Protein synthesis, in particular peptide chain elongation, is an energy-consuming biosynthetic process. AMP-activated protein kinase (AMPK) is a key regulatory enzyme involved in cellular energy homeostasis. Therefore, we tested the hypothesis that, as in liver, it could mediate the inhibition of protein synthesis by oxygen deprivation in heart by modulating the phosphorylation of eukaryotic elongation factor-2 (eEF2), which becomes inactive in its phosphorylated form. In anoxic cardiomyocytes, AMPK activation was associated with an inhibition of protein synthesis and an increase in phosphorylation of eEF2. Rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), did not mimic the effect of oxygen deprivation to inhibit protein synthesis in cardiomyocytes or lead to eEF2 phosphorylation in perfused hearts, suggesting that AMPK activation did not inhibit mTOR/p70 ribosomal protein S6 kinase (p70S6K) signaling. Human recombinant eEF2 kinase (eEF2K) was phosphorylated by AMPK in a time- and AMP-dependent fashion, and phosphorylation led to eEF2K activation, similar to that observed in extracts from ischemic hearts. In contrast, increasing the workload resulted in a dephosphorylation of eEF2, which was rapamycin-insensitive, thus excluding a role for mTOR in this effect. eEF2K activity was unchanged by increasing the workload, suggesting that the decrease in eEF2 phosphorylation could result from the activation of an eEF2 phosphatase.

Protein synthesis, in particular peptide chain elongation, is an energy-consuming biosynthetic process, accounting for a large proportion of the oxygen requirements of cells (1). Protein synthesis is regulated via the phosphorylation/dephosphorylation of translation factors and ribosomal proteins (2). The mammalian target of rapamycin (mTOR) phosphorylates eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), thereby relieving its inhibitory action on eukaryotic initiation factor 4E (eIF4E), which can then bind the mRNA cap and stimulate protein synthesis (3). The control of translation by mTOR is also exerted at elongation by regulating the phosphorylation of eukaryotic elongation factor-2 (eEF2) (4). The phosphorylation of eEF2 at Thr-56 by a specific calcium- and calmodulin-dependent eukaryotic eEF2 kinase (eEF2K) leads to its inactivation (5). The p70 ribosomal protein S6 kinase (p70S6K) lies downstream of mTOR and phosphorylates Ser-366 of eEF2K, causing inactivation (4). Therefore, mTOR activation can result in a stimulation of protein synthesis by decreasing eEF2 phosphorylation. Indeed, one of the mechanisms by which insulin stimulates protein synthesis in the heart involves mTOR activation, the effect being blocked by the mTOR inhibitor, rapamycin, and by wortmannin, which blocks phosphatidylinositol 3-kinase (PI 3-kinase) upstream of mTOR (6). The stimulation of protein synthesis by insulin also involves a mTOR-independent mechanism via the phosphorylation and inactivation of glycogen synthase kinase-3. Glycogen synthase kinase-3 phosphorylates and inactivates the guanine nucleotide exchange factor, eukaryotic initiation factor 2B (eIF2B) (7).

Increasing the workload of heart stimulates protein synthesis, leading to cardiac hypertrophy (8). The stretch of the ventricular wall could be the mechanical trigger for this effect. Therefore, protein synthesis plays an important role in the adaptation of cell size and myofibrillar content to mechanical stress. However, the signal transduction pathway leading to the stimulation of protein synthesis due to an increased workload is unknown. Like insulin, increasing the workload stimulates glycolysis via a wortmannin-sensitive pathway (9, 10). Activation of PI 3-kinase and mTOR could therefore be implicated in the stimulation of protein synthesis by increasing the workload, which was one of the aspects studied in this report. In contrast to insulin and increased workload, oxygen deprivation inhibits protein synthesis (11, 12). In skeletal muscle, an inhibition of mTOR/p70S6K signaling by transforming growth factor-β (TGF-β) leads to eukaryotic protein kinase (AMPK) activation has been proposed to explain the inhibition of protein synthesis by oxygen deprivation (13). AMPK acts as an energy and nutrient sensor in cells and is activated by an increased AMP-ATP ratio, as occurs in the absence of oxygen or in response to other cellular stresses (14, 15). Once activated, AMPK stimulates ATP-producing pathways and inhibits energy-consuming processes (14–16). In this
report, we investigated the role of mTOR and AMPK in the inhibition of protein synthesis during myocardial ischemia. We show that myocardial ischemia and increased workload modulate eEF2 phosphorylation by mechanisms independent of mTOR/p70S6K signaling.

