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The Anti-diabetic Drugs Rosiglitazone and Metformin Stimulate AMP-activated Protein Kinase through Distinct Signaling Pathways*

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Lee G. D. Fryer, Asha Parbu-Patel, and David Carling‡

From the Cellular Stress Group, Medical Research Council Clinical Sciences Centre, Hammersmith Hospital, DuCane Road, London W12 0NN, United Kingdom

AMP-activated protein kinase (AMPK) is activated within the cell in response to multiple stresses that increase the intracellular AMP:ATP ratio. Here we show that incubation of muscle cells with the thiazolidinedione, rosiglitazone, leads to a dramatic increase in this ratio with the concomitant activation of AMPK. This finding raises the possibility that a number of the beneficial effects of the thiazolidinediones could be mediated via activation of AMPK. Furthermore, we show that in addition to the classical activation pathway, AMPK can also be stimulated without changing the levels of adenine nucleotides. In muscle cells, both hyperosmotic stress and the anti-diabetic agent, metformin, activate AMPK in the absence of any increase in the AMP:ATP ratio. However, although activation is no longer dependent on this ratio, it still involves increased phosphorylation of threonine 172 within the catalytic (α) subunit. AMPK stimulation in response to hyperosmotic stress does not appear to involve phosphatidylinositol 3-phosphate kinase, protein kinase C, mitogen-activated protein (MAP) kinase kinase, or p38 MAP kinase α or β. Our results demonstrate that AMPK can be activated by at least two distinct signaling mechanisms and suggest that it may play a wider role in the cellular stress response than was previously understood.

The AMP-activated protein kinase (AMPK)[1] plays a key role in the regulation of metabolism within the muscle cell and has been implicated as a potential target in type 2 diabetes mellitus and in obesity (1–3). AMPK is a heterotrimeric complex consisting of a catalytic (α) subunit and two regulatory subunits (β and γ) (4). Isoforms of all three subunits have been identified, including two isoforms of the catalytic subunit, α1 and α2 (5). Previous studies have shown that AMPK is activated following depletion of cellular ATP together with a concomitant rise in AMP (6, 7). An increase in the AMP:ATP ratio causes increased phosphorylation of AMPK on threonine residue 172 within the α subunit by an as yet poorly characterized upstream kinase (8). In response to activation, AMPK switches off ATP-utilizing pathways and switches on ATP-producing pathways. These combined actions have led to the proposal that AMPK acts as a cellular fuel gauge (9, 10). A number of physiological and pathophysiological stimuli that lead to an increase in the AMP:ATP ratio within the cell have been demonstrated to activate AMPK, including muscle contraction, heat shock, metabolic poisoning, and ischemia (6, 11–13). Although activation of AMPK appears to be a direct consequence of an increase in the AMP:ATP ratio, it is not clear whether there are other signals, which do not involve changes in adenine nucleotide levels, that can lead to activation of AMPK.

Recently, Zhou *et al.* (14) demonstrated the activation of AMPK by metformin in both hepatocytes and skeletal muscle. Metformin, one of the most widely used oral drugs for the treatment of type 2 diabetes, decreases hyperglycemia and has beneficial effects on circulating lipids, without affecting insulin secretion (15, 16). The glucose lowering effects of metformin are attributable to both an increase in muscle glucose uptake (17) and a decrease in hepatic glucose production (16, 18). Activation of AMPK by metformin was found to be required for the decrease in glucose production and the increase in fatty acid oxidation in hepatocytes and for the increase in glucose uptake in skeletal muscle (14). In addition, we have recently shown that the stimulation of fatty acid oxidation in skeletal muscle by leptin occurs following a biphasic activation of AMPK (3). For both metformin and leptin, it was not clear whether the mechanism leading to activation of AMPK involved a significant decrease in ATP levels (3, 14). In this study we report that in muscle cells AMPK can be activated by two distinct pathways: one that involves changes in the AMP:ATP ratio and one that is independent of this ratio. Furthermore, we report the novel finding that AMPK is activated acutely by the thiazolidinedione, rosiglitazone via the AMP:ATP-dependent pathway. These results will aid further work on the potential benefit of therapeutic agents aimed at targeting AMPK in diseases such as type 2 diabetes and obesity.

EXPERIMENTAL PROCEDURES

Cell Culture—H-2K^b cells were derived from skeletal muscle of heterozygous H-2K^b tsA58 transgenic mice (19). Myoblasts were maintained under permissive conditions in Dulbecco's modified medium containing heat-inactivated fetal calf serum (20% (v/v)), chick embryo extract (2% (v/v)), L-glutamine (2% (v/v)), and penicillin/streptomycin (1% (w/v)) at 33 °C in the presence of interferon-γ. Differentiation into myotubes was induced following a switch to non-permissive growth conditions by removal of interferon-γ and incubation at 37 °C (20). For all experiments, cells were differentiated for 4 days, transferred into media containing 0.5% (v/v) fetal calf serum, and incubated overnight before use.

