Activation of the AMPK-FOXO3 Pathway Reduces Fatty Acid-induced Increase in Intracellular Reactive Oxygen Species by Upregulating Thioredoxin

Running Title: AMPK-FOXO3 and Thioredoxin Expression

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**Objective:** Oxidative stress induced by free fatty acids contributes to the development of cardiovascular diseases in patients with metabolic syndrome. Reducing oxidative stress may attenuate these pathogenic processes. Activation of AMP-activated protein kinase (AMPK) has been reported to reduce intracellular reactive oxygen species (ROS) levels. The thioredoxin (Trx) system is a major antioxidant system. In this study, we investigated the mechanisms involved in the AMPK-mediated regulation of Trx expression and the reduction of intracellular ROS levels.

**Methods and Results:** We observed that activation of AMPK by 5- aminoimidazole-4-carboxamide ribonucleotide (AICAR) significantly reduced ROS levels induced by palmitic acid (PA) in human aortic endothelial cells. Activation of AMPK increased expression of the antioxidant Trx, which mediated the ROS reduction. RT-PCR showed that AMPK regulated Trx at the transcriptional level. Forkhead transcription factor 3 (FOXO3) was identified as the target transcription factor involved in the upregulation of Trx expression. FOXO3 bound to the Trx promoter, recruited the histone acetylase p300 to the Trx promoter, and formed a transcription activator complex, which was enhanced by AICAR treatment. AMPK activated FOXO3 by promoting its nuclear translocation. We further showed that AICAR injection increased the expression of Trx and decreased ROS production in the aortic wall of ApoE -/- mice fed a high-fat diet.

**Conclusions:** These results suggest that activation of the AMPK-FOXO3 pathway reduces ROS levels by inducing Trx expression. Thus, the AMPK-FOXO3-Trx axis may be an important defense mechanism against excessive ROS production induced by metabolic stress and could be a therapeutic target in treating cardiovascular diseases in metabolic syndrome.
Oxidative stress induced by free fatty acids (FFA) plays a key role in the development of cardiovascular diseases in metabolic syndrome (1). Excessive generation of reactive oxygen species (ROS) can cause cellular injury and dysfunction by directly oxidizing and damaging DNA, proteins, and lipids, as well as by activating several cellular stress-signaling and inflammatory pathways (1). Understanding how ROS production and scavenging are regulated, and developing strategies to reduce ROS production and increase antioxidant availability are important for preventing cardiovascular diseases in metabolic syndrome.

An important signaling pathway involved in ROS regulation is the AMP-activated protein kinase (AMPK) pathway. The AMPK pathway responds to energy depletion by stimulating ATP production, and it plays an important role in controlling energy metabolism. It has been increasingly recognized that activation of this pathway could protect the cardiovascular system (2-4). Reactive oxygen species can activate the AMPK pathway (5-7). Previous studies have shown that activation of the AMPK pathway reduces intracellular ROS levels (7-10). However, the mechanisms involved are not completely understood.

The thioredoxin (Trx) system is a major antioxidant system, which promotes the reduction of proteins by cysteine thiol-disulfide exchange and plays a vital role in maintaining the cellular redox balance (11; 12). Trx, a 12 kDa redox-sensitive molecule, is the key component of the system (11; 12). Trx is ubiquitously expressed and protects the cells from ROS-induced cytotoxicity (13-15). Trx has been shown to have cardiovascular protective effects. Inhibition of endogenous Trx in the heart increases oxidative stress and cardiac hypertrophy (16), while overexpression of Trx (15; 17) or administration of exogenous Trx (18) reduce oxidative stress and protect cardiovascular system.

Given the importance of the Trx in the intracellular antioxidant defense system, we postulate that Trx is a key AMPK target that attenuates excess ROS produced by metabolic stress. Therefore, in the present study, we examined the effect of activating the AMPK pathway on Trx expression and ROS reduction in cells exposed to palmitic acid (PA).

MATERIALS AND METHODS

Cell Culture. Human aortic endothelial cells (HAECs) (Cell Applications, San Diego, California) were cultured in EGM-2 media (Cambrex, East Rutherford, New Jersey), which contained endothelial cell basic media (EBM), 2% fetal bovine serum (FBS), hydrocortisone, fibroblast growth factor 2, vascular endothelial growth factor, insulin growth factor 1, epidermal growth factor, ascorbic acid, GA-1000, and heparin. The cells were transfected with small interfering RNAs (siRNAs), plasmid DNAs, or treated with AICAR or PA at various concentrations and for the time indicated.

Preparation of Fatty Acid-albumin Complexes. Saturated palmitic acid (PA) was used in this study. We prepared lipid-containing media by conjugating PA to bovine serum albumin (BSA) using a modification of the method described previously (19). Briefly, PA was dissolved in ethanol at 200 mM and then combined with 10% FFA-free, low-endotoxin BSA, giving a final concentration of 1 to 5 mM. The pH of all solutions was adjusted to approximately 7.5, and the stock solutions were filter-sterilized and stored at -20°C until used. Control solutions containing ethanol and BSA were prepared similarly. Working solutions were prepared fresh by diluting the stock solution (1:10) in 2% fetal calf serum-EBM.
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All PA media contained 1% BSA; however, the PA-to-BSA ratio varied with the PA concentration.

