High-Fat Diet-Induced Mitochondrial Biogenesis Is Regulated by Mitochondrial-Derived Reactive Oxygen Species Activation of CaMKII.

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Calcium/calmodulin-dependent protein kinase (CaMK) activation induces mitochondrial biogenesis in response to increasing cytosolic calcium concentrations. Calcium leak from the ryanodine receptor (RyR) is regulated by reactive oxygen species (ROS), which is increased with high-fat feeding. We examined whether ROS-induced CaMKII-mediated signaling induced skeletal muscle mitochondrial biogenesis in selected models of lipid oversupply. In obese Zucker rats and high-fat–fed rodents, in which muscle mitochondrial content was upregulated, CaMKII phosphorylation was increased independent of changes in calcium uptake because sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) protein expression or activity was not altered, implicating altered sarcoplasmic reticulum (SR) calcium leak in the activation of CaMKII. In support of this, we found that high-fat feeding increased mitochondrial ROS emission and S-nitrosylation of the RyR, whereas hydrogen peroxide induced SR calcium leak from the RyR and activation of CaMKII. Moreover, administration of a mitochondrial-specific antioxidant, SkQ, prevented high-fat diet–induced phosphorylation of CaMKII and the induction of mitochondrial biogenesis. Altogether, these data suggest that increased mitochondrial ROS emission is required for the induction of SR calcium leak, activation of CaMKII, and induction of mitochondrial biogenesis in response to excess lipid availability.

Skeletal muscle, by virtue of its mass and rate of insulin-stimulated glucose transport, is an important tissue in the development of insulin resistance. Alterations in mitochondrial function and content have been implicated in the etiology of insulin resistance; therefore, a better understanding of the regulation surrounding mitochondrial biogenesis and mitochondrial energetics may provide insight into novel therapies.

Although controversial, a reduction in the number of mitochondria within muscle has been speculated to contribute to the development of insulin resistance (1). However, high-fat feeding has been shown to induce insulin resistance while increasing mitochondrial content (2,3), divorcing this proposed causal relationship. Nevertheless, a reduction in mitochondrial content could exacerbate the phenotype of insulin resistance (4), whereas the increase in mitochondrial content observed at the initiation of a high-fat diet is likely beneficial because genetic approaches that modestly increase mitochondrial content improve insulin signaling in insulin resistant animals (5). Therefore, understanding the cellular mechanisms causing mitochondrial biogenesis during a high-fat challenge may provide insight into both disease progression and potential therapeutic strategies.

Which mechanisms are responsible for mediating high-fat diet–induced mitochondrial biogenesis are currently

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unknown, but studies in myotubes and skeletal muscle have shown that raising cytosolic calcium concentrations induces mitochondrial biogenesis in a calcium/calmodulin-dependent protein kinase II (CaMKII)–dependent mechanism (6,7). In addition, the first experiments providing evidence for the nuclear translocation of peroxisome proliferator–activated receptor γ coactivator 1α involved caffeine-induced calcium release from the ryanodine receptor (RyR) and activation of CaMKII (7) independent of muscle contraction. These data suggest that sarcoplasmic reticulum (SR) calcium release from the RyR is a primary mediator of mitochondrial biogenesis in conditions without large energy turnover. Calcium release from the RyR is also regulated by reactive oxygen species (ROS) (8–10), which coincides with high-fat feeding (11,12). Whether ROS-activated CaMKII signaling is a key mechanism inducing mitochondrial biogenesis in insulin resistant muscle remains to be determined.

The current study was undertaken to determine the potential signaling events that regulate high-fat diet–induced mitochondrial biogenesis. We provide evidence that CaMKII is phosphorylated in several models of high-fat diet exposure as a result of mitochondrial ROS-induced SR calcium leak. Together, these data suggest that mitochondrial ROS-induced activation of CaMKII is responsible for the induction of mitochondrial biogenesis during a high-fat diet challenge, and we discuss the potential biological significance of alterations in mitochondrial content within this context.

