AMP-Activated Protein Kinase Activates p38 Mitogen-Activated Protein Kinase by Increasing Recruitment of p38 MAPK to TAB1 in the Ischemic Heart

Ji Li, Edward J. Miller, Jun Ninomiya-Tsuji, Raymond R. Russell III, Lawrence H. Young

Abstract—AMP-activated protein kinase (AMPK) promotes glucose transport, maintains ATP stores, and prevents injury and apoptosis during ischemia. AMPK has several direct molecular targets in the heart but also may interact with other stress-signaling pathways. This study examined the role of AMPK in the activation of the p38 mitogen-activated protein kinase (MAPK). In isolated heart muscles, the AMPK activator 5-aminoimidazole-4-carboxy-amide-1-β-D-ribofuranoside (AICAR) increased p38 MAPK activation. In AMPK-deficient mouse hearts, expressing a kinase-dead (KD) α_2 catalytic subunit, p38 MAPK activation was markedly reduced during low-flow ischemia (2.3- versus 7-fold in wild-type hearts, $P<0.01$) and was similarly reduced during severe no-flow ischemia in KD hearts ($P<0.01$ versus ischemic wild type). Knockout of the p38 MAPK upstream kinase, MAPK kinase 3 (MKK3), did not affect ischemic activation of either AMPK or p38 MAPK in transgenic mkk3$^{-/-}$ mouse hearts. Ischemia increased p38 MAPK recruitment to transforming growth factor-β-activated protein kinase 1–binding protein 1 (TAB1), a scaffold protein that promotes p38 MAPK autophosphorylation. Moreover, TAB1 was associated with the α_2 catalytic subunit of AMPK. p38 MAPK recruitment to TAB1/AMPK complexes required AMPK activation and was reduced in ischemic AMPK-deficient transgenic mouse hearts. The potential role of p38 MAPK in mediating the downstream action of AMPK to promote glucose transport was also assessed. The p38 MAPK inhibitor SB203580 partially inhibited both AICAR- and hypoxia-stimulated glucose uptake and GLUT4 translocation. Activation of p38 MAPK by anisomycin also increased glucose transport in heart muscles. Thus, AMPK has an important role in promoting p38 MAPK activation in the ischemic heart by inducing p38 MAPK autophosphorylation through interaction with the scaffold protein TAB1. (***Circ Res*****. 2005;97:872-879.)**

Key Words: ischemia ■ AMP-activated protein kinase ■ p38 MAPK mitogen-activated protein kinase ■ transforming growth factor-β–activated protein kinase 1–binding protein 1 ■ glucose transport

Energy deprivation associated with hypoxia and ischemia, exercise, and pressure overload leads to the activation of AMP-activated protein kinase (AMPK) in the heart.[1] AMPK, a serine/threonine protein kinase, acts as a fuel sensor responsible for mediating the cellular adaptation to nutritional and environmental stress.[2] AMPK is a heterotrimeric enzyme consisting of catalytic α, bridging β, and regulatory γ subunits. Regulation of AMPK activity is complex, involving allosteric activation by AMP and phosphorylation mediated by one or more upstream AMPK kinases.[3] Phosphorylation of the Thr172 site in the α subunit is essential for AMPK activation.[4]

AMPK has important metabolic actions in heart[5–8] and skeletal muscle.[9–12] Activation of AMPK by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) increases muscle glucose uptake in vivo[11] and in vitro[9–13] by a phosphatidylinositol 3-kinase–independent mechanism. AMPK has also been implicated to have a key role in the stimulation of glucose transport in the ischemic heart.[8,14] Activation of AMPK leads to translocation of GLUT4 glucose transporters to the cell surface.[13,15] However, the events downstream from AMPK that modulate GLUT4 translocation are largely unknown.

The p38 MAPK mitogen-activated protein kinase (MAPK) is an important stress kinase that is involved in inflammation, cell growth and differentiation, cell cycle, and cell death.[16,17] Available evidence from skeletal muscle and cultured cells indicates that p38 MAPK might also be involved in the regulation of glucose transport.[18,19] Muscle contraction, which increases GLUT4-mediated glucose transport, activates p38 MAPK.[19,20] Glucose uptake induced by ischemic preconditioning may also be mediated by p38 MAPK in rat heart.[21] In addition, p38 MAPK activates plasma membrane glucose uptake in 3T3-L1 adipocytes and L6 myotubes.[22,23]

The prototypical model of MAPK activation is a cascade of three kinases, consisting of MAPK, MAPK kinase

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(MAPKK), and MAPKK kinase (MAP3K).[16] However, this upstream kinase cascade is not the sole mechanism responsible for p38 MAPK activation. Recent findings indicate that interaction with the scaffold protein transforming growth factor-β–activated protein kinase 1–binding protein 1 (TAB1) may promote autophosphorylation and activation of p38 MAPK.[16] Ischemia recruits p38 MAPK to TAB1 complexes,[24] but the mechanism responsible remains uncertain.

The purpose of this study was to investigate the potential role of AMPK in p38 MAPK activation during hypoxia and ischemia. The results demonstrate that AMPK plays an important role in the activation of the p38 MAPK pathway in the ischemic heart by promoting the recruitment of p38 MAPK to macromolecular complexes containing AMPK and TAB1.

Materials and Methods

Animals

Animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care. All procedures were approved by the Yale University Animal Care and Use Committee.