AMPK Activity in H-2K^b Cells—AMPK α1 and α2 activity was measured in H-2K^b cell lysates as described previously (11). Briefly, cells were incubated for 2–180 min at 37 °C in Hepes-buffered saline (20 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$)

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‡ To whom correspondence should be addressed. Tel.: 44-208-383-4313; Fax: 44-208-383-8306; E-mail: dcarling@csc.mrc.ac.uk.

[1] The abbreviations used are: AMPK, AMP-activated protein kinase; AICA, 5-amino-4-imidazolecarboxamide; AICAR, AICA riboside; DNP, dinitrophenol; MAP, mitogen-activated protein; SAMS, the synthetic peptide corresponding to the amino acid sequence HMRSAMSGLHLVKRR.

containing 5 mM glucose in the presence or absence of 200 μM rosiglitazone, 2 mM metformin, 0.5 mM 5-amino-4-imidazolecarboxamide ribonucleoside (AICA riboside), 0.5 mM dinitrophenol (DNP), or 600 mM sorbitol, as described in the figure legends. Following this incubation, cells were rinsed in phosphate-buffered saline (5 mM NaH_2PO_4, pH 7.4, 150 mM NaCl) and lysed by addition of 0.25 ml of 50 mM Tris/HCl, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM dithiothreitol, 10% (v/v) glycerol, 1% (v/v) Triton X-100. Insoluble material was removed by centrifugation at 10,000 × *g* for 5 min, the supernatant removed, and protein concentration determined using the Bradford reagent. AMPK α1-containing complexes were immunoprecipitated from between 100 and 200 μg of protein by incubation with an anti-α1 antibody prebound to protein G-Sepharose for 2 h at 4 °C. AMPK α2-containing complexes were recovered from the supernatant of this incubation by immunoprecipitation with an anti-α2 antibody prebound to protein G-Sepharose. For total AMPK activity, lysates were immunoprecipitated using a pan-β antibody prebound to protein A-Sepharose. AMPK activity present in the immune complexes was measured by the SAMS peptide assay (21).

Western Blot Analysis—Equal amounts of H-2K^b cell lysate protein (50 μg), prepared as described above, were separated by SDS-PAGE on 6% (w/v) (for acetyl-CoA carboxylase) or 10% (w/v) (for AMPK) polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were incubated in 10 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 0.5% Tween 20, 5% (w/v) low fat milk powder for 1 h at room temperature. Membranes were probed with either an anti-AMPK phosphothreonine 172-specific antibody (Cell Signaling Technology) or an anti-phospho-acetyl-CoA carboxylase-specific antibody (Upstate Biotechnology) in this buffer at 4 °C overnight and then washed extensively with 10 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 0.5% Tween 20. The blots were incubated for 1 h at room temperature with donkey anti-rabbit IgG secondary antibody, followed by extensive washing. Blots were developed using enhanced chemiluminescence (Roche Molecular Biochemicals) and visualized using a 16-bit charge-coupled device (CCD) cooled camera (Gene Gnome; Syngene, Cambridge, UK). Quantification was carried out using GeneTools software (Syngene).

Nucleotide Extraction and Measurement—Following treatment, H-2K^b cells were washed rapidly in phosphate-buffered saline before addition of 0.25 ml of perchloric acid (5% (w/v)). Acid-insoluble material was removed by centrifugation at 10,000 × *g* for 2 min. Perchloric acid was extracted from the supernatant by three washes with 10% excess (by volume) of a 1:1 mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane. Nucleotides were separated by ion-exchange chromatography on a Mono Q PC1.6/5 column run on a SMART System (Amersham Biosciences). The column was equilibrated in 10 mM potassium phosphate buffer, pH 8, and developed with a linear gradient from 10 mM potassium phosphate to 50 mM potassium phosphate, pH 8, containing 0.2 M NaCl over 5 ml at a flow rate of 0.2 ml/min. Nucleotides were detected by their absorbance at 254 nm and compared with the elution position of standards. Areas under the AMP and ATP peaks were quantified by integration using SMART System software and used to calculate the AMP:ATP ratios.