MATERIALS AND METHODS

Radiochemicals (Amersham Biosciences); wortmannin, oligomycin (Sigma); rapamycin (Biolog) were obtained from the sources indicated. Anti-ERK1/2 antibodies, anti-eEF2, anti-eIF2B, and anti-eIF2 antibodies were from Santa Cruz Biotechnology. Anti-phospho-Thr-364E-Henseleit bicarbonate buffer containing 5 mM glucose and 2.5 mM CaCl2

...conditions... rats were perfused by the Langendorff method (9) or in buffer containing a mixture of protease and phosphatase inhibitors (18). Following incubation, extracts were prepared using a standard lysis buffer containing 10% (w/v) acrylamide.32P incorporation was calculated by phosphorimaging the dried gels. One unit of protein kinase activity corresponds to the formation of one nmol/min under the conditions of the various assays.

Immunoblotting—Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The percentage of acrylamide used for the gels depended on the protein under study. Acrylamide concentrations were 15% (w/v) for 4E-BP1, 12% (w/v) for eIF2 and 5% (w/v) for eIF2α.
and ERK1/2, and 10% (v/v) for eEF2 and eEF2B. The membranes were probed with the relevant primary antibodies, and immunoreactive bands were detected with protein G-peroxidase by enhanced chemiluminescence.

Phosphorylation Site Identification by Mass Spectrometry—Phosphorylated bands corresponding to the GST-eEF2K (M, 119000) were cut from Coomassie Blue-stained gels, concentrated, and digested with 1 µg of sequencing grade trypsin as described (20). Peptides were separated by reverse-phase narrow-bore HPLC at a flow rate of 200 µl/min, and radioactive peaks were analyzed by nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS) in a LCQ Deca XP Plus ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) (20). Metabolite Measurements and Other Methods—AMP and ATP (21), phosphocreatine (PCr), and creatine (Cr) (22) were measured in nucleotide mass spectrometry (nano-ESI-MS/MS) in a LCQ Deca XP Plus mass spectrometer (ThermoFinnigan, San Jose, CA) (20). Phosphorylation site identification was performed by nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS) in a LCQ Deca XP Plus mass spectrometer (ThermoFinnigan, San Jose, CA) (20).

RESULTS

Oxygen Deprivation Inhibits Protein Synthesis, Activates AMPK, and Increases eEF2 Phosphorylation in Rat Cardiomyocytes—Isolated cardiomyocytes were incubated for 10 min with 1 µM oligomycin, an inhibitor of oxidative phosphorylation, or under anoxic conditions (N2 replacing O2 in the gas phase). Protein synthesis was inhibited 48% by anoxia and 22% by oligomycin treatment, and there was an inverse relationship between the rates of protein synthesis and AMPK activation (Fig. 1A). Incubation of cardiomyocytes under anoxic conditions increased the phosphorylation state of Thr-56 of eEF2, the effect of anoxia being more pronounced than that of oligomycin (Fig. 1B), in line with the extent of AMPK activation.

Ischemia and Anoxia Increase eEF2 Phosphorylation in Langendorff-perfused Rat Hearts—Similarly, in Langendorff-perfused rat hearts, no-flow ischemia and anoxia induced AMPK activation (Fig. 1C) and an increase in eEF2 phosphorylation (Fig. 1D). Phosphorylation of eEF2 was detectable within 2 min and was maximal between 5 and 10 min of no-flow ischemia and correlated with AMPK activation (not shown). The phosphorylation states of initiation factors eIF-4E-BP1 and eIF2α were also known to be regulated by phosphorylation, and were unchanged under anoxia or ischemia for 10 min (not shown).