Heart Muscle Glucose Uptake

Male Sprague–Dawley rats weighing 250 to 300 g were allowed access to standard chow and water ad libitum; 2-deoxy-D-[1-^{3}H]glucose accumulation in left ventricular papillary muscles was performed as previously described.[15] Muscles were incubated with AICAR (1 mmol/L) or the p38 MAPK activator anisomycin (20 to 60 μg/mL) for variable times in oxygenated buffer or under hypoxic conditions in buffer equilibrated with 100% N_2. The p38 MAPK inhibitor SB203580 (10 μmol/L), extracellular signal–regulated kinase (ERK) inhibitor U0126 (10 μmol/L) or their vehicles were added 30 minutes before activators or hypoxia.

Mouse Heart Perfusions

Transgenic male mice (C57BL/6), expressing a kinase-dead (KD) rat α_2 isoform (K45R mutation) in heart and skeletal muscle,[12] were studied at 4 to 6 months of age. Wild-type (WT) littermates were used as controls. Mouse hearts were retrogradely perfused with Krebs–Henseleit buffer (KHB) containing 7 mmol/L glucose, 0.4 mmol/L oleate, 1% BSA, and a low fasting concentration of insulin (10 μU/mL).[8] Hearts were perfused for 30 minutes at a flow of 4 mL/min, followed by (1) moderate ischemia (0.75 mL/min for 30 minutes), (2) severe ischemia (no-flow for 15 minutes), or (3) continued baseline flow.

In Vivo Regional Ischemia

The mkk3$^{-/-}$ (MKK3 KO) mice[25] and their mkk3$^{+/+}$ WT littermates (kindly provided by Drs R. Flavell and P. Lee, Yale University, New Haven, Conn; Dr R. Davis, University of Massachusetts, Worcester, Mass) were anesthetized with IP ketamine (95 mg/kg) and xylazine (40 mg/kg), intubated, and ventilated with a respirator. After thoracotomy, a suture was placed to ligate the proximal left anterior descending coronary artery for 10 minutes.[3] Control mice underwent sham thoracotomy. Hearts were then rapidly excised, and the ischemic region of the left ventricle was freeze clamped in liquid nitrogen.

AMPK Activity and Phosphorylation

AMPK activity was measured with the synthetic SAMS peptide,[7,8] after immuno-isolation with AMPK α subunit (AMPKα) antibody[12] (kind gift of Dr M. Birnbaum, University of Pennsylvania, Philadelphia, Pa) or TAB1 polyclonal antibody (kind gift from Drs J. Ninomiya-Tsuji, North Carolina State Univesity, Raleigh, NC and J. Han, The Scripps Research Institute, La Jolla, Calif)[16,26] coupled to protein G/A Sepharose. AMPK phosphorylation was assessed by immunoblotting with an antibody to α subunits containing phosphorylated Thr172 (Cell Signaling, Beverly, Mass).[7,8]

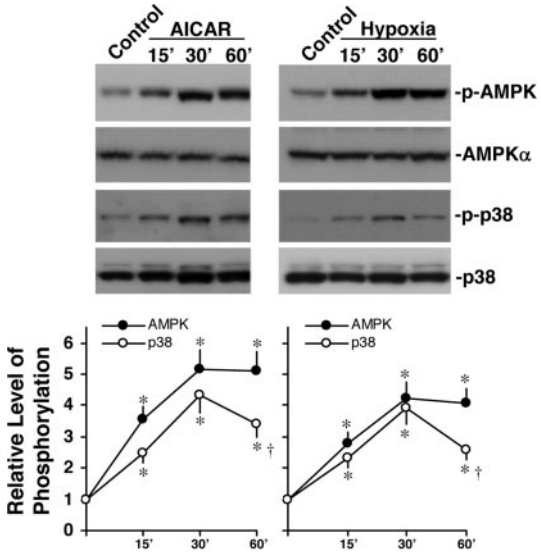

Figure 1. Effect of AICAR and hypoxia on AMPK and p38 MAPK activation. Time course of AMPK and p38 MAPK phosphorylation induced by AICAR (1 mmol/L) or hypoxia (100% N_2) in isolated rat heart papillary muscles. Immunoblots were performed using antibodies for phospho-AMPK (Thr172) (p-AMPK), AMPK α subunits (AMPKα), phospho-p38 (p-p38) MAPK, or total p38 (p38) MAPK. The graphs show phosphorylated AMPK or p38 MAPK relative to the total amount of kinase. Values are means±SE for 4 experiments. *$P<0.01$ vs control, †$P<0.01$ vs 30 minutes.

Immunoblotting

Immunoblots were performed as previously described.[15] Heart homogenate proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. For reprobing, membranes were stripped with 50 mmol/L Tris-HCl, 2% SDS, and 0.1 mol/L β-mercaptoethanol (pH 6.8). Rabbit polyclonal antibodies against phospho-p38 MAPK, total p38 MAPK, phospho-Akt, total Akt, phospho–p44/p42 ERK and total p44/p42 ERK were purchased from Cell Signaling. Rabbit polyclonal antibody against TAB1 was used as previously described.[16,26]

Cell Surface GLUT4 Labeling

After incubations, heart muscles were rinsed in ice-cold glucose-free KHB and incubated at 4°C in KHB containing 200 μmol/L Bio-LC-ATB-BGPA (4,4-O-[2-[2-[2-[2-[2-[6-(biotinylamino)hexanoyl]-amino]ethoxy]exoxy]-ethoxy]-4–1-azi-2,2,2-trifluoroethyl)benzoyl]amino-1,3-propanediyl]bis-D-glucose),[15] a kind gift from Dr G. Holman (University of Bath, Bath, UK). BGPA was cross-linked to cell surface glucose transporters by UV irradiation.[15] Cell surface GLUT4 was determined by GLUT4 immunoblotting after streptavidin-agarose isolation.[15]

Statistical Analysis

Data were expressed as means±SEM. Significance was tested by Student 2-tail t tests with Bonferroni correction or 2-way repeated measures ANOVA with post hoc analysis. A value of $P<0.05$ was considered significant.