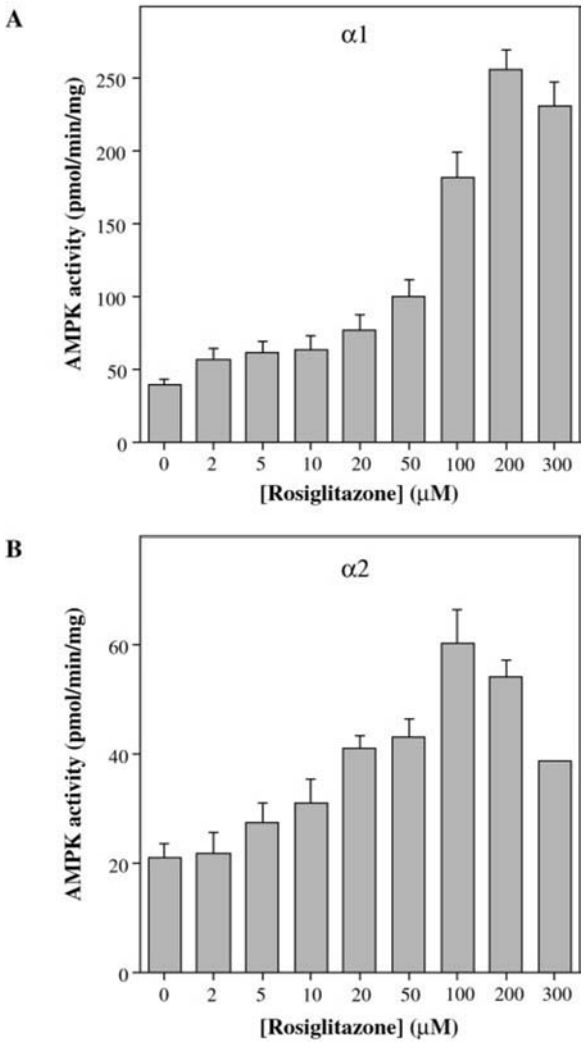

FIG. 1. **Activation of AMPK by rosiglitazone in H-2K^b muscle cells.** AMPK activity in anti-α1 (*A*) and anti-α2 (*B*) immune complexes isolated from H-2K^b cells incubated for 30 min with increasing concentrations of rosiglitazone was measured using the SAMS peptide assay. Results shown are the mean values (±S.E.) of three to five independent experiments and are plotted as pmol of phosphate incorporated into peptide per min per mg of total protein.

RESULTS

We have previously shown that AMPK is activated in H-2K^b muscle cells in response to a number of treatments (11), and recent results have identified other activators of AMPK in muscle, including metformin (14) and leptin (3). Here we show that incubation of H-2K^b muscle cells with the thiazolidinedione, rosiglitazone, leads to a marked activation of AMPK. Fig. 1 shows the effect of varying concentrations of rosiglitazone on the activity of α1- and α2-containing AMPK complexes in H-2K^b muscle cells. Activation was detected at concentrations above 5 μM, with maximal activation occurring at a concentration between 100 and 200 μM rosiglitazone following a 30-min incubation. α1-Containing complexes showed a greater stimulation by rosiglitazone (6-fold relative to control) compared with α2-containing complexes (2.5-fold). However, the activation of α2-complexes appeared more sensitive to rosiglitazone, with a maximal stimulation at 100 μM compared with 200 μM for the α1-complexes, and the concentration for half-maximal activation of AMPK was lower for α2-complexes (Fig. 1). Stimulation of AMPK by 200 μM rosiglitazone occurred rapidly, peaking after about 30 min (Fig. 2, *A* and *B*). Fig. 2*C* shows that activation of AMPK is mirrored closely by an increase in the phosphorylation state of threonine 172 within the α subunit, detected using an antibody that specifically recognizes the phosphorylated form of this residue (22). Previous studies have shown that threonine 172 is the principle activating phosphorylation site within AMPK (8). We also showed that phosphorylation of acetyl-CoA carboxylase was increased in parallel with AMPK activity and phosphorylation (Fig. 2*C*). These findings demonstrate that activation of AMPK by rosiglitazone leads directly to effects on downstream targets.

In addition to a number of cellular stresses that inhibit ATP production (10), AMPK has recently been shown to be activated in response to metformin (14). The finding that rosiglitazone also activates AMPK prompted us to address the question of whether stimulation of AMPK in muscle occurs through the same pathway in response to different stimuli. We began by investigating the possibility of additive effects on AMPK activation in response to different treatments. As we have previously reported, both α1- and α2-containing complexes are activated following incubation of H-2K^b muscle cells with AICA riboside, in response to hyperosmotic stress in the presence of 600 mM sorbitol and following incubation with the mitochon-

