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Insulin Antagonizes Ischemia-induced Thr^{172} Phosphorylation of AMP-activated Protein Kinase α-Subunits in Heart via Hierarchical Phosphorylation of Ser$^{485/491}$*

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Previous studies showed that insulin antagonizes AMP-activated protein kinase activation by ischemia and that protein kinase B might be implicated. Here we investigated whether the direct phosphorylation of AMP-activated protein kinase by protein kinase B might participate in this effect. Protein kinase B phosphorylated recombinant bacterially expressed AMP-activated protein kinase heterotrimers at Ser^{485} of the α1-subunits. In perfused rat hearts, phosphorylation of the α1/α2 AMP-activated protein kinase subunits on Ser^{485}/Ser^{491} was increased by insulin and insulin pretreatment decreased the phosphorylation of the α-subunits at Thr^{172} in a subsequent ischemic episode. It is proposed that the effect of insulin to antagonize AMP-activated protein kinase activation involves a hierarchical mechanism whereby Ser^{485}/Ser^{491} phosphorylation by protein kinase B reduces subsequent phosphorylation of Thr^{172} by LKB1 and the resulting activation of AMP-activated protein kinase.

Stimulation of the insulin and AMP-activated protein kinase (AMPK)[4] signaling pathways in heart leads to an increase in glycolysis via recruitment of GLUT4 transporters to the plasma membrane and activation of 6-phosphofructo-2-kinase (PFK-2) (1, 2). The signaling pathway for insulin requires phosphatidylinositol 3-kinase (PI3K) and, for PFK-2 activation, protein kinase B (PKB), and/or a wortmannin-sensitive and insulin-stimulated protein kinase that phosphorylates heart PFK-2 on Ser^{466} (3). PFK-2 activation in ischemia is explained by the activation of AMPK, which also phosphorylates heart PFK-2 at Ser^{466} (2).

AMPK is a heterotrimer consisting of a catalytic α-subunit together with two regulatory subunits, β and γ. Each subunit exists as multiple isoforms (α1, α2, β1, β2, γ1, γ2, γ3) giving 12 different possible combinations of holoenzyme with different tissue distribution and subcellular localization (4–6). In heart, the α2 isoform of the catalytic subunit is twice as abundant as α1 (7). The activating upstream AMPK kinase (AMPKK) phosphorylating Thr^{172} in the AMPK catalytic α-subunits was initially partially purified from rat liver and reported to contain a M_r 58,000 catalytic subunit in a M_r 195,000 complex (8). Site-directed mutagenesis showed that phosphorylation at Thr^{172} accounts for most of the activation of AMPK by AMPKK; however, new phosphorylation sites were identified as Thr^{258} and Ser$^{485/491}$ in the catalytic α1/α2-subunits, respectively (9). Site-directed mutagenesis experiments indicated that phosphorylation at these new sites was not essential for AMPK activation or activity, whereas Thr^{172} was required (9). Recently, one AMPK-activating AMPKK phosphorylating Thr^{172} was identified as the Peutz-Jeghers syndrome protein LKB1 (10, 11). LKB1 can phosphorylate the activation loop Thr residue of several members of the AMPK family (12).

In perfused heart, ischemia antagonizes insulin signaling via a drop in pH, which inhibits the tyrosine kinase activity of the insulin receptor (13). By contrast, pretreatment with insulin antagonized AMPK activation in response to ischemia (14). This effect of insulin on AMPK activation was wortmannin-sensitive and was associated with a decrease in phosphorylation of Thr^{172} in the catalytic α1- and α2-subunits (14). These findings were recently confirmed in a working mouse heart model, where it was shown that insulin decreased Thr^{172} α AMPK phosphorylation and that this was associated with PKB activation, as judged by its phosphorylation on Ser^{473} (15). Moreover, in transgenic mice overexpressing constitutively active PKB and in neonatal cardiomyocytes infected with an adenovirus expressing constitutively active PKB isoforms, Thr^{172} AMPK phosphorylation was likewise decreased (15).

Here, we investigated the molecular mechanism of the cross-talk between the insulin and AMPK signaling pathways. PKB was shown to phosphorylate the α1 and α2 AMPK subunits *in vitro*, and Ser^{485} was identified as the phosphorylation site in the α1-subunit. Moreover, perfusion of hearts with insulin activated PKB and resulted in α1/α2 AMPK subunit phosphorylation on Ser$^{485/491}$, which was accompanied by a decrease in Thr^{172} phosphorylation in a subsequent ischemic episode.