Results

AMPK and p38 MAPK Activation by Hypoxia and AICAR

We first examined the kinetics of AMPK activation in rat heart papillary muscles that were exposed to hypoxia or

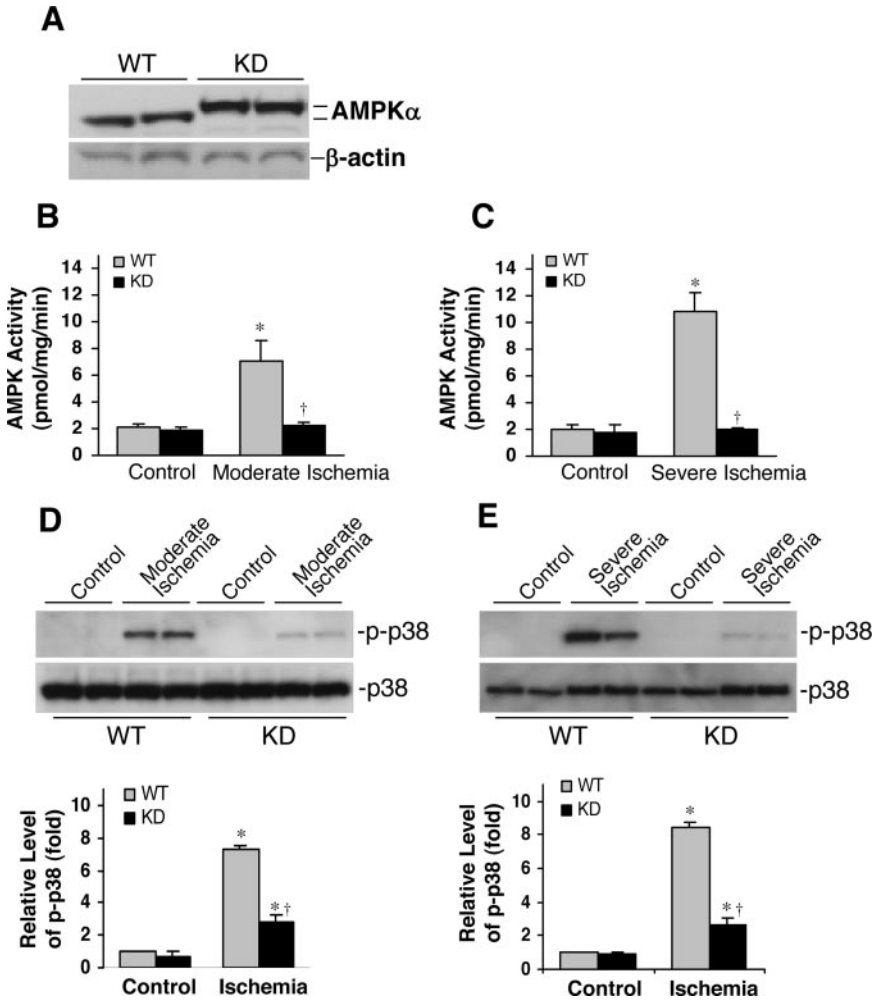

Figure 2. Effect of AMPK deficiency on ischemic heart p38 MAPK activation. AMPKα catalytic subunit immunoblots (A) of WT and AMPK-deficient (KD) mouse heart homogenates. The KD α_2 isoform migrates more slowly than WT because of its c-myc tag, and WT isoforms that are not incorporated into the heterotrimeric AMPK complex are degraded in the KD hearts.[8,12] AMPK activity in WT and KD hearts after control perfusions and after 30 minutes of moderate low-flow ischemia (B) or 15 minutes of severe no-flow ischemia (C). Stimulation of p38 MAPK phosphorylation in WT and KD hearts during moderate (D) or severe (E) ischemia. Representative immunoblots using antibodies to phospho-p38 (p-p38) MAPK or total p38 (p38) MAPK, with quantification of the content of phosphorylated p38 MAPK relative to the total amount of p38 MAPK. All values are means±SE for 5 experiments. *$P<0.01$ vs control, †$P<0.01$ vs WT ischemia.

AICAR. Both treatments stimulated phosphorylation of AMPK and p38 MAPK in an initial parallel and time-dependent manner (Figure 1). The p38 MAPK activation induced by both hypoxia and AICAR decreased after 30 minutes, despite persistent AMPK activation (Figure 1). Neither hypoxia nor AICAR treatment activated ERK or c-Jun N-terminal kinase (data not shown).

Attenuation of p38 MAPK Activation by Ischemia in AMPK-Deficient Hearts

To define the role of AMPK in the activation of p38 MAPK during ischemia, we examined the extent of p38 MAPK activation in isolated perfused AMPK-deficient transgenic mouse hearts during ischemia. As previously reported,[8] the KD α_2 AMPK isoform effectively replaced native heart AMPK α isoforms (Figure 2A). AMPK activity increased 3-fold ($P<0.01$) during low-flow ischemia (Figure 2B) and 5-fold ($P<0.01$) during no-flow ischemia (Figure 2C) in the WT hearts but failed to increase in the KD hearts (Figure 2B and 2C). Interestingly, p38 MAPK phosphorylation increased 7-fold ($P<0.01$) during low-flow ischemia in WT hearts but only 2.3-fold in KD hearts ($P<0.01$ versus ischemic WT) (Figure 2D). Similar results were seen during more severe no-flow ischemia, which activated p38 MAPK 8-fold ($P<0.01$) in the WT hearts but only 2-fold in the KD hearts ($P<0.01$ versus ischemic WT) (Figure 2E).