Rapamycin Has No Effect on the Rate of Protein Synthesis and eEF2 Phosphorylation in Rat Cardiomyocytes and Langendorff-perfused Rat Hearts—In isolated cardiomyocytes, rapamycin inhibited mTOR as it blocked Thr-389 phosphorylation of p70S6K induced by a mixture of insulin (0.1 µM), leucine (1 mM), and glucose (10 mM) (Fig. 2A). The phosphorylation of Thr-389 of p70S6K by mTOR is required for its activation (24). Rapamycin did not mimic the effect of oxygen deprivation to inhibit protein synthesis in cardiomyocytes (Fig. 2C), suggesting that this inhibition did not result from a reduction in mTOR/p70S6K signaling. Moreover, the level of eEF2 phosphorylation in ischemic-perfused hearts was unaffected by rapamycin, although there was a small increase in eEF2 phosphorylation in the controls (Fig. 2B). The failure of rapamycin to inhibit basal protein synthesis, despite a slight increase in eEF2 phosphorylation, indicates that the mTOR/p70S6K pathway was not active and could not be implicated in the control of eEF2 phosphorylation under these conditions.

AMPK Phosphorylates and Activates eEF2K—The upstream kinase that phosphorylates Thr-56 of eEF2 is a highly specific calcium-and-calmodulin-dependent protein kinase called eEF2K (5). When measured in the presence of calcium, eEF2K was activated 3-fold in extracts prepared from isolated Langendorff-perfused rat hearts subjected to 10 min of no-flow ischemia (Fig. 3A). However, in the absence of calcium, some eEF2K activity was detectable, as observed in crude extracts from certain cells (25). Recombinant human

GST-eEF2K was phosphorylated by AMPK in a time- and AMP-dependent fashion to a stoichiometry of 0.7 mol of phosphate incorporated per mol of enzyme (Fig. 3B). Phosphorylation resulted in a 2–2.5-fold increase in eEF2K activity that was restricted to the total activity measured in the presence of calcium (Fig. 3C). In an attempt to identify phosphorylation sites, GST-eEF2K was maximally phosphorylated with AMPK and [γ-32P]MgATP. Following SDS-PAGE, bands corresponding to eEF2K were digested with trypsin, and the resulting peptides were separated by reverse-phase HPLC. A radioactive peak was detected, and its labeling was increased by AMP (not shown). Analysis of the labeled peak by nano-ESI-MS/MS allowed the identification of Ser-78 as one phosphorylation site for AMPK in the fragmentation spectrum of the phosphopeptide eEF2K Ser-78 [YSSG]SPSAS[SFHFX]K, where “p” represents the phosphorylated residue (not shown).

Regulation of eEF2 Phosphorylation by Anoxia under Working Conditions—Since eEF2K is a Ca2+-dependent protein kinase, basal eEF2 phosphorylation could be different in a model of isolated working heart, in which a higher calcium transient has been observed. Therefore, isolated working hearts were perfused with an afterload of 100 cm of H2O and a preload of 20 cm of H2O (physiological conditions) under normoxia and were then submitted to anoxia. Heart function was monitored continuously with a pressure transducer, indicating a drop in aortic pressure within the first minutes of anoxia (Fig. 4A). After 5 min of anoxia, AMPK was maximally activated about 4-fold (Fig. 4B), and at 10 min of anoxia, the AMP:ATP ratio was increased 2–3-fold (Fig. 4C), whereas the PCr/Cr ratio was reduced by about 30% (Fig. 4D). An increase in eEF2 phosphorylation was observed after 5 and 10 min of anoxia (Fig. 4E) as in unloaded hearts (Fig. 1D).
**eEF2 Is Dephosphorylated during Increased Heart Work**—In perfused working hearts, we also studied the effects of increased heart work on eEF2 phosphorylation, as this condition is known to increase protein synthesis (8). Heart work was increased by raising the afterload from 60 (low load) to 120 cm of H2O (high load). In contrast to anoxia, an acute increase in pressure load resulted in a decrease in eEF2 phosphorylation, which occurred within 1 min and persisted over 15 min (Fig. 5A). This could explain the increase in protein synthesis in response to an increased workload (8), as eEF2 phosphorylation inhibits protein synthesis. The effect of increasing the workload to dephosphorylate eEF2 was unaffected by wortmannin and rapamycin (Fig. 6A), which are inhibitors of PI3-kinase and mTOR respectively. Surprisingly, Thr-389 phosphorylation of p70S6K was also decreased under high load conditions, suggesting that mTOR was inhibited (Fig. 6B). This contrasts with the situation in rat cardiomyocytes treated with insulin, in which p70S6K is activated and protein synthesis is stimulated, both effects being blocked by rapamycin (6) (see also Fig. 2A). No significant decrease in eEF2K activity was observed after 15 min of high load (Fig. 5B), suggesting that the decrease in eEF2 phosphorylation might be due to the activation of an eEF2 phosphatase. Taken together, these results show a decrease in eEF2 phosphorylation in response to increasing the workload, for which mTOR/p70S6K does not seem to play a role.