RESEARCH DESIGN AND METHODS

Animals

Male lean (n = 8; 224 ± 10 g; blood glucose 5.0 ± 0.2 mmol/L) and obese Zucker (n = 8; 310 ± 6 g; blood glucose 13.1 ± 1.4 mmol/L) rats were purchased from Charles River Laboratories. Male Sprague-Dawley rats (n = 15 in total, weighing ~500 g) were bred onsite at the University of Guelph. Sprague-Dawley rats (n = 5 per group) were randomly assigned to either an ad libitum control diet (D05092806BM; Research Diets) or an ad libitum high-fat diet (60% kcal) (D05120801M; Research Diets) for 4 weeks. Additional Sprague-Dawley rats (n = 5) fed a control diet were used for soleus muscle incubation experiments. Male C57B1/6 mice were purchased from Charles River Laboratories and randomly assigned (n = 5 per group) to either a low-fat control diet (ssniff) or a high-fat high-sucrose diet (Research Diets) with or without water supplemented with 250 nmol/kg body weight Skulachev ion (SkQ) (plastoquinonyl decyltriphenylphosphonium) for 16 weeks. The SkQ was donated by O. Fedorkin (Mitotech). This study was approved by the Animal Care Committees at the University of Guelph, University of Waterloo, and Maastricht University.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) copy number was determined in the red gastrocnemius muscle using real-time PCR as previously reported (13).

Sarco(endo)plasmic Reticulum Ca^{2+}-ATPase Activity and Calcium Uptake

Maximal sacro(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) activity and Ca^{2+} uptake were determined in red tibialis anterior homogenates as previously reported (14).

Mitochondrial Hydrogen Peroxide Emission

Measurement of red gastrocnemius muscle mitochondrial hydrogen peroxide (H_2O_2) emission was determined fluorometrically (Lumina; Thermo Scientific) in a constantly stirring cuvette at 37°C (Peltier controlled) as previously described (15).

Isolated Soleus Muscles

Incubations were performed as previously described (16). Briefly, excised muscles (n = 5) were immediately stripped, and one strip was used for each of the four conditions: Media 199 only (control), media supplemented with dantrolene (Dan) (10 μmol/L in DMSO; 0.3% DMSO final concentration), media supplemented with 3 mmol/L H_2O_2 [as previously reported (17)], or media supplemented with both Dan and H_2O_2 (Dan + H_2O_2) for 20 min.

Western Blotting

Western blotting in red gastrocnemius muscles using commercially available antibodies was performed as previously reported (13,16,18). CaMKII was detected at ~55–60 kDa, representing the δ/γ isoform. 4-Hydroxynonenal (4HNE) (Abcam) and oxidized CaMKII (Millipore) were purchased, whereas the SERCA1a antibody (A52) was a gift (Dr. David MacLennan, University of Toronto). Ponceau staining was used to ensure constant loading.

S-nitrosylation of the RyR

The presence of RyR nitrosylated tyrosine residues was determined as previously reported (19). Briefly, the RyR was immunoprecipitated and separated using SDS-PAGE, transferred to polyvinylidene fluoride, and probed for RyR and nitrosylated tyrosine residues (Cayman).

Statistical Analysis

Data were analyzed by unpaired Student t test (Figs. 1 and 2) or ANOVA and Fisher least significant difference post hoc test (Figs. 3 and 4). $P < 0.05$ was considered significant. All data are reported as mean ± SEM.

