresed for 130 min at 100 V using a MiniProtein electrophoresis system (BioRad). After electrophoreses, gels were wrapped in plastic wrap and exposed to a phosphor screen (Eastman Kodak Company) overnight. Images were captured and quantified as described above.

Statistical Analysis

A one-way 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), and all values were expressed as means ± SE.

RESULTS

Body and Epididymal Fat Pad Mass

Body and epididymal fat pad mass data have been published by us in a companion paper (37). Body and epididymal fat pad mass of the HF animals were heavier than CON and HFX animals ($p<0.05$).

CAP Protein Concentration

Plasma membrane CAP protein concentration in the HF group was reduced compared to CON and HFX groups ($p<0.05$, Fig. 1). CAP protein concentration was not different between CON and HFX animals (Fig. 1).

c-Cbl Protein Concentration and Tyrosine Phosphorylation

Plasma membrane c-Cbl protein concentration was not different among groups in the absence or presence of insulin (Fig. 2A). In the absence of insulin, c-Cbl tyrosine phosphorylation was similar among groups (Fig. 2B). In the presence of insulin c-Cbl tyrosine phosphorylation was elevated above basal levels in both CON and HFX animals ($p<0.05$) but not in the HF group.

APS, Flotillin and TC10 Protein Concentration

APS (Fig. 3A), Flotillin (Fig. 3B) and TC10 (Fig. 3C) protein concentration were similar in the absence or presence of insulin among groups.

aPKCζ Kinase Activity Toward TC10

In the absence of insulin, the substrate specific kinase activity of aPKCζ was similar among groups (Fig. 4). In the presence of insulin threonine phosphorylation of TC10 by aPKCζ in CON and HFX animals was greater than that of the HF animals ($p<0.05$).

DISCUSSION

The classical PI3K dependent and novel CAP/c-Cbl insulin signaling cascades were believed to independently contribute to insulin-stimulated GLUT4 translocation. However, it has been

proposed that these two pathways may work in partnership (24–27). Of interest, aPKCζ/λ may be recruited to the plasma membrane in a TC10-dependent mechanism as it has been reported that both aPKCζ/λ and TC10 can form complexes with the plasma membrane-associated proteins Par3 and Par6 (24,28–30). Furthermore, Kotani *et al*. (48) have reported that when Par3 is overexpressed in 3T3-L1 adipocytes that insulin-stimulated aPKCζ/λ activation is inhibited which in turn results in decreased GLUT4 translocation. Collectively, this data raises the possibility that the PI3K dependent and CAP/c-Cbl signaling cascades may not only work in concert but may actually have components of the signaling cascades that "cross-talk" in response to insulin stimulation.

Supporting this contention we observed that insulin-stimulated aPKCζ kinase activity toward TC10 was reduced by high-fat feeding but normalized by aerobic training. The functional significance of this observation remains to be determined but may be related to the role that TC10 plays in the cortical actin remodeling that is critical for docking/fusion of GLUT4 vesicles to the plasma membrane in response to insulin stimulation (16,17,49). Liu et al. (50) have observed in differentiated rat L6 muscle cells that insulin recruits aPKCζ to the plasma membrane and mediates glucose transport through actin remodeling. Moreover, we have recently shown that in these high-fat fed animals insulin-stimulated plasma membrane aPKC ζ and λ protein concentration and insulin-stimulated aPKCζ/λ activities were less than that of the normal diet animals, and that exercise training increased both insulin-stimulated plasma membrane association and activation of aPKCζ/λ in the high-fat fed skeletal muscle (11), which complemented our observation that insulin-stimulated 3-MG transport rates in HF animals were less than CON and HFX animals but were not different between CON and HFX (37).

It has been reported that aPKCζ/λ interacts with GLUT4 containing vesicles (24,51) and aPKCζ regulates munc18 (a protein of the GLUT4 vesicular trafficking machinery) (52). We have previously demonstrated that insulin-stimulated plasma membrane GLUT4 protein concentration is reduced by high-fat feeding and increased in the high-fat fed-exercise trained animals (11). Accordingly, it seems entirely plausible to suggest that aPKCζ serves to couple the activation of the PI3K dependent and CAP/c-Cbl signaling cascades. Specifically, the PI3K dependent insulin signaling cascade's primary role may lie in initiating GLUT4 vesicular translocation while the CAP/c-Cbl insulin signaling cascade might predominantly assist in the trafficking, docking and fusion of GLUT4 vesicles to the plasma membrane. However, the "cross talk" that occurs between these two pathways appears necessary to orchestrate the coordinated movement of GLUT4 containing vesicles among cellular compartments in response to insulin stimulation.