MATERIALS AND METHODS

[γ-^{32}P]ATP was from Amersham Biosciences. Other reagents and recombinant bacterially expressed wild-type kinase-inactive (D157A) and S485A and S485D mutant AMPK heterotrimers were from sources cited previously (9, 16, 17). Recombinant active PKB was purchased from Calbiochem, and recombinant active LKB1 was from Upstate Biotechnology. Anti-phospho-Thr^{172} and anti-phospho-Ser$^{485/491}$ α-sub-

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[4] The abbreviations used are: AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PFK-2, 6-phosphofructo-2-kinase; ANOVA, analysis of variance; HPLC, high performance liquid chromatography.

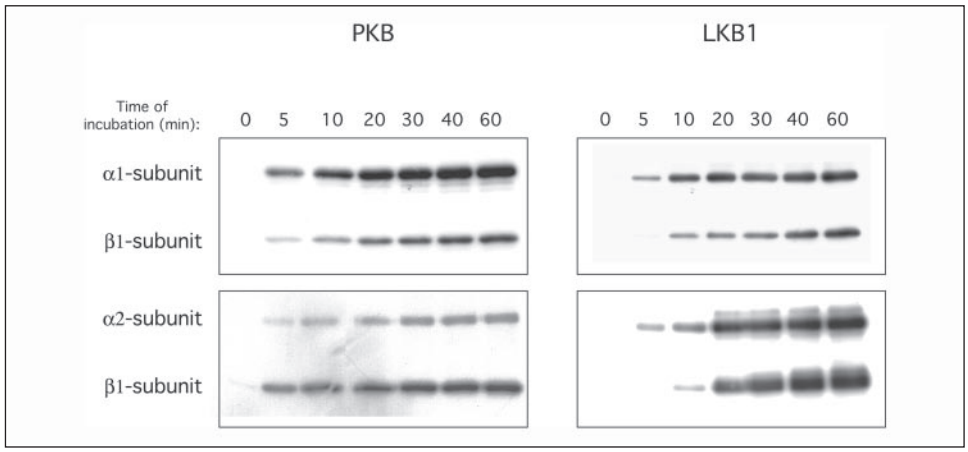

FIGURE 1. **Phosphorylation of bacterially expressed AMPK complexes by PKB.** Purified bacterially expressed AMPK $\alpha1\beta1\gamma1$ and $\alpha2\beta1\gamma1$ complexes were incubated with PKB or LKB1 and [γ-^{32}P]MgATP as described under "Materials and Methods." Aliquots were removed at the indicated times for SDS-PAGE, gel drying, and autoradiography. The autoradiograms shown are representative of three separate experiments.

unit antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-$\alpha1/\alpha2$ AMPK subunit antibodies were generously donated by Prof. Grahame Hardie (University of Dundee).

Perfusion of Isolated Rat Hearts—Hearts from fed male Wistar rats were perfused by the Langendorff method (18) as described previously (16). Hearts were freeze-clamped at the indicated times for the preparation of extracts for immunoblotting and enzyme assay. Samples of frozen left ventricles were homogenized in 9 volumes of lysis buffer on ice, using an Ultra-Turrax homogenizer (2). All animal experiments were approved by the Ethical Committee of the Faculty of Medicine, Université catholique de Louvain.

In Vitro Phosphorylation and Enzyme Assays—Purified bacterially expressed recombinant AMPK complexes(5 μg) were incubated in the presence of recombinant active PKB (0.4 unit/ml), recombinant active LKB1 (0.08 unit/ml) and 0.1 mM [γ-^{32}P]MgATP (specific radioactivity: 1000 cpm/pmol) in a final volume of 50 μl of 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol at 30 °C. The units of PKB and LKB1 are as quoted by the suppliers and refer to the phosphorylation of relevant peptide substrates. At the indicated times, aliquots (5 μl) were removed for SDS-PAGE. The activity of PKB- or LKB1-phosphorylated AMPK was assayed in a final volume of 25 μl of 50 mM Hepes, pH 7.2, with 0.2 mM SAMS peptide, 0.2 mM AMP, and 0.1 mM [γ-^{32}P]MgATP (specific radioactivity: 1000 cpm/pmol). Aliquots (5 μl) were removed and spotted onto Whatman P81 papers for measurement of ^{32}P incorporation (16). PKB was immunoprecipitated from heart extracts and assayed as described previously (19). One unit of protein kinase activity corresponds to the incorporation of 1 nmol of phosphate into the appropriate peptide substrate per min under the assay conditions.