**siRNA and Plasmid DNA Transfection.** Gene expression was silenced with specific siRNAs, including AMPK siRNA (Santa Cruz Biotechnology, Santa Cruz, California), Trx siRNA (Santa Cruz Biotechnology), and forkhead transcription factor 3 (FOXO3) siRNA (Dharmacon, Chicago, Illinois). Various FOXO3a plasmids (Addgene Inc, Cambridge, Massachusetts), including wild type (HA-FOXO3a WT), constitutively active (HA-FOXO3a TM) and dominant-negative (HA-FOXO3a TM deltaDB) DNAs, were used in this study. Transfection of HAECs or human smooth muscle cells (HSMCs) with siRNAs or plasmid DNAs was carried out with LipofectAMINE™ 2000 (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Transfected cells were then treated with PA and AICAR at the designated concentrations for the indicated amount of time. The efficiency of transfection was confirmed by western blot.

**Quantitative ROS Detection.** Intracellular ROS levels were detected with the oxidant-sensitive fluorogenic probes 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) and dihydroethidium bromide (DHE) (Invitrogen, Carlsbad, California). Treated cells on the coverslip were incubated with 5 µM CM-H_2DCFDA or 2 µM DHE in serum-free medium for 30 minutes at 37°C. The slides were examined under a Leica DMLS epifluorescence microscope (Leica Microsystems, Bannockburn, Illinois), the images were captured with a Leica DC 100 digital camera using identical acquisition settings, and the data was analyzed with Image-Pro Plus V4.5 software (Media Cybernetics, Inc, Bethesda, Maryland). Fluorescence was detected and normalized to cell number. The mean fluorescent intensity was calculated randomly from 5 visual fields per coverslip. Relative ROS levels were compared and expressed as the percentage of the no-treatment controls.

Tissue ROS levels were detected with DHE. Fresh segments of thoracic aorta were frozen in optimal cutting temperature (OCT) compound. Cryosections (6 µm) were equilibrated with Krebs-HEPES buffer (130 mM NaCl, 5.6 mM KCl, 2 mM CaCl_2, 0.24 mM MgCl_2, 11 mM glucose, and 8.3 mM HEPES; pH 7.4) at 37°C for 30 minutes. Cryosections were incubated with 2 µM DHE at 37°C for 30 minutes, and then stained with the nuclear counterstain DAPI (4′,6-diamidino,2-phenylindol) (0.1 µg/mL) at room temperature for 5 minutes. Fluorescence was detected and all images were captured with identical acquisition parameters. Values of red ethidium fluorescence were normalized to blue DAPI fluorescence. The mean fluorescent intensity randomly counted from 3 visual fields per vessel was calculated.

**Immunofluorescent Staining and Immunohistochemistry.** Cells were grown on glass coverslips and treated with PA and AICAR. Treated cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.2% Triton X-100 for 5 minutes. The coverslips were blocked with 1% BSA, incubated with the primary antibody, washed with PBS, incubated with Texas Red-labeled secondary antibody, and then stained with 0.1 µg/mL DAPI at room temperature for 5 minutes. Fluorescence was detected.

For immunohistochemical analysis, formalin-fixed, paraffin-embedded aortic sections were deparaffinized and rehydrated before antigen retrieval in citrate buffer (92-98°C for 12 minutes). Endogenous peroxidase activity was quenched by incubating the slides with 3% hydrogen peroxide for 10 minutes, and nonspecific staining was
reduced by blocking with 5% normal blocking horse serum. The sections were incubated with the primary antibodies at 4°C overnight, and then incubated with second antibody and detected with 3,3-diaminobenzidine (DAB) with the VECTASTAIN ABC kit (Vector Laboratories Inc; Burlingame, California). Nuclei were counterstained with hematoxylin. Slides treated only with normal IgG were used as negative controls. The images were captured and analyzed with Image-Pro Plus V4.5 software (Media Cybernetics, Inc). The signal density was normalized to vascular area. The mean intensity was calculated randomly from 3 visual fields per vessel.

**Western Blot Analysis.** Treated cells were collected and lysed as described previously (20). The NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Rockford, Illinois) was used to separate and prepare nuclear and cytoplasmic proteins from cultured HAECs. Protein samples (15 µg per lane) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were blocked, incubated with primary antibody, washed, and incubated with the secondary HRP-labeled antibody. Bands were visualized with Enhanced Chemiluminescence (Amersham Biosciences, Piscataway, New Jersey). Protein bands, including β-actin, were quantified by densitometry with the Quantity One imaging program (Bio-Rad, Hercules, California). The relative protein levels were normalized to β-actin and expressed as the percentage of the no-treatment control.