Association of AMPK With TAB1

To determine the potential mechanisms responsible for AMPK activation of p38 MAPK in the ischemic heart, we first assessed the extent of p38 MAPK activation in ischemic hearts from transgenic mkk3$^{-/-}$ (MKK3 KO) versus mkk3$^{+/+}$ WT controls. MKK3 is a specific upstream kinase of p38 MAPK.[25] Both AMPK (Figure 3A) and p38 MAPK (Figure 3B) were normally activated by in vivo regional ischemia in MKK3 KO hearts, confirming that ischemia does not stimulate p38 MAPK through the MKK3 pathway.[24]

We then examined whether AMPK might interact with TAB1 to increase p38 MAPK activation during ischemia. TAB1 is a scaffold protein that binds components of the transforming growth factor-β–initiated signal transduction cascade and promotes p38 MAPK autophosphorylation.[16] Immunoblots of TAB1 immunoprecipitates demonstrated that TAB1 was associated not only with p38 MAPK (Figure 3C, second panel) but also with AMPK (Figure 3D, first panel). The TAB1 immunoprecipitates contained the α_2 isoform of the catalytic subunit of AMPK (Figure 3D, first panel) but not the lower abundance α_1 isoform (data not shown). Control immunoprecipitates performed with nonimmune rabbit IgG did not reveal any p38 MAPK or AMPK, supporting the specificity of the TAB1 immunoprecipitates (Figure 3C and 3D).

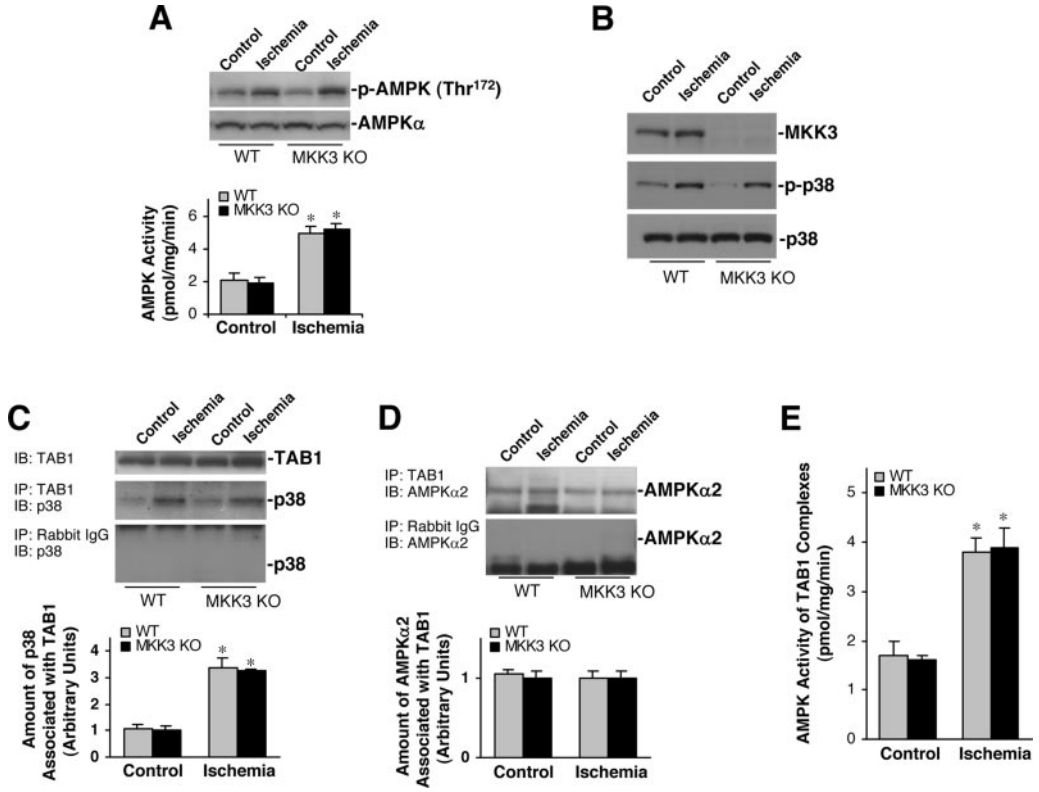

Figure 3. Mechanisms of p38 MAPK activation in the ischemic heart: interaction of AMPK with TAB1. A, AMPK activation in hearts from WT and MKK3 KO mice following 10 minutes of regional ischemia or control sham operation. Top, Representative phospho-AMPK (Thr172) (p-AMPK) or pan-α AMPK (AMPKα) immunoblots. Bottom, AMPK activity measured after immunoprecipitation with AMPKα antibody. Values are means±SE for 5 experiments. *P<0.01 vs control. B, Representative MKK3, phospho-p38 (p-p38) MAPK, or total p38 (p38) MAPK immunoblots of homogenates from control or ischemic WT or MKK3 KO hearts. C, Representative TAB1 immunoblots of homogenates (first panel) or p38 MAPK immunoblots of TAB1 or nonimmune rabbit IgG immunoprecipitates (second and third panels, respectively). The graph quantifies the effect of ischemia on the relative amount of p38 associated with TAB1 in WT and MKK3 KO hearts. Values are means±SE for 3 experiments. *P<0.01 vs control. D, Representative AMPKα_2 immunoblots of TAB1 or nonimmune rabbit IgG immunoprecipitates (first and second panels, respectively). The graph quantifies the relative amount of AMPKα_2 associated with TAB1 in WT and MKK3 KO hearts. Values are means±SE for 3 experiments. E, AMPK activity contained in TAB1/AMPK complexes after immunoprecipitation with TAB1 antibody. Values are means±SE for 3 experiments. *P<0.01 vs control.