Aerobic exercise training is well recognized as an intervention that improves glucose metabolism in skeletal muscle (53–56). In the high-fat fed rodent model aerobic exercise training reverses not only whole body insulin resistance (34,35) but also improves impairments in the classical PI3K dependent insulin signaling cascade as evidenced by increased PI3K activity, Akt2 activity, aPKCζ/λ activity, aPKCζ/λ translocation, GLUT4 expression and GLUT4 translocation (11,37,57,58). In contrast, the effects of chronic aerobic training on the CAP/c-Cbl insulin signaling pathway have not been well studied. Bernard et al. (39) have reported that exercise training increases CAP protein concentration and c-Cbl tyrosine phosphorylation in normal rat skeletal muscle, and increases CAP and TC10 protein concentration in obese Zucker rat skeletal muscle (59). Consistent with these findings we observed that chronic aerobic exercise normalized CAP protein concentration and c-Cbl tyrosine phosphorylation in high-fat fed rodent skeletal muscle. This training adaptation likely contributes to enhancing the formation of the CAP/c-Cbl complex which facilitates GLUT4 to traffic to the plasma membrane through TC10 activation (16,21–24). Skeletal muscle APS, c-Cbl, and TC10 protein concentration were unaltered by either exercise training or a high-fat

diet, which is also in agreement with previous reports (32,39), and suggest that neither exercise training nor dietary content affects the expression of these proteins.

In conclusion, the present study provides the first evidence that in skeletal muscle the CAP/c-Cbl insulin signaling cascade may directly interact with components of the PI3K dependent insulin signaling cascade. Additionally, we demonstrate that chronic aerobic exercise training can reverse high-fat diet-induced impairments in components of the CAP/c-Cbl insulin signaling cascade in skeletal muscle.

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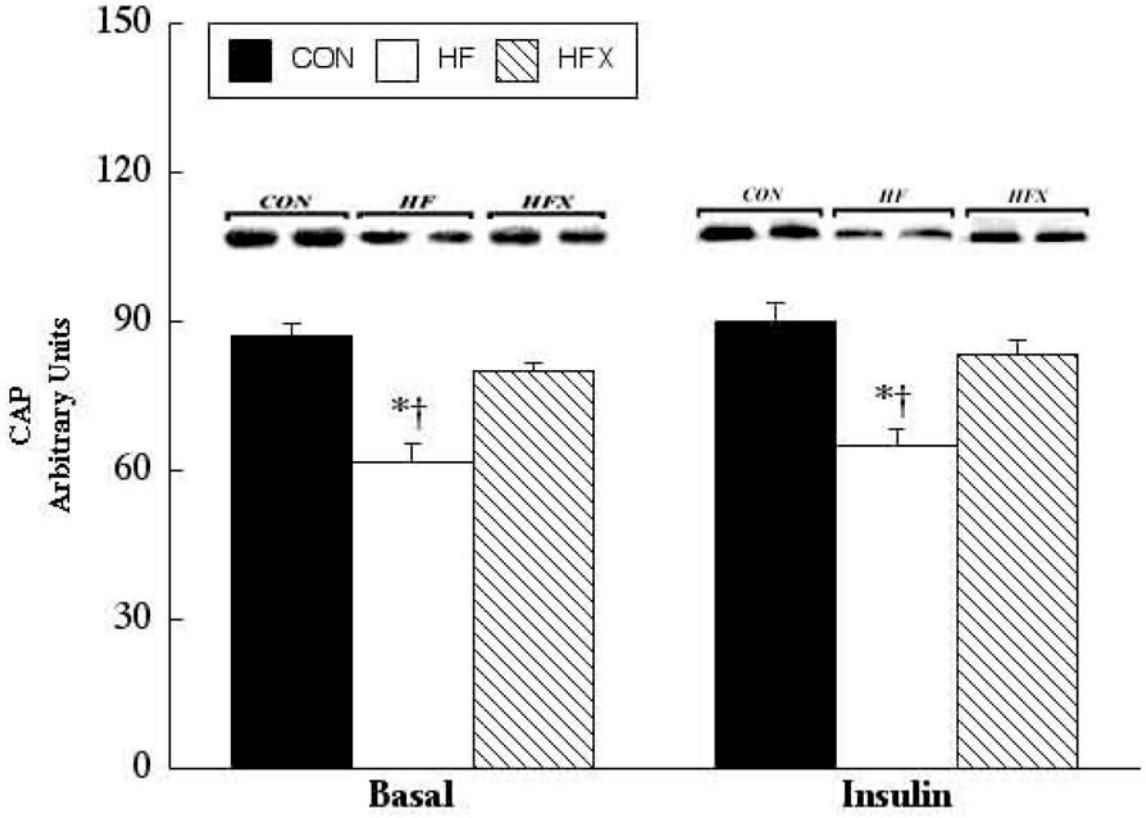