RESULTS

We first examined the phosphorylation state of CaMKII in a chronic model of obesity, namely obese Zucker rats, which are known to have higher levels of skeletal muscle mitochondrial content (2,20). We have confirmed these findings because mtDNA was increased ($P < 0.05$) approximately twofold in obese animals (Fig. 1*A*), whereas markers of the electron transport chain were increased to varying degrees (Fig. 1*B*). In addition, we found that although total CaMKII was not different in the muscle of obese animals, CaMKII threonine (Thr) 287 phosphorylation was increased approximately twofold (Fig. 1*C*). The

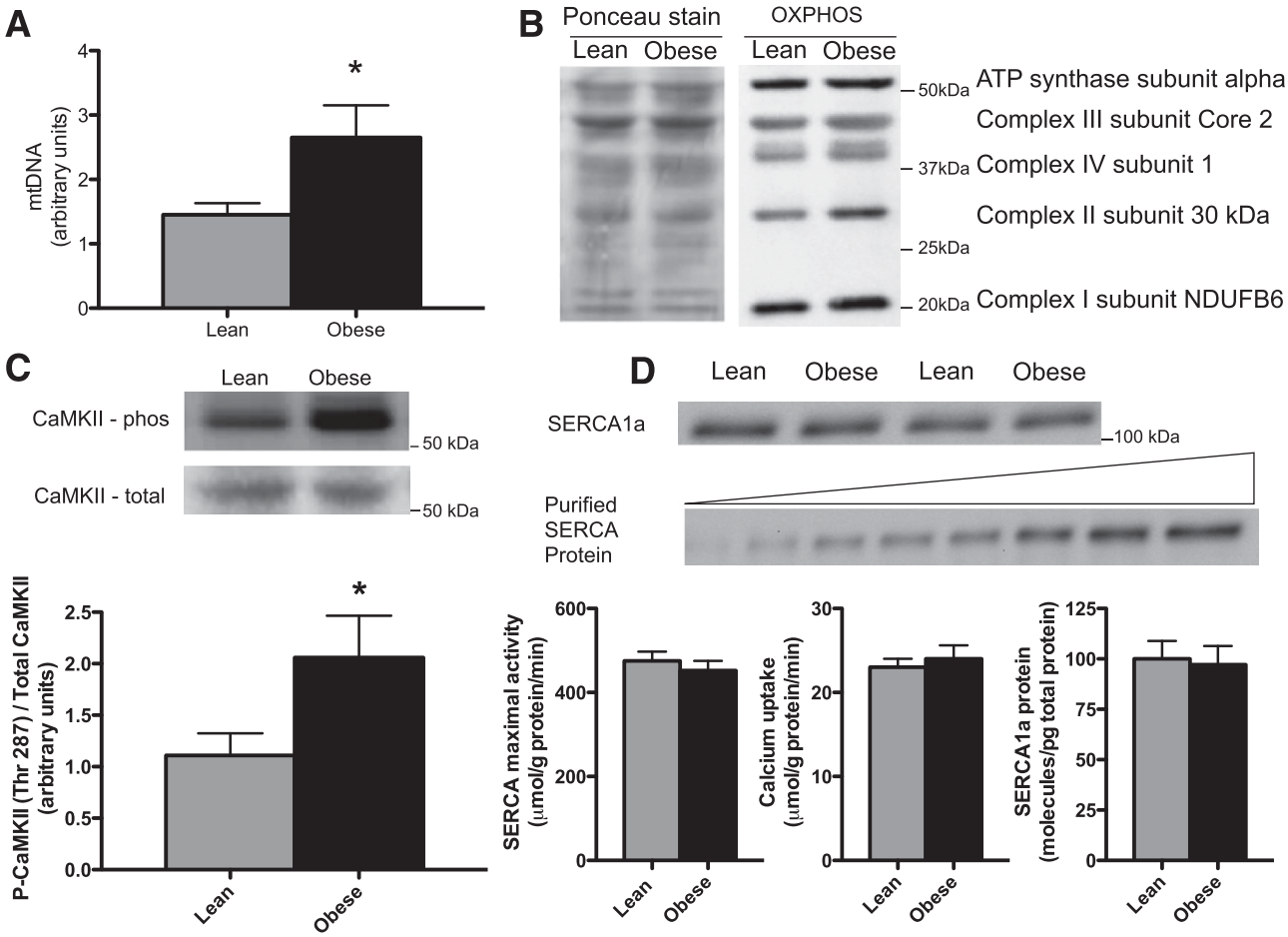

Figure 1—Mitochondrial content, CaMKII, and calcium uptake in obese Zucker rats. mtDNA (*A*) was increased in obese Zucker rats, and this was further supported by a representative Western blot for subunits of the electron transport chain using the MitoSciences OXPHOS antibody cocktail (*B*) (a representative Ponceau stain of the entire membrane is shown to confirm constant loading). The induction of mitochondrial biogenesis was affiliated with phosphorylation of CaMKII (*C*) (detected at ~60 kDa) in the absence of changes in SERCA activity, calcium uptake, or SERCA protein content (*D*) (detected at ~110 kDa). Total and phosphorylated CaMKII were detected at ~60 kDa using antibodies purchased from Cell Signaling. Data are mean ± SEM. $n = 8$. *Significantly different ($P < 0.05$) from lean animals.

increased phosphorylation of CaMKII was not associated with changes in maximal SERCA activity, calcium uptake, or the expression of SERCA protein (Fig. 1*D*), suggesting that SR calcium leak, and not uptake, may be altered in these animals.