**Quantitative RT-PCR.** Total RNA from treated cells was extracted with Trizol (Invitrogen) according to the manufacturer’s protocol. The mRNAs were reverse-transcribed with the iScript cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR (qRT-PCR) was performed with the iCycler iQ RT-PCR detection system (Bio-Rad). Primers were designed with Beacon Designer 2.0 software (Premier Biosoft International, Palo Alto, California). We used the following primers for human Trx: forward 5'-GCTTGGCAAATGTCAAGC-3' and reverse 5'-TTGGCTCCAGAAAATTCACC-3'. mRNA levels were acquired by normalizing the threshold cycle (Ct) of Trx to the Ct of β-actin. The relative levels of mRNA were compared and expressed as the percentage of the no-treatment control.

**Chromatin Immunoprecipitation (ChIP) Assay.** We used the ChIP assay kit (Upstate Biotechnology, Lake Placid, New York) as described previously.(20) The immunoprecipitated DNA and the input DNA were quantified with the qRT-PCR detection system (Bio-Rad). The relative levels of DNA were normalized to the input DNA and expressed as the percentage of the no-treatment control. The PCR products were also separated on a 1.5% agarose gel. The following primers were used for the FOXO binding site in the 5'-flanking region of the human Trx gene: for site 6 forward primer 5'-CCGACTAAACCCTGCTGTGTC-3' and reverse primer 5'-CTCCGGAATTTACCGTGACC-3'.

**Immunoprecipitation.** Immunoprecipitation was conducted as described previously(21). Treated cells were lysed for 60 minutes in ice-cold extraction buffer containing 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 2 mM Na3VO4, 50 mM β-glycerophosphate, and a protease inhibitor mixture (Amersham Biosciences). Cleared cell lysates were incubated with the appropriate antibody precoupled to protein A/G-agarose beads (Santa Cruz Biotechnology) at 4°C overnight. The beads were washed twice with extraction buffer and then twice with extraction buffer containing 0.5 M LiCl. Proteins were eluted either in kinase buffer for the kinase assay or
in SDS sample buffer for Western blot analysis.

**Kinase Assays.** AMPK was precipitated from cell lysates with an anti-AMPK antibody. AMPK-containing beads were incubated with recombinant FOXO3 in kinase assay buffer supplemented with 100 µM ATP for 20 minutes at 30°C. Samples were separated on a 10% SDS-PAGE and transferred to PVDF membranes. Anti-serine and anti-threonine antibodies were used to detect phosphorylated serines and threonines incorporated into FOXO3.

**Trx Activity Assay.** Trx activity was measured with the insulin disulfide reduction assay as described elsewhere(22). Total cellular protein was extracted with lysis buffer (20 mM HEPES pH 7.9, 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 1 mg/ml Protease Inhibitor Cocktail III). Cellular protein extracts were incubated with buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 1 mg/ml BSA, 2 mM DTT) at 37°C for 15 minutes before they were incubated with Trx reductase (Sigma, Saint Louis, Missouri) in the reaction buffer (0.3 mM insulin, 200 µM NADPH, 1 mM EDTA, and 20 mM HEPES pH 7.6) at 37°C for 20 minutes. The reaction was terminated by adding stop mix (6 M guanidine HCl and 1 mM DTNB in 0.2M Tris-HCl, pH 8.0), and the absorption at 412 nm was measured. Relative Trx activities were quantified after normalization with total protein and expressed as the percentage of the no-treatment control.

**Animal Study.** Four-week-old apolipoprotein-E knockout (ApoE -/-) male mice (Jackson Laboratory, Bar Harbor, ME) were fed a high-fat diet (Research Diets Inc, New Brunswick, New Jersey) for 4 weeks and then subcutaneously injected with AICAR (0.5 mg/g body weight /day) or an equivalent volume of normal saline for 2 days. Mice were euthanized 24 hours later. The aortas were irrigated with PBS, collected, and preserved at −80°C until used; alternatively, the aortas were fixed in 4% paraformaldehyde for the immunochemistry assay or OCT compound for ROS detection. All experiments were approved by the Animal Care Research at Baylor College of Medicine.

**Statistical Analysis.** All quantitative variables are presented as the means±SEM from 3 separate experiments. We compared the differences of 3 or more groups with 1-way ANOVA. Two-tailed P<0.05 was considered statistically significant.

**RESULTS**

**AMPK Reduced ROS Levels Induced by PA in Endothelial Cells.** We first tested whether activation of the AMPK pathway could reduce FFA-induced ROS production. HAECs were incubated with increasing amounts of AICAR in the presence or absence of PA; ROS levels were detected in the treated cells. As shown in Figure 1A, AICAR treatment alone had minimal effects on basal ROS levels. Palmitic acid significantly increased intracellular ROS levels, an observation consistent with previous reports(23). The PA-induced increase in intracellular ROS levels was reduced by AICAR in a dose-dependent manner with up to a 60% reduction at the highest dose (500 µM). This result indicates that activation of AMPK can reduce intracellular ROS levels. Additionally, suppression of AMPK by specific siRNAs not only increased basal ROS levels, but also augmented the PA-induced increase in ROS levels (Figure 1B). Furthermore, the AICAR-induced reduction in ROS levels was abolished by AMPK siRNA. These data suggest that the AMPK pathway is capable of reducing intracellular ROS levels under basal conditions and when induced by PA.