Phosphorylation Site Identification by Mass Spectrometry—Bacterially expressed recombinant $\alpha1\beta1\gamma1$ AMPK (5 μg) and $\alpha1$D157A$\beta1\gamma1$ kinase-inactive AMPK were incubated as described above with 0.1 mM [γ-^{32}P]MgATP (specific radioactivity: 1000 cpm/pmol) and PKB (0.4 unit/ml) or LKB1 (0.2 unit/ml) for 60 min at 30 °C for phosphorylation site determination by mass spectrometry (9).

Immunoblotting—The total content of the catalytic subunits of AMPK ($\alpha1/\alpha2$ AMPK) and the phosphorylated forms of AMPK were determined in total tissue lysates by Western blotting (20, 21) using antibodies against total $\alpha1/\alpha2$ AMPK and phosphospecific antibodies against Thr^{172} and Ser$^{485/491}$. Proteins were separated by SDS-PAGE in gels containing 10% (w/v) acrylamide and transferred to polyvinylidene fluoride membranes. The membranes were incubated in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 1 h and probed with anti-phospho-Ser^{485}/Ser^{491} $\alpha1/\alpha2$ AMPK or anti-phospho-Thr^{172} AMPK rabbit antibodies. As loading controls, anti-$\alpha1/\alpha2$ AMPK antibodies raised in sheep that do not distinguish the phosphorylated proteins were used. After incubation with anti-rabbit IgG conjugated to IR dye 800 (Rockland Inc., Philadelphia, PA) and anti-sheep IgG conjugated to Alexa 680 (Molecular Probes, Leiden, Netherlands), the membranes were scanned in two different channels using the Odyssey IR imager (Li-Cor Biosciences). Band intensities were quantified using the Odyssey software, and signals for the anti-phospho-AMPK antibodies were expressed relative to those obtained with the total AMPK antibodies.

Other Methods—Protein concentration was estimated by the method of Bradford (22) with bovine serum albumin as a standard. Statistical significance of differences was evaluated using a Student's two-sided *t* test or by two-way ANOVA with time and insulin treatment as independent factors. Differences were judged to be significant at $p < 0.05$.

RESULTS

In Vitro Phosphorylation of Bacterially Expressed AMPK by PKB and LKB1—Phosphorylation of recombinant bacterially expressed AMPK complexes by PKB and LKB1 was investigated by ^{32}P incorporation from [γ-^{32}P]ATP into $\alpha1\beta1\gamma1$ and $\alpha2\beta1\gamma1$ complexes. In the presence of PKB and LKB1, both the $\alpha1$- and $\alpha2$-subunits were phosphorylated almost to completion in a time-dependent manner (Fig. 1). PKB phosphorylated the $\alpha2$-subunits to a lesser extent than the $\alpha1$-subunits. By contrast, phosphorylation of the $\alpha2$-AMPK subunits by LKB1 was greater than that observed for the $\alpha1$-subunits. Concerning the β-subunits, it is likely that phosphorylation following LKB1 treatment represents autophosphorylation as already shown for phosphorylation by AMPKK (9). By contrast, the phosphorylation of $\beta1$ by PKB suggests the presence in this isoform of transphosphorylation sites for PKB, since the $\beta2$-subunits were not phosphorylated by PKB in $\alpha1\beta2\gamma1$ AMPK (data not shown).

Identification of Phosphorylation Sites for LKB1 and PKB in the AMPK $\alpha1$-Subunits—Bacterially expressed $\alpha1\beta1\gamma1$ AMPK heterotrimers were incubated with [γ-^{32}P]ATP and LKB1 or PKB. After 60 min of incubation, the reactions were stopped for SDS-PAGE. Bands corresponding to the α-subunits were cut from Coomassie Blue-stained gels and "in-gel" digested with trypsin. The resulting peptides were separated by reverse-phase HPLC. Incubation with LKB1 led to the labeling of three major peaks containing peptides phosphorylated on Thr^{258}, Ser^{485}, and Thr^{172} (Fig. 2*A*). A fourth peak that eluted between fractions 35 and 40 was previously shown to contain a Thr^{172}-phosphorylated peptide with a missed cleavage for trypsin (9). Phosphorylation at both Thr^{172} and Ser^{485} after treatment with LKB1 was confirmed by immu-