Before determining a potential mechanism that could alter calcium leak, given the chronic nature of the obese Zucker rat model, we first determine whether increased CaMKII phosphorylation occurred following high-fat feeding. High-fat feeding in male rats increased fasting blood glucose levels (5.8 ± 0.3 vs. 7.1 ± 0.5 mmol/L), body weight (463 ± 37 vs. 527 ± 13 g), and subunits of the electron transport chain (~25%) (Fig. 2*A*). In addition, CaMKII Thr 287 phosphorylation was increased (~50%) (Fig. 2*B*). Collectively, these observations confirm the findings in the obese Zucker rat model. Moreover, we found that high-fat feeding in male rats increased H_2O_2 emission from mitochondria approximately twofold (Fig. 2*C*), and this coincided with an increase in 4HNE (Fig. 2*D*), suggesting the induction of oxidative stress. Of note, these observations were only observed in male rats because high-fat feeding in female rats did not increase mitochondrial ROS emission, phosphorylation of CaMKII, or markers of mitochondrial content (Supplementary Fig. 1). Combined, these data suggest that mitochondrial ROS may contribute to the observed high-fat diet–induced mitochondrial biogenesis in male rats.

Given the emergence of protein cysteine modifications as fundamental molecular events regulating a host of cellular processes, we speculated that mitochondrial ROS emission regulates the induction of mitochondrial biogenesis following a high-fat diet. In mouse heart cell lysates, exogenous provision of H_2O_2 directly activates CaMKII through oxidation (21). We therefore determined the oxidation status of CaMKII following high-fat feeding; however, this was not altered (Fig. 2*E*). Because CaMKII was phosphorylated independent of apparent changes in calcium uptake, we next focused on the potential for ROS