**AMPK Increases the Expression of the Antioxidant Trx.** We next investigated whether the AMPK pathway could reduce ROS levels through Trx. We first examined whether the AMPK pathway could regulate
Trx expression. As shown in Figure 2A, activation of the AMPK pathway by AICAR significantly upregulated expression of Trx in the absence and presence of PA. Although PA itself transiently increased Trx expression (data not shown), prolonged PA exposure decreased Trx expression. Importantly, knockdown of AMPKα by its specific siRNA inhibited both basal and AICAR-induced Trx expression (Figure 2B), implicating that the AMPK pathway is involved in upregulating Trx. Consistent with this expression pattern, AICAR significantly increased total cellular Trx activity in the absence and presence of PA (Figure 2C). Taken together, these data suggest that activation of AMPK increases Trx expression.

FOXO3 is Required for the AMPK-induced Upregulation of Trx. We investigated the mechanisms responsible for the AMPK-mediated upregulation of Trx. The 5' flanking region of the human Trx gene contains consensus binding sites for many transcription factors. We identified FOXO3 as one of these transcriptional factors that may mediate AMPK-induced Trx transcription. Silencing FOXO3 with siRNA significantly prevented the AICAR-induced expression of Trx at both the protein (Figure 5A) and mRNA level (Figure 5B), indicating that FOXO3a is involved in the AMPK-induced upregulation of Trx. Furthermore, overexpression of constitutively active FOXO3a (FOXO3a CA) significantly increased Trx expression in the absence or presence of AICAR, but domain negative FOXO3a (FOXO3a DN) dramatically decreased Trx expression (Figure 5C), further suggesting that FOXO3a is capable of upregulating Trx expression. Together, these data support the critical role of FOXO3 in the AMPK-induced upregulation of Trx.

FOXO3 Binds Directly to the Trx promoter, Recruits p300 and Forms a Transcription Activator Complex on the Trx Promoter. To examine whether FOXO3 directly induces Trx transcription, we examined whether FOXO3 binds to the Trx promoter in vivo. FOXO3 binds to the consensus site 5′-(C/G)(A/T)AAA(C/T)A-3′ (24). The promoter region in the Trx gene contains 6 putative FOXO binding sites (tgAAAGAgtga at -1346/-1342, tgAAAGAagga at -1339/-1335, gaAAACAcaga at -1236/-1232, caAAATAccgc at -859/-855, ggAAACActga at -807/-803, and tgAAAGAacag at -613/-609) (Figure 6A). Results of the ChIP assay performed with a FOXO3 antibody showed that FOXO3 strongly bond to site 6 (Figure 6B). Importantly, the binding of FOXO3 to the Trx promoter was significantly increased by AICAR treatment (Figure 6B, P<0.001),
further suggesting that FOXO3 may mediate AMPK-induced Trx transcription.

We examined how the transcription factor FOXO3 binds to the *Trx* promoter and induces gene expression. One possible mechanism is chromatin remodeling in which transcription factors bind to a promoter and recruit histone acetylases (e.g., p300) that acetylate histones, unwind chromatin, and induce gene transcription. To test whether this mechanism applied to the FOXO3-mediated transcription activation of the *Trx* gene, we first examined whether p300 could bind to the *Trx* promoter. By using the ChIP assay, we found that AICAR treatment significantly increased p300 binding to the *Trx* promoter (Figure 6C). We then examined whether FOXO3 was associated with p300 in vivo. By using a communoprecipitation assay, we observed that FOXO3 was associated with p300 and that this association was increased by AICAR treatment (Figure 6D), indicating that p300 recruitment to the *Trx* promoter may be mediated at least in part by FOXO3. Furthermore, the double-ChIP assay (Figure 6E) showed that FOXO and p300 were in the same transcription complex in the *Trx* promoter, and this association was increased by AICAR treatment. Together, these results suggest that activated FOXO3 may recruit p300 and form a transcription activation complex in the *Trx* promoter, a process that can be promoted by the AMPK pathway.

### AMPK Increases FOXO3 Nuclear Translocation.

We investigated the potential mechanisms by which AMPK regulates FOXO3. The immunostaining assay (Figure 7A) and Western blot (Figure 7B) showed that AICAR significantly increased the translocation of FOXO3 from the cytoplasm to the nucleus, which was prevented by AMPKα siRNA. We also examined whether AMPK could directly phosphorylate FOXO3. The *in vitro* kinase assay, with purified AMPK as the kinase and recombinant FOXO3 as the substrate, showed that AMPK directly phosphorylated FOXO3 at serine and threonine sites and that AICAR increased threonine phosphorylation of FOXO3 (Figure 7C). These results indicate that FOXO3 may be phosphorylated by AMPK and subsequently translocate into the nucleus where it binds the *Trx* promoter and increases *Trx* transcription.