Figure 1.
Plasma membrane CAP protein concentration obtained from normal diet, control (CON); high fat diet, control (HF); high fat diet, exercise trained (HFX) animals. *, Significantly different from CON ($p<0.05$). †, Significantly different from HFX ($p<0.05$). Values are means ± SE.

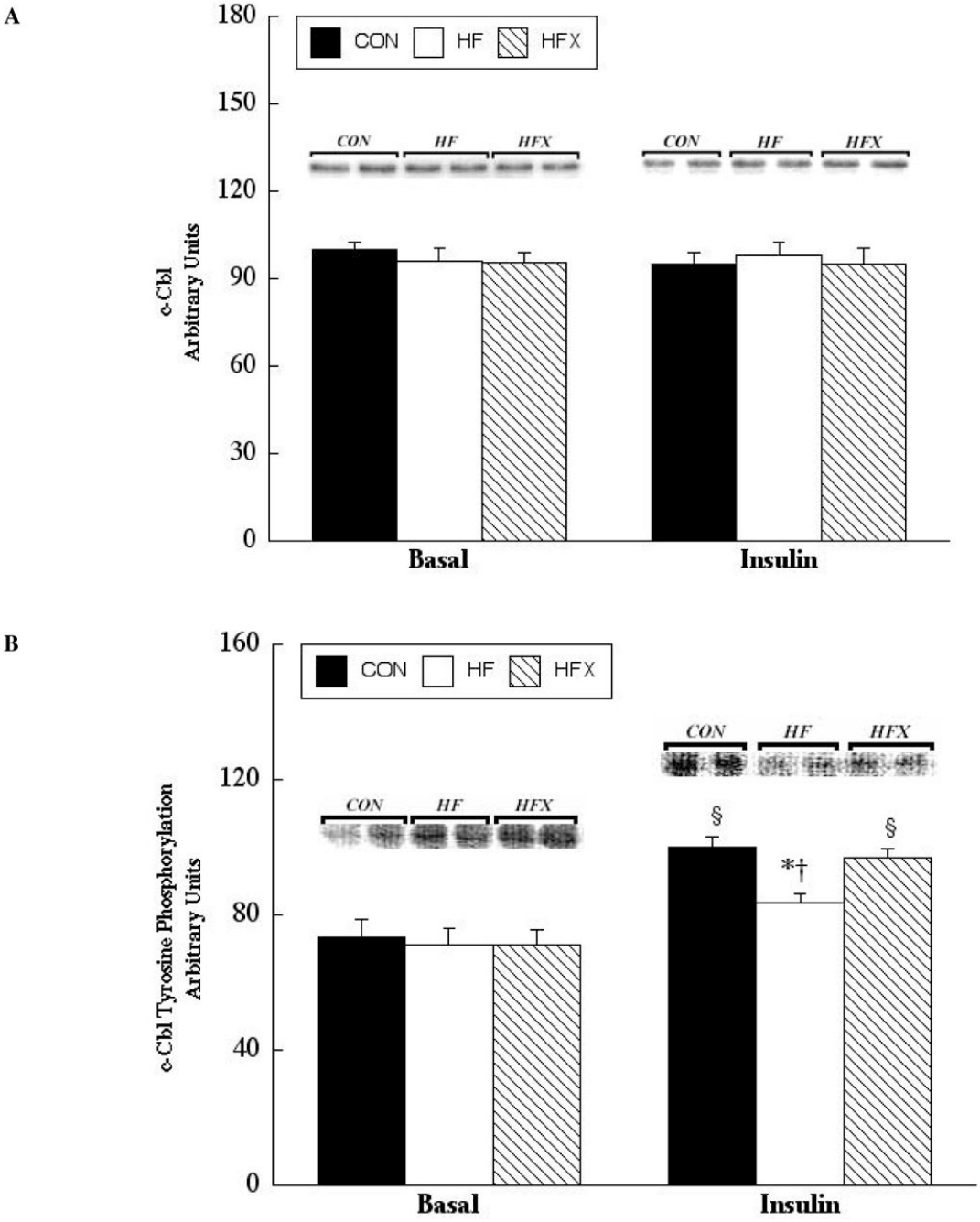

Figure 2.