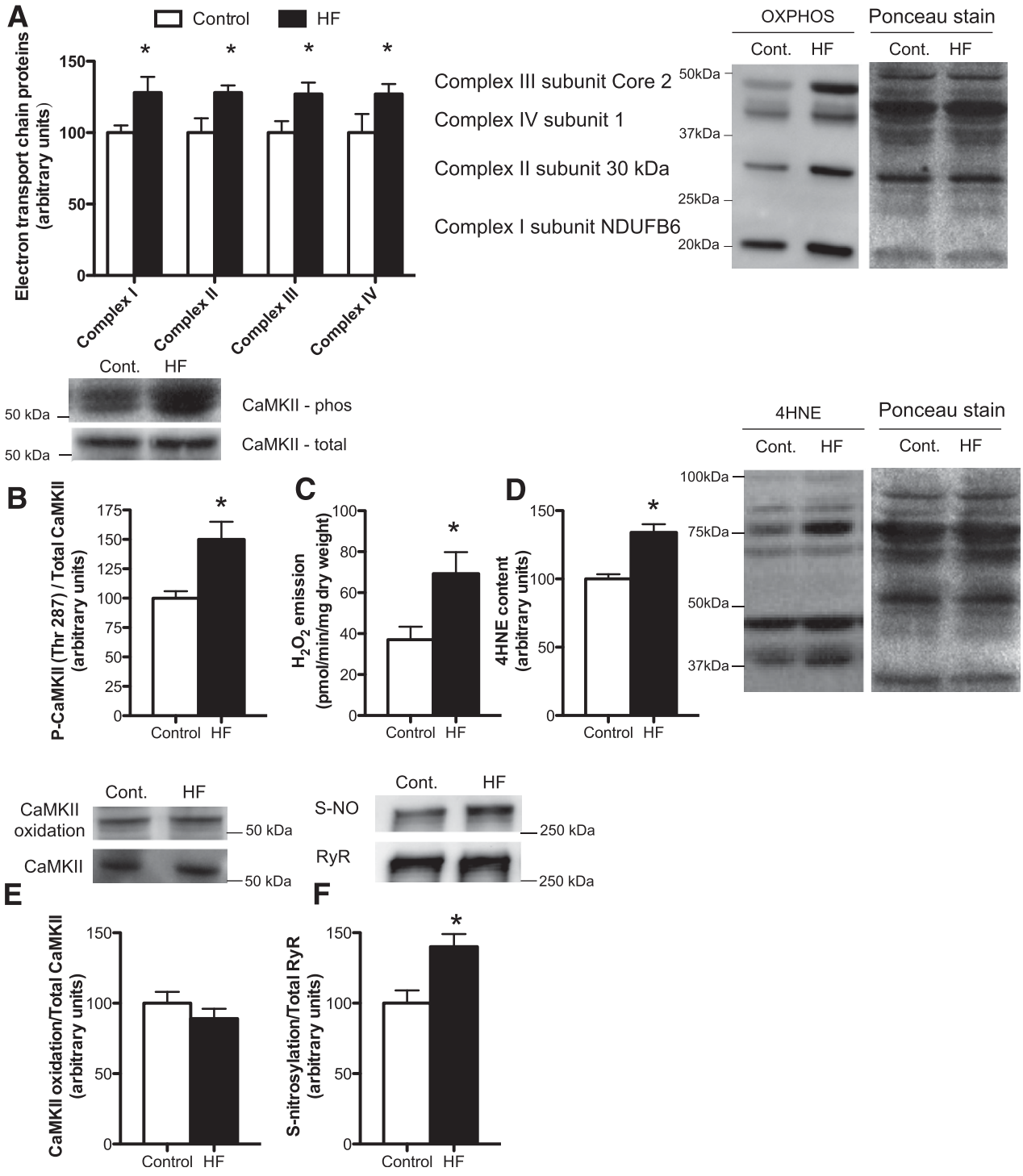

Figure 2—High-fat diet (HF)–induced mitochondrial biogenesis is associated with CaMKII phosphorylation and oxidative stress. Representative Western blots show an increase in subunits of the electron transport chain using the MitoSciences OXPHOS antibody cocktail (*A*) (a representative Ponceau stain of the entire membrane is shown to confirm constant loading) in association with phosphorylation of CaMKII (*B*). In addition, HF increased rates of H_2O_2 emission from permeabilized muscle fibers (*C*) and 4HNE content (*D*) (a representative Ponceau stain of the entire membrane is shown to confirm constant loading). HF did not alter the oxidation of CaMKII (*E*) but increased S-nitrosylation (S-NO) of the RyR (*F*). Complex I was detected at ~20 kDa, complex II at ~30 kDa, complex IV at ~39 kDa, complex III at ~47 kDa, and complex V at ~53 kDa. The 4HNE blot detected several bands at ~37 kDa, 45 kDa, 60 kDa, 75 kDa, and 80 kDa. Data are mean ± SEM. $n = 5$. *Significantly different ($P < 0.05$) from chow-fed controls.