Interestingly, ischemia increased the amount of p38 MAPK associated with TAB1/AMPK complexes (Figure 3C, second panel and bar graph; P<0.01 versus control). Ischemia-stimulated recruitment of p38 MAPK to TAB1/AMPK complexes was not altered in MKK3 KO hearts compared with ischemic WT hearts (Figure 3C, second panel and bar graph). However, the amount of p38 MAPK detected in TAB1 immunoprecipitates from ischemic AMPK-deficient KD hearts was markedly reduced compared with ischemic WT hearts (Figure 4A, second panel and bar graph; P<0.01 versus WT ischemia).

The association between AMPK and TAB1 did not appear to depend on the activation of AMPK. There were similar amounts of AMPK in TAB1 immunoprecipitates in control and ischemic hearts (Figures 3D and 4B), as well as in KD and WT hearts (Figure 4B). However, the AMPK activity associated with TAB1/AMPK complexes was greater in ischemic hearts (Figure 3E; P<0.01 versus control). In contrast, there was no AMPK activation in TAB1/AMPK complexes in ischemic KD hearts (Figure 4C; P<0.01 versus WT ischemia).

Taken together, these findings indicate that ischemic activation of AMPK within TAB1/AMPKα_2 complexes may play a critical role in enhancing the recruitment of p38 MAPK to the scaffold protein TAB1, which then promotes p38 MAPK autophosphorylation.

Inhibition of AMPK-stimulated Glucose Transport by p38 MAPK Inhibitor

The AMPK activator AICAR stimulates glucose transport in heart and skeletal muscle,[10,13] and AMPK has an important role in promoting glucose transport during ischemia and hypoxia.[8,12,14] However, the downstream mechanism responsible for increased glucose transport remains uncertain, leading us to examine whether the AMPK-stimulated glucose uptake might be mediated by downstream p38 MAPK activation. AICAR and hypoxia both increased deoxyglucose uptake in isolated rat heart muscles (P<0.01 for each) (Figure 5A). Preincubation with the p38 MAPK inhibitor SB203580 reduced AICAR- and hypoxia-stimulated glucose uptake by 47±5% (P<0.05) and 56±9% (P<0.05), respectively (Figure 5A).

AMPK activation of glucose uptake enhances GLUT4 translocation to the plasma membrane,[13] but there is also evidence that p38 MAPK may activate GLUT4 present on the cell surface, rather than stimulate translocation.[23,27] To distinguish between these possibilities, cell surface GLUT4

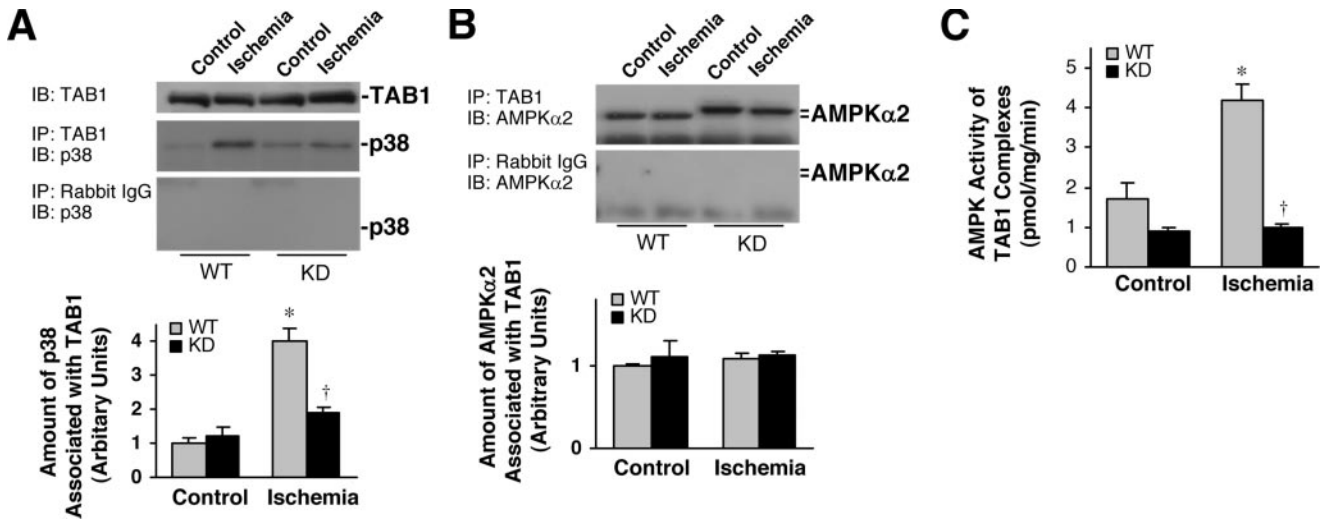

Figure 4. Effect of AMPK deficiency on the recruitment of p38 MAPK to TAB1 in ischemic hearts. The association among TAB1, p38 MAPK, and AMPK in homogenates from isolated WT or KD mouse hearts after 15 minutes of global no-flow ischemia or control perfusions. A, Representative TAB1 immunoblots of homogenates (first panel) and p38 MAPK immunoblots of TAB1 or nonimmune IgG immunoprecipates (second and third panels, respectively). The graph quantifies the effect of ischemia on the relative amount of p38 MAPK associated with TAB1 in WT and KD hearts. Values are means±SE for 3 experiments. *$P<0.01$ vs control, †$P<0.01$ vs WT ischemia. B, Representative AMPKα_2 immunoblots of heart homogenates after immunoprecipitation with TAB1 antibody (first panel) or nonimmune rabbit IgG (second panel). The graph quantifies the relative amount of AMPKα_2 associated with TAB1 in WT and KD hearts. Values are means±SE for 3 experiments. C, AMPK activity associated with TAB1 immunoprecipitate complexes. Values are means±SE for 3 experiments. *$P<0.01$ vs control, †$P<0.01$ vs WT ischemia.