### AICAR Increases Trx Expression and Decreases ROS Levels In Vivo.

Finally, we examined whether AMPK activation could affect the expression of Trx and, thus, reduce ROS levels in vivo. We used high-fat diet fed ApoE-/- mice, a model that can produce similar metabolic disturbance, ROS overproduction and vascular changes as seen in metabolic syndrome. These mice were injected with either saline or AICAR (0.5 mg/g body weight/day) for 2 days; ROS levels and Trx expression in the aorta were compared. As shown in Figure 8, the AICAR injection decreased ROS levels (Figure 8A) and increased expression of Trx in the aortic wall (Figure 8B), suggesting that activation of the AMPK pathway may enhance Trx expression and subsequently reduce ROS levels in the vascular wall.

### DISCUSSION

In the present study, we showed that activation of AMPK reduces ROS levels by inducing expression of the antioxidant Trx. The transcriptional factor FOXO3 mediated the induction of transcription. AMPK activates FOXO3 by promoting its nuclear translocation, *Trx* promoter binding, and subsequently transcription complex formation. Taken these findings together, we propose a pathway of the AMPK-mediated reduction of intracellular ROS (Figure 8C).

Fatty acids are fuels that are used to efficiently generate ATP primarily through β-oxidation. However, when fatty acids are present in excessive amounts, along with increased oxidation and energy generation, they produce increased ROS, which
contribute significantly to the pathogenesis of microvascular and macrovascular complications in diabetes (25; 26). Strategies to decrease intracellular ROS levels and oxidative damage may have therapeutic potential in treating diabetes and its complications.

Our finding that activation of the AMPK pathway reduced intracellular ROS levels is consistent with previous reports (7-10). The AMPK pathway acts as a fuel gauge by switching on catabolic pathways for ATP generation when energy is depleted—a process coupled to the increase in ROS production. It has been shown that ROS can activate the AMPK pathway (5-7). The ability of AMPK to reduce ROS levels counterbalances the overproduction of ROS during fatty acid consumption. Reducing fatty acid-induced increases in ROS levels in endothelial cells may be an important mechanism in AMPK-mediated cardiovascular protection. Additionally, AMPK regulates endothelial function (2), angiogenesis (27), and cell cycle regulation (28). Moreover, AMPK also inhibits vascular inflammation (3), prevents endothelial injury induced by hyperglycemia and free fatty acids (4), and reduces myocardial infarction (29). Thus, upregulating this pathway may provide therapeutic benefits by not only reducing lipid storage and insulin resistance, but also preventing cardiovascular complications in metabolic syndrome.

We studied the mechanisms involved in the AMPK-mediated reduction in ROS. Decreasing intravascular ROS levels can be achieved by preventing the generation of or removing excess reactive species. Previous studies suggest that activation of the AMPK pathway normalizes hyperglycemia-induced ROS production by inducing manganese superoxide dismutase (MnSOD) (8; 30). We have shown for the first time that the AMPK pathway can decrease fatty acid-induced increases in intracellular ROS levels by upregulating Trx, a novel additional mechanism that explains AMPK’s effects on reducing intracellular ROS. Trx is ubiquitously expressed in endothelial cells and protects the cells from ROS-induced cytotoxicity (15). Trx can also bind and inhibit apoptosis signal-regulating kinase 1 (ASK1) (32), an upstream kinase in the cellular stress-sensitive pathways (ie, JNK and p38 pathways). Thus, upregulation of the Trx system by the AMPK pathway may be an important protective mechanism against excessive oxidative stress and the activation of stress-signaling pathways in the body.

Regarding how the AMPK pathway induces Trx expression, our study suggests that FOXO3 may be a target transcription factor that mediates AMPK’s effects on Trx expression and ROS reduction. FOXO transcription factors are important regulators of metabolism, cell-cycle progression, apoptosis and oxidative stress resistance. Recent findings suggest that ROS can activate FOXO (33-37). Although FOXOs mediate ROS-induced apoptosis (37; 38) under lethal conditions, they can increase cell survival in response to physiologic oxidative stress (31; 39-42), a function that is required for long-term regenerative potential and cell longevity (41; 43).

The mechanisms whereby FOXO3 reduces ROS levels are not well defined. It has been shown that FOXO3 may be involved in the induction of catalase (44). Our study shows that FOXO3 reduces intracellular ROS levels by directly inducing the antioxidant Trx. When activated by AMPK, FOXO3 directly binds to the Trx promoter and forms a transcriptional complex on the Trx promoter, which may lead to activation of Trx transcription. However, further site-directed mutagenesis studies are needed to determine whether FOXO3 indeed targets the site 6 and induces the transcriptional complex formation in the Trx promoter and whether this site is important for FOXO3 mediated Trx promoter
transactivation. Together, these findings indicate the importance of FOXO3 in reducing ROS levels and protecting cells.

Increasing evidence suggests that AMPK can directly phosphorylate FOXO3, which mediates AMPK’s ability to reduce cell stress and increase cell survival (45; 46). Greer and colleagues have recently shown that AMPK directly phosphorylates at least 6 residues in the C-terminal domain of FOXO3, which activates the FOXO3 transcription factor (45). Our results support their findings. We showed that activation of AMPK by AICAR induced the nuclear translocation of FOXO3 and the binding of FOXO3 to the Trx promoter. Further studies will be necessary to define the detailed mechanisms of FOXO3 regulation by the AMPK pathway in response to metabolic stress.