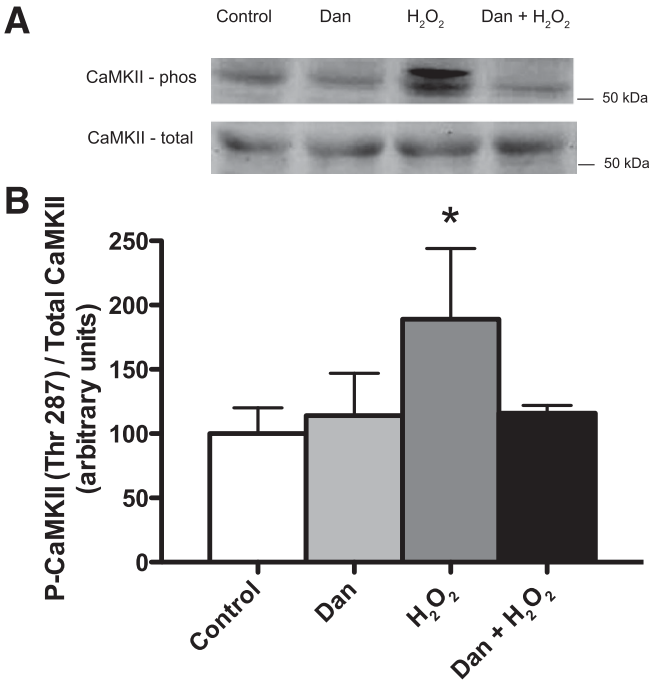

Figure 3—H_2O_2 induces calcium release from the RyR and the phosphorylation of CaMKII. Soleus muscle incubations showing 3 mmol/L exogenous H_2O_2 causes phosphorylation of CaMKII, whereas the presence of Dan, a specific inhibitor of the RyR, prevents this response. Representative Western blots (*A*) and quantified values representing mean ± SEM (*B*) are shown. Total and phosphorylated CaMKII were detected at ~60 kDa using antibodies purchased from Cell Signaling. Data are mean ± SEM. $n = 5$. *Significantly different ($P < 0.05$) from all other conditions.

to enhance SR calcium leak. In muscle, the RyR is highly responsive to oxidative changes, which result in enhanced calcium leak or release (8–10). We therefore determined whether high-fat feeding altered the extent of S-nitrosylation of the RyR as a marker of oxidative changes. This approach revealed a ~40% increase in nitrosylated tyrosine residues on the RyR (Fig. 2*F*), suggesting that oxidative changes on the RyR may activate CaMKII. To further test this notion, we used an isolated soleus muscle incubation preparation to determine whether ROS-induced activation of the RyR would increase CaMKII phosphorylation. We show that exogenous provision of H_2O_2 increased CaMKII phosphorylation ~75%, but this was prevented with the addition of the RyR inhibitor Dan (Fig. 3*A* and *B*). RyR1 is also located on the inner mitochondrial membrane and, under certain conditions, could be a calcium extrusion pathway (22), which presumably could activate cytosolic CaMKII. However, it is unlikely that the mitochondrial RyR is responsible for CaMKII activation with H_2O_2 treatment based on the relatively low abundance of mitochondrial RyR (estimated to represent ~10% of that found in SR) and the differences in mitochondrial versus SR calcium stores. These data indicate, therefore, that H_2O_2-induced SR calcium leak activates CaMKII phosphorylation.