content was determined using the exofacial bis-glucose photolabeling reagent Bio-LC-ATB-BGPA.[15] AICAR and hypoxia treatment increased cell surface GLUT4 content 2.4-fold ($P<0.05$) and 2.5-fold ($P<0.05$), respectively (Figure 5B). SB203580 reduced AICAR- and hypoxia-stimulated cell surface GLUT4 by 46±5% ($P<0.05$) and 41±6% ($P<0.05$), respectively (Figure 5B). Total membrane GLUT4 content was not different between treated muscles and controls (Figure 5B), indicating that GLUT4 translocation was responsible for increased glucose uptake. The plasma membrane marker α_1-Na/K-ATPase was not detectable in the Bio-LC-ATB-BGPA–binding fractions, demonstrating the specificity of proteins cross-linked by this reagent (Figure 5B).

Stimulation of Glucose Transport by the p38 MAPK Activator Anisomycin

To examine whether activation of p38 MAPK stimulated glucose uptake and GLUT4 translocation, heart muscles were incubated with anisomycin, a pharmacological activator of p38 MAPK.[28] Anisomycin activated p38 MAPK in a time- and dose-dependent manner (Figure 6A), without stimulating phosphorylation of AMPK or Akt (Figure 6B), known mediators of GLUT4 translocation.[1] Anisomycin significantly increased deoxyglucose uptake (Figure 6C; $P<0.05$ versus control). SB203580 blocked anisomycin-stimulated p38 MAPK activation (Figure 6D, first panel) and deoxyglucose uptake (Figure 6C). Anisomycin-treatment also increased cell surface GLUT4 content (Figure 6D, second panel and bar graph; $P<0.05$ versus control), and this action was also blocked by SB203580 (Figure 6D, second panel and bar graph; $P<0.05$ versus anisomycin alone). The total membrane GLUT4 content did not differ between treated and control muscles (Figure 6D, third panel), indicating that anisomycin-stimulated GLUT4 translocation.

Discussion

AMPK is emerging as an important signaling pathway in the ischemic heart, stimulating glucose uptake[8,13,14] and glycolysis,[6] and preventing high-energy phosphate depletion, injury, and apoptosis during ischemia reperfusion.[8] AMPK interacts with a number of signaling pathways, including endothelial NO synthase, ribosomal S6 kinase, and mammalian target of rapamycin.[1] This study demonstrates that AMPK plays an important role in the activation of p38 MAPK during hypoxia and ischemia. First, AICAR rapidly stimulated p38 MAPK phosphorylation with a time course that paralleled AMPK phosphorylation. Second, AMPK and p38 MAPK phosphorylation was temporally concordant in hypoxic heart muscles. Third, AMPK deficiency markedly impaired p38 MAPK activation during ischemia in transgenic KD mouse hearts. In addition, the results indicate that AMPK activation of p38 MAPK involves the interaction of AMPK with the scaffold protein TAB1, which promotes p38 MAPK autophosphorylation.[16] An important finding in this study was that the recruitment of p38 MAPK to TAB1/AMPK complexes during ischemia required AMPK activation and was diminished in AMPK-deficient KD hearts. In contrast, p38 MAPK activation was normal in MKK3 knockout hearts, and the AMPK activity of TAB1/AMPK complexes was similar in ischemic mkk3$^{-/-}$ and mkk3$^{+/+}$ hearts. Thus, the interaction of activated AMPK with TAB1 appears to induce the recruitment of p38 MAPK to TAB1 complexes and likely plays an important role in the MKK3-independent activation of p38 MAPK in the ischemic heart.

The MAPKs play a central role in orchestrating both acute and long-term changes in the cell in response to cellular stress.[17] The p38 MAPK is activated by diverse stimuli, including ischemia, exercise, muscle contraction, oxidative