CONCLUSIONS

In summary, using both in vitro and in vivo experiments we have shown that activation of the AMPK pathway significantly reduced PA-induced intracellular ROS levels by increasing the expression of the antioxidant Trx. The transcriptional factor FOXO3 mediated AMPK’s effect on Trx expression. AMPK upregulated Trx transcription by increasing the nuclear translocation of FOXO3 and by promoting its binding to the Trx promoter. The AMPK-FOXO pathway has protective effects against cellular superoxide levels induced by metabolic stress and could be a therapeutic target when treating cardiovascular diseases in metabolic syndrome.

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Figure 1. Activation of the AMP-activated protein kinase (AMPK) pathway reduced intracellular reactive oxygen species (ROS) levels induced by palmitic acid (PA). A) AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) significantly reduced PA-increased intracellular ROS levels in human aortic endothelial cells (HAECs). The cells were treated with AICAR in the absence or presence of PA for 24 hours and then incubated with CM-H₂DCFDA. Fluorescence was detected and normalized to cell number. The mean fluorescent intensity was calculated randomly from 5 fields per coverslip. Relative ROS levels were compared with the no-treatment control and expressed as the percentage of the control. Shown are representative microscopic scans and the quantitative analysis of fluorescent intensity from 3 independent experiments. Data represent mean±SEM. *P<0.05, **P<0.01, ***P<0.001. B) AMPK was involved in the AICAR-induced reduction in ROS levels. HAECs were transfected with AMPK siRNA, and then treated with AICAR in the absence or presence of PA for 24 hours. The effectiveness of AMPKα knockdown was examined by anti-AMPKα antibody. ROS were detected with CM-H₂DCFDA. Representative staining and the quantitative analysis are shown. Data represent the mean±SEM (n=3). **P<0.01 ***P<0.001. AMPKα siRNA prevented AICAR-induced reduction of ROS.

Figure 2. Activation of the AMP-activated protein kinase (AMPK) pathway upregulated thioredoxin (Trx). A) AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) induced Trx expression in the absence or the presence of palmitic acid (PA). Human aortic endothelial cells (HAECs) were treated with increasing amounts of PA, AICAR, or PA and AICAR for 24 hours. Trx expression was examined by an anti-Trx antibody on a Western blot and was normalized with β-actin. The relative levels of Trx were compared and expressed as the percentage of the control. Representative blots and quantitative analysis from 3 independent experiments are shown. **P<0.01, ***P<0.001 vs no treatment control or as indicated. B) Involvement of the AMPK pathway in basal and AICAR-regulated Trx expression. HAECs were transfected with AMPK siRNAs, and then treated with AICAR in the absence or presence of PA for 24 hours. Trx expression was measured by an anti-Trx antibody on a Western blot and was normalized with β-actin. The relative levels of protein were compared and expressed as the percentage of the control. Representative blots and quantitative analysis from 3 independent experiments are shown. **P<0.01 ***P<0.001 vs the no-treatment control or as indicated. Knockdown of AMPKα by siRNA downregulated basal Trx protein levels and reversed the AICAR-induced induction of Trx. C) AICAR enhanced total Trx activity. HAECs were treated with increasing amounts of PA, AICAR, or PA and AICAR for 24 hours. Relative Trx activity in the cell lysate was assessed, normalized, and expressed as the percentage of the no-treatment controls. Data represent the mean ±SEM (N=3). *P<0.05, **P<0.01, ***P<0.001 vs the no-treatment control or as indicated.

Figure 3. Thioredoxin (Trx) mediated the AMP-activated protein kinase (AMPK)–induced reduction in reactive oxygen species (ROS) levels. Human aortic endothelial cells (HAECs) were transfected with Trx siRNA, and then treated with AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) in the absence or the presence of palmitic acid (PA) for 24 hours. The effectiveness of Trx knockdown was examined by anti-Trx antibody. Intracellular ROS levels were detected with CM-H₂DCFDA. Shown are representative microscopic scans and the quantitative analysis of fluorescent intensity from 3 experiments. Data represent the mean±SEM.
Knockdown of Trx by siRNA increased basal ROS levels, enhanced PA-induced ROS levels, and prevented the AICAR-induced reduction in ROS levels.

**Figure 4. AMP-activated protein kinase (AMPK) induced thioredoxin (Trx) expression at the transcriptional level.** A) AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) induced a significant dose-dependent increase in Trx mRNA. Human aortic endothelial cells (HAECs) were treated with AICAR in the presence of palmitic acid (PA) for 24 hours. Trx mRNA levels were examined by RT-PCR and normalized with β-actin mRNA. The relative levels of mRNA were compared and expressed as the percentage of the control. Data represent the mean±SEM (N=3). *P<0.05, **P<0.01, ***P<0.001 versus PA treatment. B) Involvement of the AMPK pathway in basal and AICAR-regulated Trx expression. HAECs were transfected with AMPK siRNAs, and then treated with AICAR in the absence or the presence of PA for 24 hours. Trx mRNA levels were examined by RT-PCR. Data represent the mean±SEM (N=3). **P<0.01, ***P<0.001. The basal and AICAR-upregulated Trx mRNA was reversed by AMPK siRNA.