Although these experiments suggest that ROS may be a key mediator of CaMKII phosphorylation following high-fat diets, other events could mediate these effects in vivo. Therefore, we found it important to directly test the notion that mitochondrial ROS could cause mitochondrial biogenesis in a high-fat model. To accomplish this, we fed mice a high-fat diet in the presence and absence of a mitochondrial targeted antioxidant, SkQ. We show that high-fat feeding increased the expression of the subunits of complex I and II ~50% (Fig. 4*A* and *B*, respectively), whereas trends for increases in complexes III and IV ($P = 0.10$; data not shown) were seen, supporting our previous work showing that SkQ prevents high-fat diet–induced increases in citrate synthase activity (12). These increases in markers of mitochondrial content were once again accompanied by increased CaMKII phosphorylation (Fig. 4*C*). Of importance, consuming a mitochondrial targeted antioxidant prevented diet-induced CaMKII phosphorylation (Fig. 4*C*) and the induction of mitochondrial biogenesis (Fig. 4*A* and *B*). In contrast, consumption of SkQ exaggerated the induction of AMP-activated protein kinase (AMPK) phosphorylation (Fig. 4*D*), suggesting that phosphorylation of AMPK is not sufficient for high-fat diet–induced mitochondrial biogenesis, supporting previous reports in AMPK knockdown mice (23). Altogether, these data suggest that mitochondrial ROS-induced SR calcium leak and activation of CaMKII are responsible for high-fat diet–induced mitochondrial biogenesis.

DISCUSSION

Given the current data showing that mitochondrial ROS emission is primarily responsible for promoting high-fat diet–induced mitochondrial biogenesis, two potential hypotheses exist regarding the physiological ramifications of increasing mitochondrial biogenesis within this context: *1*) increasing fatty acid oxidation and *2*) decreasing mitochondrial ROS production. Regarding the first hypothesis, similar to exercise training (24), an increase in mitochondrial content may promote a higher reliance on fatty acids as a fuel source. Regarding the second hypothesis, ROS-induced mitochondrial biogenesis may dissipate protonmotive force over a greater mitochondrial volume and, therefore, may decrease mitochondrial ROS emission rates. In support of this hypothesis, it has been previously shown that skeletal muscle with the lowest mitochondrial content has the highest propensity to emit mitochondrial-derived H_2O_2 (25). Although in the current study and in others (11,12) mitochondrial H_2O_2 emission is increased following a high-fat diet, in theory, this could be exacerbated if mitochondrial biogenesis did not occur, but this remains to be determined. In addition, increasing the sensitivity of mitochondria to ADP, as proposed to occur following the induction of mitochondrial biogenesis (24), would increase the ability of ADP to dissipate protonmotive force and decrease mitochondrial ROS emission rates in vivo. Given the increase in maximal ROS emission rates known to occur following high-fat feeding, the