(A) Plasma membrane c-Cbl protein concentration, **(B)** Plasma membrane tyrosine phosphorylation of c-Cbl obtained from normal diet, control (CON); high fat diet, control (HF); high fat diet, exercise trained (HFX) animals. *, Significantly different from CON ($p<0.05$). †, Significantly different from HFX ($p<0.05$). §, Significantly different from basal condition ($p<0.05$). Values are means ± SE.

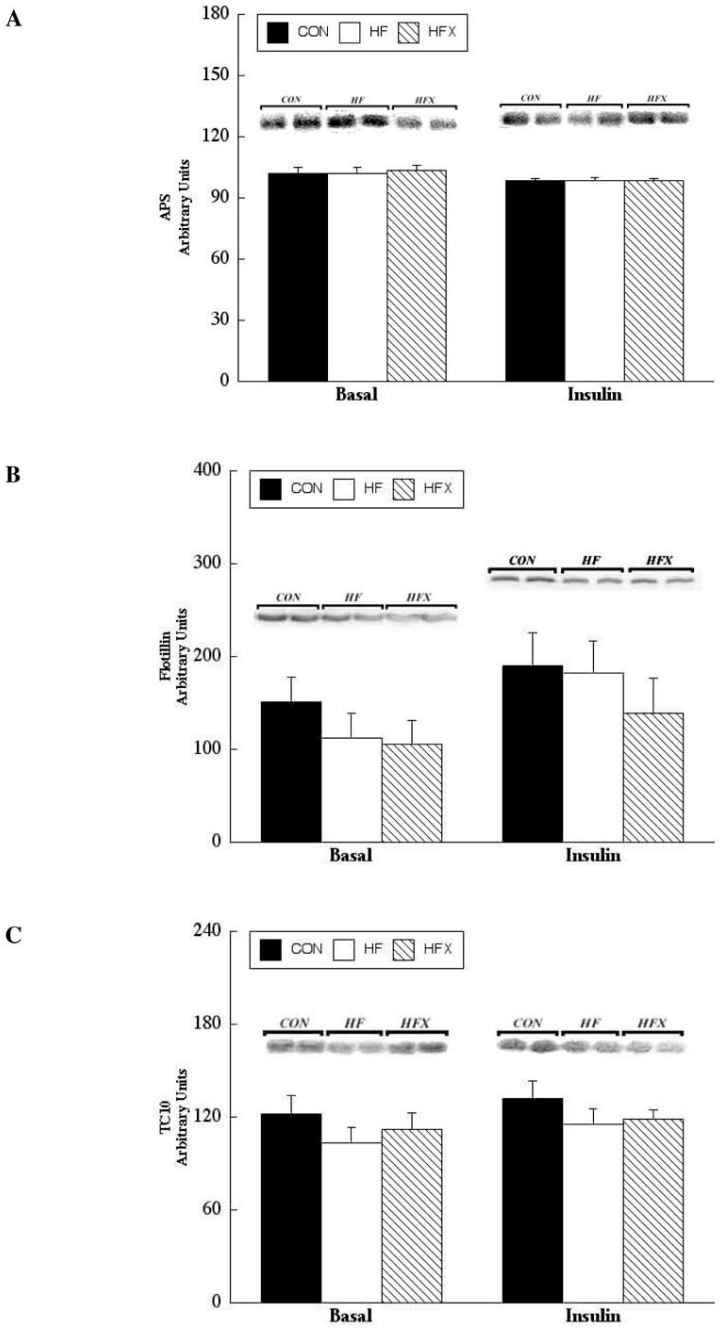

Figure 3.