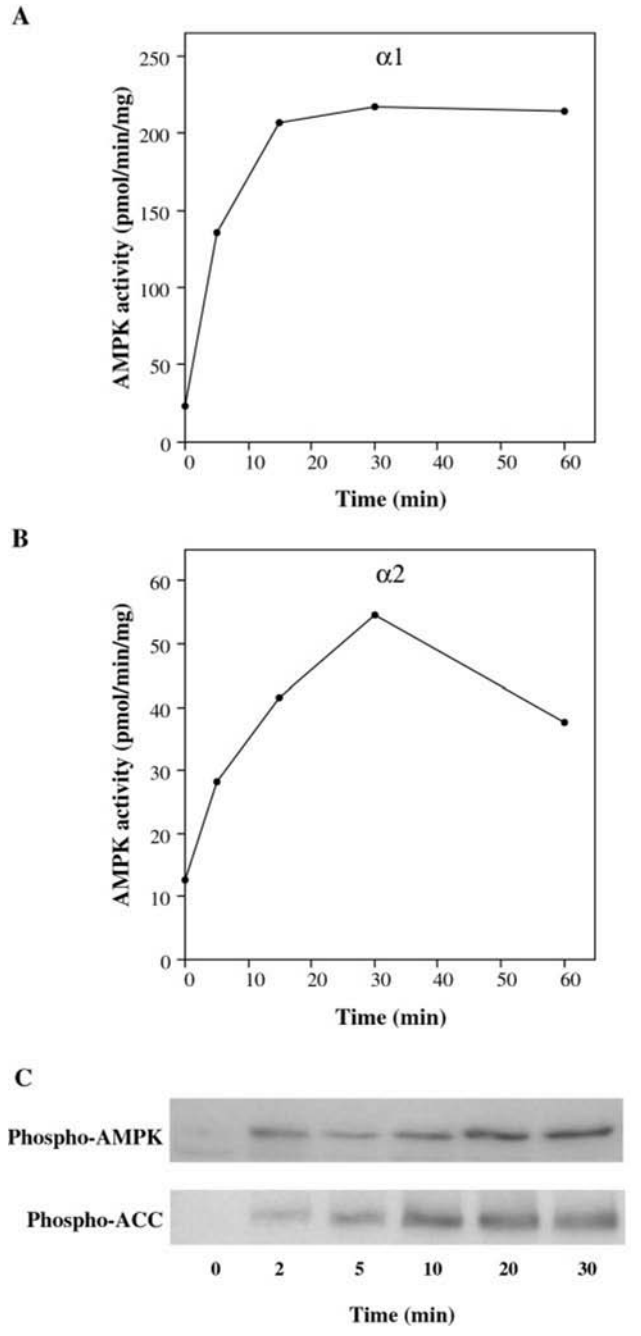

FIG. 2. **Time course of activation of AMPK and phosphorylation of AMPK and acetyl-CoA carboxylase in response to rosiglitazone.** H-2K^b cells were treated with 200 μM rosiglitazone for varying times, and AMPK activity in anti-α1 (*A*) and anti-α2 (*B*) immune complexes was determined. Results shown are the average values from two independent experiments, which varied by less than 15%. *C*, protein samples of H-2K^b cell lysates treated with 200 μM rosiglitazone for the times indicated were analyzed by SDS-PAGE and blotted with antibodies specific for either phosphothreonine 172 within the AMPK α subunit (*upper panel*) or phospho-acetyl-CoA carboxylase (*ACC*, *lower panel*).

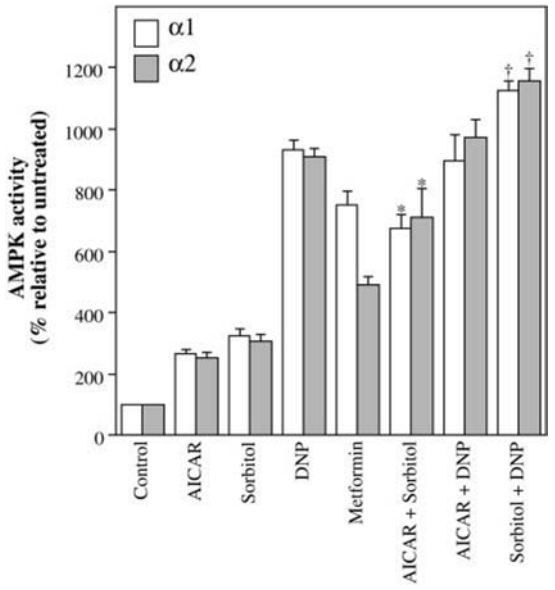

FIG. 3. **Activation of AMPK by different stimuli.** H-2K^b cells were incubated in the presence or absence of either 0.5 mM AICA riboside (*AICAR*), 600 mM sorbitol, 0.5 mM DNP, 0.5 mM AICAR plus 600 mM sorbitol, 0.5 mM AICAR plus 0.5 mM DNP, or 600 mM sorbitol plus 0.5 mM DNP for 30 min. Cells were incubated with 2 mM metformin for 180 min. Following incubation, AMPK activity in α1- (*open bars*) and α2- (*shaded bars*) immune complexes was measured using the SAMS peptide assay. Results are the mean of three to seven independent experiments and are expressed as a percentage of the activity in the absence of any additions (control value). Significant differences in AMPK activity between the effect of AICAR or sorbitol alone, or in combination, are denoted by * ($p < 0.05$). Similarly, significant differences in AMPK activity between the effect of DNP or sorbitol alone, or in combination are denoted by † ($p < 0.05$).