Increasing the workload resulted in a transient increase in 4E-BP1 phosphorylation, which was rapamycin-insensitive (Fig. 6C). The anti-phospho antibody used in these experiments recognizes Thr-36 and probably cross-reacts with Thr-45 (the surrounding sequences are almost identical). However, there was no change in eIF2α phosphorylation state (not shown). In a previous study, we showed that the stimulation of glycolysis by increasing the workload, like the metabolic effects of insulin, were wortmannin-sensitive and did not require AMPK (10). The MEK/ERK and PI3-kinase pathways are both stimulated in response to insulin and implicated in the phosphorylation and inactivation of eEF2K. Therefore, we checked two downstream targets of these two pathways (ERK and eIF2B respectively) to exclude their implication in the dephosphorylation of eEF2 observed during the increased workload. The phosphorylation state of eIF2B tended to increase slightly on increasing the workload, and no changes in the phosphorylation states of ERK1/2 were detected (not shown).

**DISCUSSION**

In this report, we show that AMPK activation correlates with an increase in eEF2 phosphorylation in anoxic cardiomyocytes. In perfused working hearts, we also studied the effects of increased heart work on eEF2 phosphorylation, as this condition is known to increase protein synthesis (8). Heart work was increased by raising the afterload from 60 (low load) to 120 cm of H2O (high load). In contrast to anoxia, an acute increase in pressure load resulted in a decrease in eEF2 phosphorylation, which occurred within 1 min and persisted over 15 min (Fig. 5A). This could explain the increase in protein synthesis in response to an increased workload (8), as eEF2 phosphorylation inhibits protein synthesis. The effect of increasing the workload to dephosphorylate eEF2 was unaffected by wortmannin and rapamycin (Fig. 6A), which are inhibitors of PI3-kinase and mTOR respectively. Surprisingly, Thr-389 phosphorylation of p70S6K was also decreased under high load conditions, suggesting that mTOR was inhibited (Fig. 6B). This contrasts with the situation in rat cardiomyocytes treated with insulin, in which p70S6K is activated and protein synthesis is stimulated, both effects being blocked by rapamycin (6) (see also Fig. 2A). No significant decrease in eEF2K activity was observed after 15 min of high load (Fig. 5B), suggesting that the decrease in eEF2 phosphorylation might be due to the activation of an eEF2 phosphatase. Taken together, these results show a decrease in eEF2 phosphorylation in response to increasing the workload, for which mTOR/p70S6K does not seem to play a role.

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and ischemic heart. The increase in eEF2 phosphorylation during ischemia occurred before any change in the phosphorylation state of 4E-BP1 and was not mimicked by rapamycin. This suggests that the inhibition of mTOR signaling is not involved in the AMPK-induced inhibition of basal protein synthesis, confirming previous observations in hepatocytes (17) and cardiomyocytes (26). In hepatocytes incubated with amino acids, p70S6K becomes activated, and this effect was antagonized by AMPK activation (27, 28). Also, AMPK activation by 5-amino-4-imidazolecarboxamide riboside in rat skeletal muscle in vivo inhibited both the mTOR pathway and protein synthesis (13). Therefore, AMPK activation inhibits mTOR signaling and protein synthesis, but only if the pathway is first switched on, e.g. by amino acids or insulin.

In extracts from anoxic hepatocytes (17) or ischemic-perfused hearts (Fig. 3A), eEF2K was activated, suggesting that AMPK might phosphorylate and activate eEF2K. In some cells, treatments that lead to a rise in cAMP activate eEF2K by increasing the calcium-independent activity (29), whereas in other systems, the total activity measured in the presence of calcium is increased (30). The situation is further complicated by the proposed existence of isozymes of eEF2K (31), which could exhibit different kinetic properties. Here we show that in ischemic hearts, AMPK activation leads to an increase in eEF2K activity, which is mainly reflected in an increase in the total, calcium-dependent activity of the extracts (Fig. 3A). However, we cannot exclude that an inhibition of eEF2 phosphatase by AMPK could also contribute toward an increase in overall phosphorylation of eEF2. In vitro, AMPK phosphorylated a
human recombinant GST-eEF2K (Fig. 3B), which led to eEF2K activation (Fig. 3C). Moreover, eEF2K activation was restricted to the calcium-dependent activity (Fig. 3C), similar to the activation of eEF2K seen in ischemic hearts (Fig. 3A). When the AMPK-phosphorylated human GST-eEF2K was digested with trypsin and analyzed by HPLC and mass spectrometry, Ser-78 was identified as a phosphorylation site for AMPK in a peak whose labeling was increased in the presence of AMP. This serine residue is conserved in eEF2K from human, mouse, and rat. However, the HPLC profile indicated the existence of other sites that could account for the change in activity.