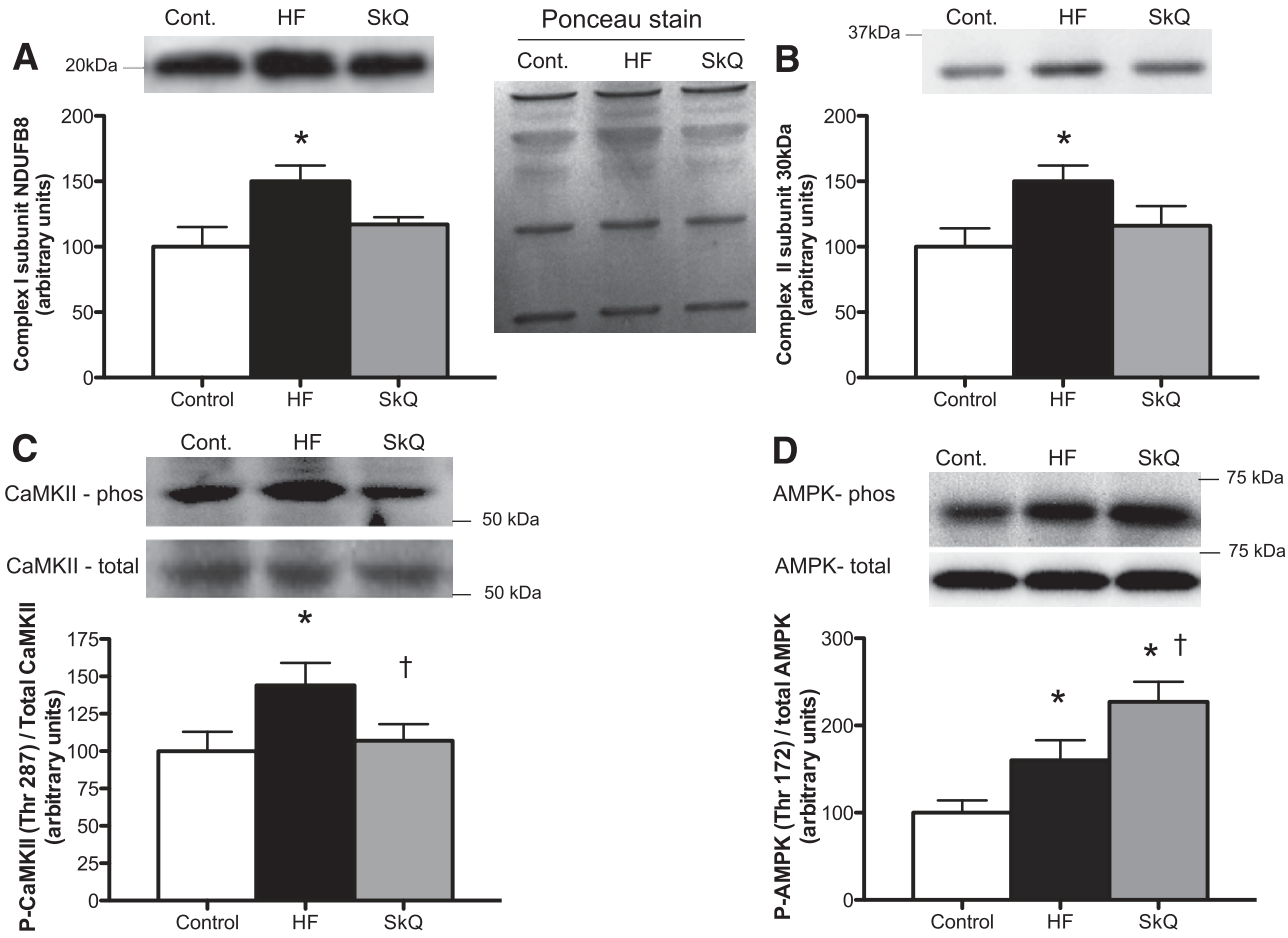

Figure 4—High-fat diet (HF)–induced mitochondrial biogenesis and phosphorylation of CaMKII are prevented by the administration of mitochondrial-targeted antioxidant SkQ. Representative Western blots show increases in complex I (*A*) (band detected at ~20 kDa) and II (*B*) (band detected at ~30 kDa) subunits of the electron transport chain in association with phosphorylation of CaMKII (*C*) (bands detected at ~60 kDa). A representative Ponceau stain of the entire membrane is shown to confirm constant loading. These changes were prevented with the administration of SkQ. In contrast, AMPK (band detected at ~63 kDa) phosphorylation was induced to a greater extent with the administration of SkQ (*D*). Complexes I and II were detected with monoclonal antibodies from MitoSciences, and total and phosphorylated CaMKII and AMPK were detected using antibodies purchased from Cell Signaling. Data are mean ± SEM. $n = 5$. *Significantly different ($P < 0.05$) from control; †significantly different from HF animals.

adaptations in mitochondrial content are not sufficient to regain metabolic homeostasis.

The current study was designed to elucidate the molecular mechanisms responsible for the induction of mitochondrial biogenesis. We believe that this information provides insight into how changes in mitochondrial content can influence cellular homeostasis because most cellular processes are regulated through feedback control. Given the current data showing that mitochondrial ROS emission is primarily responsible for promoting high-fat diet–induced mitochondrial biogenesis, we speculate that an increase in mitochondrial content is an attempt by the cell to attenuate mitochondrial ROS emission or is a tool/signal mediating molecular adaptations aimed at increasing rates of fatty acid oxidation. Activation of CaMKII appears to be tightly affiliated with these observations, suggesting that calcium-mediated signaling events play a primary role in high-fat diet–induced mitochondrial biogenesis.

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