drial uncoupling agent, DNP (Fig. 3). Maximal activation of both α1- and α2-containing complexes by DNP was achieved at a concentration of 0.5 mM. Increasing the concentration above 0.5 mM did not result in any further increase in AMPK activity (data not shown). Incubation of cells in the presence of AICA riboside and 0.5 mM DNP did not increase AMPK activity relative to cells incubated with DNP alone (Fig. 3). In contrast, treatment of cells with 0.5 mM DNP and 600 mM sorbitol resulted in a small, but consistent, and statistically significant, increase in the activity of both α1- and α2-containing complexes above the maximal stimulation seen with DNP alone (Fig. 3). Hyperosmotic stress had a large additive effect on the stimulation of AMPK by AICA riboside (Fig. 3). AICA riboside is converted within the cell to the monophosphorylated form, ZMP, which in some cells can accumulate to high levels and mimic the actions of AMP on AMPK (23, 24). The additive effects of hyperosmotic stress and DNP or AICA riboside suggest that alternate mechanisms of activating AMPK exist. Conversely, the finding that there is no additive effect on AMPK with AICA riboside and DNP treatment is consistent with them both acting through a similar mechanism. In agreement with a recent report (14) we found that incubation of muscle cells with 2 mM metformin led to a significant increase in the activity of both α1- and α2-containing complexes. Consistent with the previous study, we also found that in muscle cells metformin had a greater effect on α1-containing complexes (7.5-fold activation) than α2-complexes (4.9-fold activation).

The results above suggested to us that alternate pathways for activation of AMPK exist. To explore further this possibility we determined the levels of adenine nucleotides in cells treated with different stimuli that activate AMPK. As can be seen from the ion-exchange chromatograms shown in Fig. 4, incubation with DNP (0.5 mM) causes a marked increase in the level of AMP compared with untreated control cells. A similar increase in AMP is seen following incubation of H2-K^b cells with rosiglitazone (200 μM). In contrast to these treatments, hyperosmotic stress and metformin do not lead to a detectable change in nucleotide levels compared with untreated control cells (Fig. 4). The intracellular AMP:ATP ratios following different treatments were determined by integration of the AMP and ATP peaks and are listed in Table I. To exclude the possibility that the effects of hyperosmotic stress and metformin on AMPK

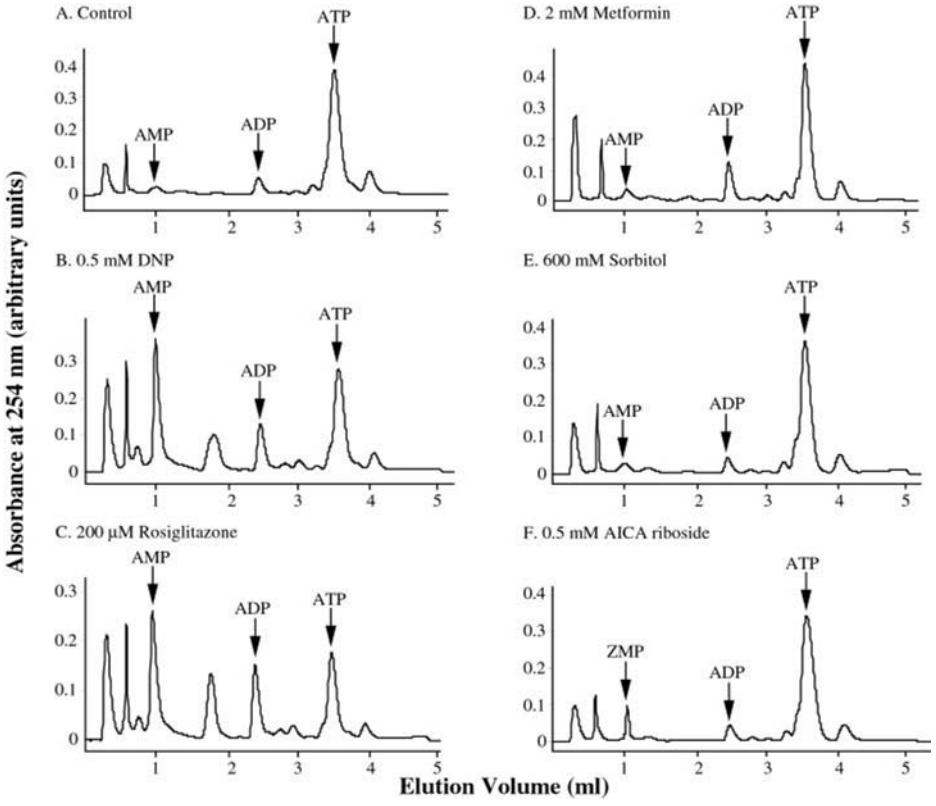

FIG. 4. **Analysis of adenine nucleotide levels in H-2K^b cells.** The effect of different treatments (as described previously) on cellular nucleotides was determined by ion-exchange chromatography. In each case, a representative trace is shown. The position at which AMP, ADP, and ATP standards elute are indicated on each trace by *arrows*. In *F*, the elution position of ZMP, rather than AMP, is shown.