In addition to activation by AMPK, a fall in intracellular pH, as occurs during ischemia (32), could participate in increasing the phosphorylation state of eEF2, as eEF2K activity rises as the pH drops below 7 (33). The increase in eEF2 phosphorylation is therefore likely to be due to a combination of phosphorylation/activation of eEF2K by AMPK and stimulation of eEF2K activity as a result of the fall in pH. In cortical neurons, oxidative stress induced by H$_2$O$_2$ treatment has been shown to inhibit protein synthesis by a process involving the phosphorylation of eEF2, which it was proposed could have been secondary to a rise in intracellular calcium (34). However, it is noteworthy that AMPK is activated in CCL13 cells treated with H$_2$O$_2$ (20).

Calcium levels are constantly varying in cardiac myocytes, whereas they are at basal levels in most other cells. Therefore, a mechanism that is independent of calcium would be important to ensure increased phosphorylation of eEF2, since a decreased calcium transient is observed within a few minutes in the absence of oxygen, the effect being more pronounced when the intracellular pH falls (35, 36). In contrast, increasing the workload increases the calcium transient of cardiomyocytes. However, perfusion under working conditions did not result in eEF2 phosphorylation, and the effect of anoxia on eEF2 phosphorylation was still apparent. This suggests that an additional mechanism maintains eEF2 in its dephosphorylated form in normoxic conditions, thereby counteracting the effect of calcium on eEF2K activity and maintaining protein synthesis.

In contrast to the situation in hypoxia, an acute increase in workload resulted in a decrease in eEF2 phosphorylation. This could be involved in the stimulation of protein synthesis in response to an increased workload (8) and reinforces the hyp-
phosphorylation and inactivation of eEF2K by p90rsk/p70S6K1 shown that protein-serine/threonine phosphatase 2A and the trophic response to an increased hemodynamic load (38). It was proposed to be protein-serine/threonine phosphatase 2A. Therefore, we suggest that the decrease in eEF2 phosphorylation state during increased workload are controlled by two downstream of glycogen synthase kinase-3 in the insulin-signaling pathway, did not decrease on increasing the workload, which contrasts with the effect of insulin in decreasing eEF2 phosphorylation (7, 37). Furthermore, there was no change in the phosphorylation of eIF2B, which lies down-stream of glycogen synthase kinase-3 in the insulin-signaling pathway, did not decrease on increasing the workload, which contrasts with the effect of insulin in decreasing eEF2 phosphorylation (7, 37). Furthermore, there was no change in the phosphorylation of eIF2B, which plays a role in the inhibition of protein synthesis via eEF2K activation and the inhibition of protein synthesis in cardiac myocytes (25, 26). Also, the phosphorylation state of eIF2B, which is a suppressor of the mTOR pathway, did not decrease on increasing the workload, which suggests that AMPK is not required for the acute adaptation of the myocardium to changes in pressure load (10). The decrease in eEF2 phosphorylation by increasing the workload was wortmannin- and rapamycin-insensitive, and no changes in the phosphorylation states of ERK1/2 were detected (not shown).

In conclusion, during ischemia AMPK not only participates in the stimulation of glycolysis but is also involved in the inhibition of protein synthesis via eEF2K activation and the subsequent phosphorylation and inactivation of eEF2. In contrast, glycolysis and protein synthesis (at least for eEF2 phosphorylation) during increased workload are controlled by two separate pathways, namely PI 3-kinase activation for the stimulation of glycolysis and activation of a phosphatase for eEF2 (Fig. 7).

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Myocardial Ischemia and Increased Heart Work Modulate the Phosphorylation State of Eukaryotic Elongation Factor-2
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