**Figure 5. Forkhead transcription factor 3 (FOXO3) mediated the AMP-activated protein kinase (AMPK)–induced upregulation of thioredoxin (Trx).** A) Involvement of FOXO3 in basal and AICAR (5-aminoimidazole-4-carboxamide ribonucleotide)-regulated Trx protein expression. Human aortic endothelial cells (HAECs) were transfected with FOXO3 siRNA, and then treated with AICAR in the absence or the presence of palmitic acid (PA) for 24 hours. The effectiveness of FOXO3 knockdown was examined by anti-FOXO3 antibody. Trx protein was measured by Western blot. Representative data and quantitative analysis from 3 independent experiments are shown. *P<0.05, ***P<0.001. FOXO3 siRNA decreased basal Trx protein levels and reversed the AICAR-mediated induction of Trx protein. B) Involvement of FOXO3 in basal and AICAR-regulated Trx mRNA expression. HAECs were transfected with FOXO3 siRNA, and then treated with AICAR in the absence or the presence of PA for 24 hours. Trx mRNA was examined by RT-PCR. Data represent the mean±SEM (N=3). ***P<0.001. FOXO3 siRNA decreased basal Trx mRNA levels and reversed the AICAR-mediated induction of Trx mRNA. C) Overexpression of FOXO3 increased Trx protein levels. HAECs were transfected with wild type (HA-FOXO3a WT), constitutively active (HA-FOXO3a CA), or dominant-negative (HA-FOXO3a DN) FOXO3a. The transfection effectiveness was determined by anti-HA antibody. The expression of Trx, FOXO3, and β-actin were examined. Representative blots from 3 independent experiments are shown.

**Figure 6. AMP-activated protein kinase (AMPK) promoted forkhead transcription factor 3 (FOXO3) binding to the thioredoxin (Trx) promoter and formation of the FOXO3/p300 transcription complex in the Trx promoter.** A) Depiction of FOXO binding sites in the Trx promoter. B) AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) increased binding of FOXO3 to the Trx promoter. Human aortic endothelial cells (HAECs) were treated with AICAR and palmitic acid (PA) for 24 hours. FOXO3-DNA complexes were cross-linked by formaldehyde and immunoprecipitated with anti-FOXO3 antibody. Bound FOXO3 sites in the Trx promoter were detected by qPCR and normalized with input DNA. Relative DNA was compared and expressed as the percentage of the no-treatment controls. Representative blots and quantitative analysis from 3 independent experiments are shown. Data represent the mean±SEM. *P<0.05, ***P<0.001 versus PA treatment. C) AICAR (5-aminoimidazole-4-
carboxamide ribonucleotide) increased recruitment of p300 to the Trx promoter. Human aortic endothelial cells (HAECs) were treated with AICAR and palmitic acid (PA) for 24 hours. The protein-DNA complex was immunoprecipitated with an anti-p300 antibody. The FOXO binding site in the DNA-protein complex was amplified by PCR. Representative blots and qPCR analysis from 3 independent experiments are shown. Data represent the mean ±SEM (N=3).

**P<0.01***P<0.001. D) AICAR increased FOXO3 and p300 association. The FOXO3 and p300 complex was coimmunoprecipitated with an anti-FOXO3 antibody, and p300 was detected by an anti-p300 antibody. Alternatively, the complex was immunoprecipitated with an anti-p300 antibody, and FOXO was detected by an anti-FOXO3 antibody. Representative blots and quantitative analysis from 3 independent experiments are shown. *P<0.05, **P<0.01, ***P<0.001.

E) AICAR increased binding of the FOXO3 and p300 complex in the Trx promoter. HAECs were treated with AICAR and PA for 24 hours. The protein-DNA complex cross-linked by formaldehyde was first immunoprecipitated with an anti-FOXO3 antibody and then with an anti-p300 antibody. The FOXO site on the Trx promoter was detected by PCR. Representative blots and quantitative RT-PCR analysis from 3 independent experiments are shown. Data represent the mean ±SEM (N=3). ***P<0.001