TABLE I
AMP:ATP ratios in H-2K^b cells following various treatments

Adenine nucleotides from perchloric acid extracts of H-2K^b cells were analyzed by ion-exchange chromatography. The areas under the AMP and ATP peaks were integrated and used to calculate the AMP:ATP ratio. In each case the values are the means ± S.E. from three to four independent measurements. It was not possible to calculate the value for cells treated with AICA riboside since the ZMP peak masked the AMP peak.

Treatment	AMP:ATP ratio
Control	0.05 ± 0.01
200 μM rosiglitazone	1.10 ± 0.16
0.5 mM dinitrophenol	1.19 ± 0.29
600 mM sorbitol	0.05 ± 0.01
2 mM metformin	0.08 ± 0.01
0.5 mM AICA riboside	Not determined

activation were due to a very small increase in the AMP:ATP ratio, we determined the concentration of DNP required to produce an equivalent increase in AMPK activity to these treatments and measured the AMP:ATP ratio under these conditions. At the concentration of DNP (0.0375 mM) at which AMPK activity is stimulated to the same extent as following hyperosmotic stress (3-fold), the AMP:ATP ratio more than doubled (0.05 to 0.13). Similarly, at the concentration of DNP (0.2 mM) required to activate AMPK to the equivalent level as produced by metformin, the AMP:ATP ratio (0.29) was almost 6-fold that of the control value. Consistent with previous studies in muscle cells (11), incubation with AICA riboside resulted in the appearance of a ZMP peak. Since ZMP elutes from the column at almost the same position as AMP in our system, we were unable to calculate the AMP:ATP ratio following incubation with AICA riboside. However, as can be seen from the chromatogram (Fig. 4), AICA riboside treatment does not cause a detectable change in the levels of ATP and ADP compared with control cells.

Phosphorylation of threonine 172 within the α subunit of AMPK was markedly increased following treatment with DNP (Fig. 5*A*) and rosiglitazone (Fig. 2*C*), both of which increase the AMP:ATP ratio. In addition, hyperosmotic stress and metformin, which do not affect nucleotide levels, also increased threonine 172 phosphorylation, indicating that both the nucleotide-dependent and -independent mechanisms of activating AMPK involve phosphorylation at this site. Hyperosmotic stress in addition to DNP caused a slight increase in phosphorylation compared with the individual treatments, but direct quantification of the chemiluminescence did not reveal any statistical significance for this observation (Fig. 5*B*). Since the effects of hyperosmotic stress and DNP on AMPK activity appear to be additive, these findings suggest that additional phosphorylation sites may be involved in the activation of AMPK.

While the mechanisms leading to activation of AMPK following an increase in the AMP:ATP ratio are reasonably well understood (7, 25, 26), the mechanisms that operate to activate AMPK in response to hyperosmotic stress or metformin, treatments that do not alter the intracellular levels of adenine nucleotides, are unknown. Whereas the effects of metformin on intracellular signaling pathways have not been studied extensively, hyperosmotic stress has been shown previously to activate a number of components of signaling pathways, including phosphatidylinositol 3-kinase (27), protein kinase C (28), mitogen-activated protein (MAP) kinase, and MAP kinase kinase (reviewed in Ref. 29). We therefore tested the effect of a number of inhibitors of these pathways on the activation of AMPK by hyperosmotic stress. Wortmannin was used to block signaling through phosphatidylinositol 3-kinase, PD 98059 to block the MAP kinase pathway, SB 202190 to inhibit the p38 α and β MAP kinase isoforms and the bisindoylmaleimide, Ro318220, to inhibit protein kinase C activity (30). As can be seen in Fig. 6, none of the inhibitors used had any significant effect on AMPK activity, suggesting that activation in response to hyperosmotic stress does not signal through any of these established pathways. Further studies will be necessary to elucidate