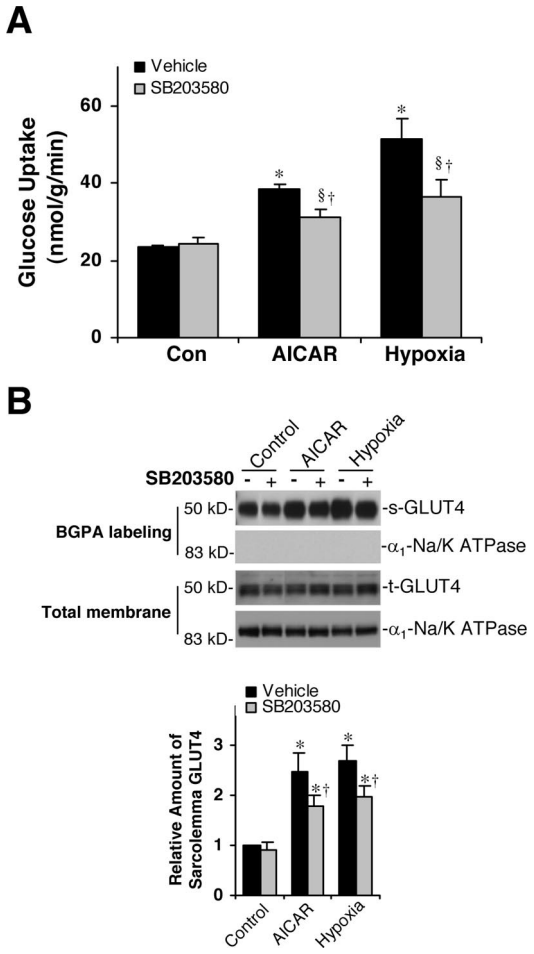

Figure 5. Inhibition of AICAR- and hypoxia-stimulated glucose uptake and GLUT4 translocation by SB203580. Heart muscles were preincubated for 30 minutes with p38 MAPK inhibitor SB203580 (10 μmol/L) before incubation with or without AICAR (1 mmol/L) for 60 minutes or hypoxia (100% N_2) for 60 minutes. A, 2-Deoxy-[1-^{3}H]glucose was added during the last 30 minutes to measure glucose uptake. Values are means±SE of 4 experiments. *P<0.01 vs control, §P<0.05 vs control, †P<0.05 vs AICAR or hypoxia alone. B, Cell surface GLUT4 was labeled with Bio-LC-ATB-BGPA, isolated on streptavidin-agarose, and immunoblotted with specific GLUT4 antibodies. Representative immunoblots showing cell surface (streptavidin isolates) and total membrane GLUT4 (first and third panels) or as a negative control, α_1-Na/K-ATPase (second and fourth panels). The ratios of surface to total GLUT4 are expressed as means±SE for 3 experiments, each including 2 to 3 pooled muscles. *P<0.05 vs control, †P<0.05 vs AICAR or hypoxia alone.

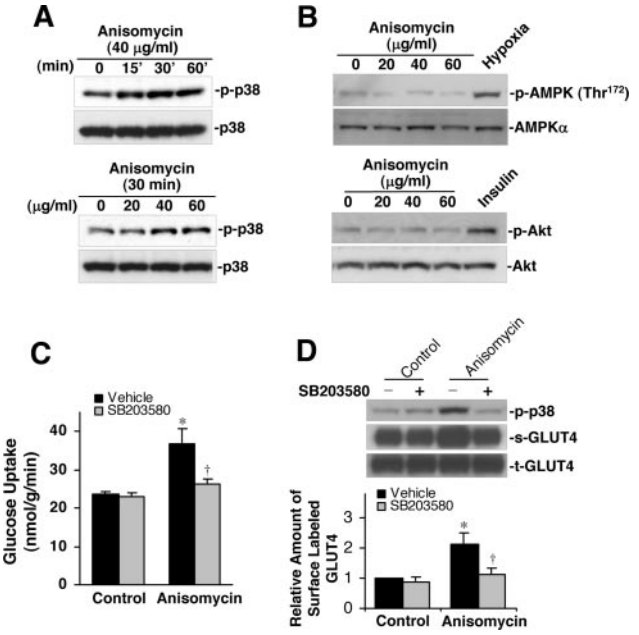

Figure 6. Anisomycin stimulation of p38 MAPK and glucose transport. A, Effects of incubation time and anisomycin dose on p38 MAPK phosphorylation in rat heart muscles. Representative phospho-p38 (p-p38) MAPK and total p38 (p38) MAPK immunoblots. B, Effects of anisomycin (40 μg/mL) on AMPK and Akt phosphorylation in heart muscles. Representative phospho-AMPK (Thr172) (p-AMPK), total AMPK (AMPKα), phospho-Akt (Ser^{473}) (p-Akt), and total Akt (Akt) immunoblots. As positive controls, heart muscles were incubated for 30 minutes either under hypoxic conditions for p-AMPK or with insulin (10 mU/mL) for p-Akt. C, Heart muscles were incubated with or without anisomycin (40 μg/mL) for 30 minutes with or without preincubation with the p38 MAPK inhibitor SB203580 (10 μmol/L) before measuring 2-deoxy-[1-^{3}H]glucose uptake. Values are means±SE for 5 experiments. *P<0.05 vs control, †P<0.05 vs anisomycin alone. D, Anisomycin-stimulated GLUT4 translocation to cell surface membrane. Heart muscles were incubated with anisomycin (40 μg/mL) for 30 minutes with or without preincubation with SB203580 (10 μmol/L). Representative immunoblot of heart homogenates using phospho-p38 antibody (p-p38,first panel). Cell surface GLUT4 was labeled with Bio-LC-ATB-BGPA, isolated on streptavidin-agarose, and immunoblotted with GLUT4. Representative immunoblots showing cell surface GLUT4 (s-GLUT4, second panel) and total membrane GLUT4 (t-GLUT4, third panel). The graph quantifies the relative amount of surface to total GLUT4. Values are means±SE for 3 experiments, each including 2 to 3 pooled muscles. *P<0.05 vs control, †P<0.05 vs anisomycin alone.

stress, heat shock, UV irradiation, and inflammation.[29] Interestingly, many of these stimuli also activate AMPK.[1] The current results using transgenic AMPK-deficient hearts are the first to provide direct evidence that AMPK is an upstream trigger that activates the p38 MAPK pathway. In AMPK-deficient compared with WT hearts, p38 MAPK phosphorylation was decreased by 67% during moderate low-flow ischemia and by 75% during severe no-flow ischemia. The dominant-negative AMPK protein is expressed only in cardiac myocytes; therefore, the residual p38 MAPK activation observed might have occurred in endothelial, smooth muscle, or other nonmyocytic cells within the KD hearts. To the extent that this occurred, these results would have underestimated the role of AMPK in cardiac myocyte p38 MAPK activation during ischemia. The activation of p38 MAPK also decreased after 30 minutes of AICAR and hypoxia treatments, despite persistent AMPK-activation most likely reflecting the upregulation of MAPK phosphatase-1.[30]