(A) Plasma membrane APS protein concentration, **(B)** Plasma membrane Flotillin concentration, **(C)** Plasma membrane TC10 concentration obtained from normal diet, control (CON); high fat diet, control (HF); high fat diet, exercise trained (HFX) animals. Values are means ± SE.

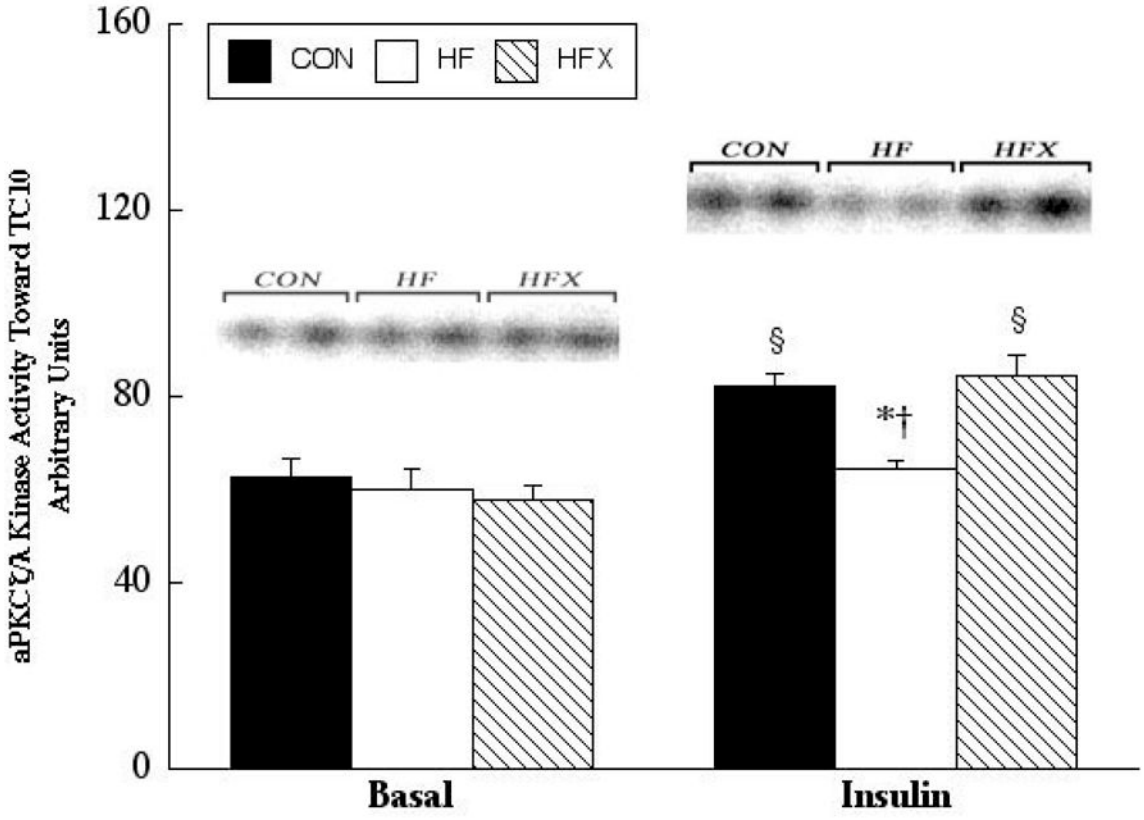

Figure 4.

Plasma membrane aPKCζ/λ kinase activity toward TC10 obtained from normal diet, control (CON); high fat diet, control (HF); high fat diet, exercise trained (HFX) animals. *, Significantly different from CON ($p<0.05$). †, Significantly different from HFX ($p<0.05$). §, Significantly different from basal condition ($p<0.05$). Values are means ± SE.