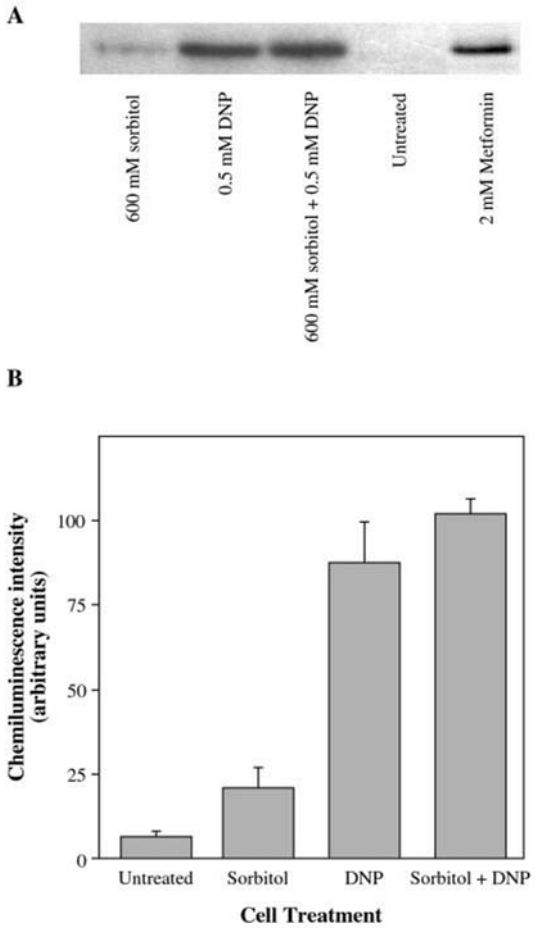

FIG. 5. **Phosphorylation of AMPK on threonine 172.** Following various treatments, H-2K^b cell lysates were resolved by SDS-PAGE and blotted with antibodies specific for phosphothreonine 172 within the AMPK α subunit. A representative blot is shown in *A*. The signal intensities following treatment with 600 mM sorbitol, 0.5 mM DNP, or a combination of the two, from three to four individual experiments were quantified, and the results are shown in *B*.

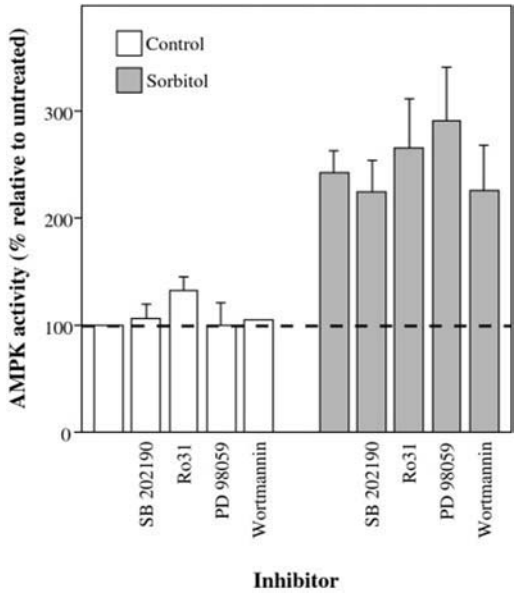

FIG. 6. **Effect of selected inhibitors on AMPK activation by hyperosmotic stress.** H-2K^b cells were preincubated for 30 min in the presence or absence of either 10 μM SB 202190, 2 μM Ro318220, 10 μM PD98059, or 100 nM wortmannin. Following this, cells were incubated for a further 30 min in the presence (*shaded bars*) or absence (*open bars*) of 600 mM sorbitol. Total AMPK activity in cell lysates was measured following immunoprecipitation using a pan-β antibody. Results are the mean values ($\pm$S.E.) of three to six independent experiments and are plotted as a percentage of the control activity (measured in the absence of inhibitor or sorbitol) represented by the *dashed line*.

the mechanisms by which this nucleotide independent activation of AMPK occur.

DISCUSSION

To our knowledge our results provide the first evidence that rosiglitazone, a member of the thiazolidinedione class of anti-diabetic drugs, activates AMPK in muscle through a mechanism involving an increase in the AMP:ATP ratio. The effect of rosiglitazone on cellular adenine nucleotide levels is dramatic, producing a similar increase in the AMP:ATP ratio as that elicited by a high concentration of DNP. Although the precise mechanism by which rosiglitazone leads to a change in nucleotide levels is unknown, thiazolidinediones have been reported to acutely inhibit mitochondrial fuel oxidation in skeletal muscle (31), but the basis for this is not understood. Whether inhibition of fuel oxidation is sufficient to account for the change in nucleotide levels observed in our current study remains to be established, but it seems likely that such a mechanism will play at least some role in the effect. Although we have only studied the effect of rosiglitazone on AMPK, all the thiazolidinedones were found to inhibit mitochondrial oxidation in muscle (31), suggesting that activation of AMPK may be a general effect of the thiazolidinedione class of compounds.