These experiments also provide novel insight into the mechanism through which AMPK leads to p38 MAPK pathway activation. The upstream kinases responsible for p38 MAPK activation, MKK3, and MKK6 phosphorylate p38 MAPK on the prototypical Thr180 and Tyr182 sites in the Thr-Gly-Tyr motif within the activation domain of the kinase.[29] Because this sequence is not an AMPK phosphorylation motif, we reasoned that AMPK-induced p38 MAPK phosphorylation must be mediated either indirectly or upstream to MKK3, which is the predominant upstream kinase

in heart muscle.[24] However, we found that p38 MAPK activation was not impaired in MKK3 KO mouse hearts during regional ischemia in vivo, consistent with previous results in isolated perfused globally ischemic hearts.[24] In addition, ischemia does not appear to activate MKK6 in the heart (J.L., unpublished data, 2005).

Autophosphorylation appears to be an important mechanism mediating p38 MAPK activation in the ischemic heart, and recent studies have implicated the scaffold protein TAB1 in this process.[24] Perhaps the most significant findings of this study are that AMPK was present in TAB1 immunoprecipitates and that the ischemia-stimulated recruitment of p38 MAPK to TAB1/AMPK complexes was impaired in AMPK-deficient KD hearts. The α_2 isoform of the AMPK catalytic subunit was present in the TAB1 immunoprecipitates. Whether this finding reflects the greater abundance of α_2 compared with α_1 in the heart or the specific interaction of the α_2 subunit with TAB1 is uncertain.

Interestingly, the amount of AMPK associated with TAB1 was similar in control and ischemic WT hearts, as well as in KD hearts, suggesting that the association of AMPK with TAB1 does not require AMPK activation. However, AMPK activation was found to be critical for the recruitment of p38 MAPK to TAB1 in the ischemic hearts. Taken together, these findings indicate that the ischemic activation of AMPK associated with the scaffold protein TAB1 is responsible for promoting the recruitment of p38 MAPK to the TAB1/AMPK–containing macromolecular complex, which mediates p38 MAPK autophosphorylation. These results expand on emerging evidence that scaffold proteins have an important role in regulating the activation and potential compartmentalization of MAPK.[29]

These experiments also suggest that the p38 MAPK pathway might contribute to AMPK activation of glucose transport in heart muscle. AMPK has an important role in triggering glucose transport in heart[13,14] and skeletal muscle[12] during ischemia and hypoxia. Stimulation of glucose transport is mediated primarily by translocation of GLUT4 transporters from intracellular storage vesicles to their physiologically active sites on the cell surface membranes.[31] The p38 MAPK activator anisomycin increased deoxyglucose uptake and cell surface GLUT4 content, without activating either the AMPK or phosphatidylinositol 3-kinase/Akt pathways known to trigger GLUT4 translocation. These results are consistent with p38 MAPK having a role in glucose transport and GLUT4 translocation in the heart. Prior studies have also implicated p38 MAPK in the stimulation of glucose transport and attributed this action to the intrinsic activation of glucose transporters in the plasma membrane.[22,23,32] We found that the p38 MAPK inhibitor SB203580 reduced AICAR-stimulated glucose uptake and GLUT4 translocation, consistent with results in isolated skeletal muscle,[33] perfused hearts,[21] and cardiomyocytes.[34] Dominant negative p38 MAPK expression also has been reported to inhibit AICAR-stimulated glucose transport in Clone 9 cells, presumably by modulating intrinsic GLUT1 activity.[32] Although these results support the hypothesis that AMPK stimulation of glucose transport may be partially mediated by p38 MAPK, caution is warranted in view of potential confounding features of SB203580. These include inhibition of nucleoside transport and AICAR activation of AMPK in some[33,35] but not all cell types,[32,33] interference with glucose transport,[36] and partial inhibition of upstream AMPK kinase activity (J.L., unpublished results, 2005).

AMPK plays a critical role in preventing ischemic injury in perfused mouse hearts,[8] but the role of p38 MAPK is unclear. There is evidence suggesting that p38 MAPK activation might be cardioprotective in ischemic preconditioning.[21,37–39] However, other reports suggest that p38 MAPK might play a role in promoting cardiomyocyte apoptosis after ischemia.[40] Whether activation of the p38 MAPK is beneficial or detrimental is known to depend on the specific stimulus and cell type.[41] Our results do not address whether p38 MAPK, as a downstream effector of AMPK, contributes to the cardioprotective effect of AMPK. However, to the extent that the p38 MAPK pathway might partially mediate glucose transport during ischemia, this aspect of p38 MAPK action could contribute to AMPK preventing ischemic injury.

We used both isolated rat heart muscles and transgenic mouse hearts to address the interaction between AMPK and p38 MAPK. The pharmacological activator AICAR reproducibly activates AMPK in isolated rat heart muscles[15] but is less effective in perfused hearts, leading us to select rat muscles for AICAR experiments. The transgenic mouse model enabled us to address the role of AMPK in p38 MAPK activation and the recruitment of p38 MAPK to TAB1 during various degrees of in vitro global ischemia and in vivo regional ischemia and proved important to expand on the results of the initial AICAR experiments.

Thus, the findings of this study indicate that the ischemic activation of AMPK within TAB1/AMPK complexes leads to the recruitment of p38 MAPK to a TAB1 macromolecular complex, where it undergoes autophosphorylation. The molecular mechanism through which AMPK interacts with TAB1 to recruit p38 MAPK to the macromolecular complex is of great interest and remains to be elucidated by future research.

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