Figure 7. AMP-activated protein kinase (AMPK) promoted forkhead transcription factor 3 (FOXO3) nuclear translocation. A) Effects of AMPK on FOXO3 cellular location. Human aortic endothelial cells (HAECs) cultured on coverslips were transfected with AMPK siRNA, and then treated with AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) in the absence or presence of PA for 24 hours. Treated cells were stained with an anti-FOXO3 antibody-Texas Red (red) and DAPI (blue). Merged image shows colocalization. Representative images from 3 independent experiments are shown. AICAR increased FOXO3 nuclear translocation, which was prevented by AMPK siRNA. B) HAECs were transfected with AMPK siRNA, and then treated with AICAR in the absence or presence of PA for 24 hours. Nuclear and cytoplasmic proteins were extracted and the levels of FOXO3 were examined with an anti-FOXO3 antibody and normalized with β-actin or lamin A/C. Representative blots and quantitative analysis from 3 independent experiments are shown. Data represent the mean±SEM (N=3). **P<0.01, ***P<0.001. C) AMPK directly phosphorylated FOXO3 in vitro. HAECs were treated with AICAR and PA for 24 hours. AMPK was precipitated from the cell lysate using an anti-AMPK antibody and then incubated with recombinant FOXO3 in kinase assay buffer supplemented with ATP. Samples were separated on SDS-PAGE gel and transferred to PVDF membranes. Anti-serine (Anti-Ser) and anti-threonine (Anti-Thr) antibodies detected phosphorylation incorporated into the FOXO3. AICAR increased FOXO3 phosphorylation. Representative blots and quantitative analysis from 3 independent experiments are shown.

Figure 8. AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) increased thioredoxin (Trx) expression and decreased reactive oxygen species (ROS) levels in apolipoprotein-E knockout (ApoE -/-) mice fed a high-fat diet (HFD). ApoE -/- mice were fed a HFD for 4 weeks and then injected with either 0.9% saline or AICAR (0.5 mg/g body weight /day) for 2 days. A) Superoxide O₂⁻ in the aortas was detected with DHE staining (red), and nuclei were counterstained with DAPI (blue). The relative ROS levels were estimated from the ratio of ethidium/DAPI fluorescence. The mean fluorescent intensity randomly selected from 3 fields per vessel was calculated. Results are means ±SEM (n=5). **P<0.01 versus no AICAR control. B) Trx protein was detected with immunohistochemical staining. The signal density was normalized
with vascular area and expressed as arbitrary units per \( \mu m^2 \). The mean fluorescent intensity randomly selected from 3 fields per vessel was calculated. Representative staining and quantitative analysis from each group are shown (n=5). Results are means ±SEM (n=5). \*P<0.05 versus no AICAR control. C) Schematic diagram of the possible mechanism for the AMP-activated protein kinase (AMPK)-mediated reduction in ROS levels. Activation of the AMPK pathway triggers phosphorylation and nuclear translocation of the transcription factor FOXO3, which can bind to the \( Trx \) promoter and promote Trx transcription. Increased Trx expression leads to a reduction in ROS levels.
Fig. 2

A

B

C

AMPK-FOXO3 and Thioredoxin Expression
Fig. 3

AMPK-FOX3 and Thioredoxin Expression

Trx siRNA (100nM) - + - + - + - + +
AICAR (250uM) - - + + - - + + +
PA (0.3 mM) - - - + + + + +

Extracellular ROS (% of control)

Trx siRNA (100nM) - + - + - + - + +
AICAR (250uM) - - + + - - + + +
PA (0.3 mM) - - - + + + + +

Trx protein levels (% of control)

Trx siRNA (100nM) - +

* *** **

β-actin

Trx
Fig. 4

A

![Graph A: Trx mRNA expression with varying concentrations of AICAR and PA.](image)

B

![Graph B: Trx mRNA expression with AMPKα siRNA, AICAR, and PA.](image)
AMPK-FOXO3 and Thioredoxin Expression

**Fig. 6**

**A**

- **Promoter**
  - -1577
  - 1
  - 23
  - 45
  - ATG
  - UTR

- **Sequence Sites**
  - -1546/-1342
  - -1339/-1335
  - -1236/-1232
  - -859/-855
  - -807/-803
  - -613/-609

- **FOXO Binding Sites**

**B**

- **Input**
- **FOXO 3 Bound**

- **Graph**
  - Trx promoter DNA
  - % of Control:
    - PA (0.3 mM)
    - AICAR (nM)
    - 0
    - +
    - 250
    - 500
  - **Bars**
  - **Legend**
  - **Significance**
AMPK-FOXO3 and Thioredoxin Expression

C

E

D

D
AMPK-FOXO3 and Thioredoxin Expression

**Fig. 7**

A

|                      | DAPI | FOXO3 | Merge |
|----------------------|------|-------|-------|
| AMPKα siRNA (100nM) | -    | +     | -     |
| AICAR (250μM)       | -    | -     | +     |
| PA (0.3 mM)          | -    | -     | +     |

B

|                      | Nuclear | Cytoplasmic |
|----------------------|---------|-------------|
| AMPKα siRNA (100nM) | -       | -           |
| AICAR (250μM)       | -       | +           |
| PA (0.3 mM)          | -       | -           |
Fig. 8

A

DHE

DAPI

HFD

HFD + AICAR

B

IgG

Trx

Trx

Negative Control

HFD

HFD + AICAR

C

AICAR

AMPK ↑

FOXO 3 ↑

Trx ↑

ROS ↓

Tissue ROS level

(DHE fluorescence relative to DAPI)

Trx Staining

(Arbitrary units/mg)

HFD

HFD + AICAR

HFD

HFD + AICAR