The thiazolidinediones are a relatively new class of anti-diabetic drug that have been shown to reduce plasma glucose and insulin levels and improve some of the abnormalities of lipid metabolism associated with type 2 diabetes (32). The beneficial actions of the thiazolidinediones have been attributed largely to their effects on the transcription factor peroxisome proliferator-activated receptor γ in adipose tissue (32, 33). An increase in insulin sensitivity in peripheral tissues, *e.g.* skeletal muscle, has also been noticed with thiazolidinedione therapy (34) and in skeletal muscle cell cultures (35). Indeed, studies on transgenic models of lipoatrophy have suggested that thiazolidinediones do not require significant levels of adipose tissue to improve insulin sensitivity and that there are important direct effects of troglitazone on other tissues, most probably skeletal muscle (36). Our results demonstrating activation of AMPK by rosiglitazone raise a number of questions regarding the mechanisms underlying the beneficial effects of the drug in the treatment of type 2 diabetes. AMPK has been shown to be involved in the regulation of gene expression in the liver (14, 37). In skeletal muscle, long term activation of AMPK (38) or overexpression of a constitutively active form of AMPK (39) has been shown to increase the expression of a number of proteins that lead to an increase in insulin sensitivity. These effects overlap with some of the actions of the thiazolidinediones, including an increase in glucose transporter levels in muscle cells (40). Furthermore, rosiglitazone has been reported to decrease cholesterol synthesis in a number of cell lines in a peroxisome proliferator-activated receptor γ-independent manner (41). 3-Hydroxy-3-methylglutaryl Co-A reductase, a key enzyme in the synthesis of cholesterol, is phosphorylated and inactivated by AMPK and was one of the first targets identified for the kinase (42).

Here we show that rosiglitazone activates both α1- and α2-containing AMPK complexes, and this leads to a marked increase in the phosphorylation of acetyl-CoA carboxylase. Since there have been no reports to suggest that the activation of AMPK occurs in the absence of downstream effects, it seems likely that the phosphorylation of all its downstream targets

will be increased following stimulation of AMPK by rosiglitazone. In this study we have addressed the acute effects of rosiglitazone on AMPK, and it remains to be determined what the longer term effects on the kinase are. However, repeated acute stimulation of AMPK by AICA riboside has been shown to have long term effects in muscle (38), and it is possible that similar results would be obtained with rosiglitazone. Our results, therefore, raise the intriguing possibility that a number of the beneficial effects of the thiazolidinediones may be mediated through activation of AMPK.

The classical pathway for activation of AMPK involves an increase in the intracellular AMP:ATP ratio (9, 10). However, a number of recent studies have hinted that other pathways also lead to activation of the kinase. Activation of β2-adrenergic receptors in 3T3-L1 adipocytes (43) or Gq-coupled receptors in Chinese hamster ovary cells (44) have been demonstrated to increase AMPK activity. Recently, leptin was found to stimulate AMPK in a biphasic fashion, through both AMP-dependent and -independent mechanisms (3). Activation of AMPK in hepatocytes by metformin was shown to occur without changing ATP levels, although AMP levels were not determined in this study (14). In our current study we demonstrate that both hyperosmotic stress and metformin activate AMPK without increasing the AMP:ATP ratio. These results provide the first unequivocal evidence that AMPK can be activated by alternate mechanism(s) that do not require changes in the energy status of the cell. Although hyperosmotic stress and metformin both activate AMPK without altering the AMP:ATP ratio, we are unable to determine whether they act through the same, or different, mechanisms. Indeed, it is possible that stimuli that activate AMPK in parallel with an increase in the AMP:ATP ratio may also activate these alternate pathway(s), although the results of the additivity experiments would argue against this, at least for the effects of hyperosmotic stress and DNP.

In addition to its allosteric regulation by AMP and ATP, AMPK is activated by phosphorylation catalyzed by an, as yet unidentified, upstream kinase termed AMPK kinase (AMPKK) (8). The principle activating phosphorylation site within AMPK has been identified as threonine 172 within the activation segment of the α subunit (8). Previous studies have shown that phosphorylation at this site is essential for AMPK activity (45, 46). Consistent with this, we found that all the stimuli we tested caused an increase in phosphorylation at this site. However, we were not able to detect increased phosphorylation of this site in cells subjected to both hyperosmotic stress and DNP over cells treated with DNP alone, suggesting that other phosphorylation sites may be involved in the activation process. In a previous study (46) we provided evidence that in addition to threonine 172, phosphorylation of other sites on both the α and β subunits are involved in the regulation of AMPK activity. At present, the identities of the upstream kinases in the AMPK cascade remain poorly characterized, and we cannot determine whether the same protein kinase, or distinct kinases, phosphorylate threonine 172 in response to different stimuli. Clearly, identification of the upstream kinases is essential for determining the mechanisms underlying regulation of AMPK by phosphorylation, which in turn will facilitate elucidation of the different pathways of activation. Our preliminary results, using inhibitors of components of known signaling pathways, indicate that activation of AMPK by hyperosmotic shock does not require activation of phosphatidylinositol 3-kinase, p38 α or β, MAP kinase kinase, or protein kinase C.

The results of our current study have significant implications for the understanding of the mechanisms leading to activation of AMPK. The finding that AMPK is activated by stimuli that do not increase the AMP:ATP ratio opens up the possibility that many other signaling pathways may feed into the AMPK cascade. Activation of AMPK by metformin and rosiglitazone, two widely used anti-diabetic drugs, via these different mechanisms may have important consequences for the treatment of type 2 diabetes and strengthens the idea that specific activators of AMPK could provide useful agents for alleviating the abnormalities that occur in the metabolic syndrome.

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