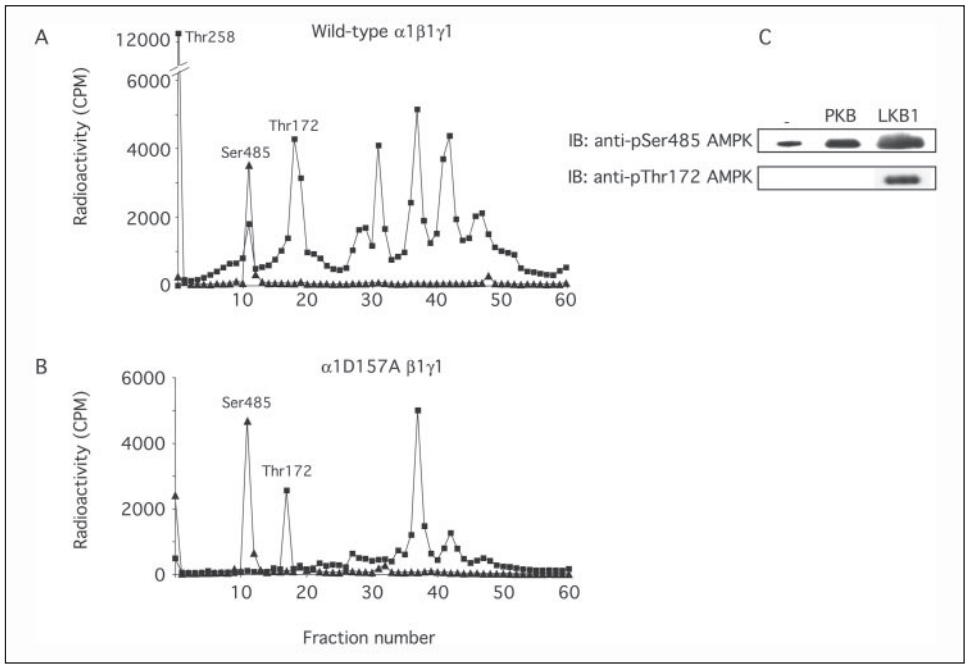

FIGURE 2. **HPLC profiles of ^{32}P-labeled peaks from α1-subunits phosphorylated by LKB1 or PKB and digested with trypsin.** Bacterially expressed wild-type α1β1γ1 (*A*) and kinase-inactive α1D157Aβ1γ1 AMPK (*B*) were phosphorylated *in vitro* by PKB (▲) or by LKB1 (■) and [γ-^{32}P]ATP (specific radioactivity: 1000 cpm/pmol) for 60 min, as described under "Materials and Methods." Following SDS-PAGE, bands corresponding to the α1-subunits were cut from SDS-polyacrylamide gels and digested with trypsin. Peptides were separated by reverse-phase narrowbore HPLC in a linear acetonitrile gradient, and radiolabeled peaks were analyzed by nano-electrospray ionization tandem mass spectrometry (see "Materials and Methods"). The identity of phosphorylation sites in the radiolabeled peaks is indicated. The phosphorylation state of Ser^{485} and Thr^{172} in the α1-subunit after phosphorylation by PKB and LKB1 was confirmed by immunoblotting with phospho-specific antibodies (*C*).

noblotting (Fig. 2*C*). Moreover, our findings indicate that, as well as corresponding to a site for AMPKKs, Ser^{485} and Thr^{258} can also be autophosphorylation sites, since their phosphorylation by LKB1 was abolished in α1 D157Aβ1γ1 kinase-inactive AMPK, with no effect on Thr^{172} labeling (Fig. 2*B*). Therefore, phosphorylation of AMPK by LKB1 leads first to phosphorylation of Thr^{172}, which is required for AMPK activation, and then to the phosphorylation of Ser^{485} in the α1-subunit. By contrast, treatment with PKB resulted in the labeling of a single radioactive peak. Fragmentation of the phosphorylated peptide in the mass spectrometer (23) allowed the identification of Ser^{485} as the phosphorylated residue for PKB in the tryptic α1-peptide ^{483}SGSISNYR^{490}. The identification of Ser^{485} as a target of PKB was confirmed by immunoblotting (Fig. 2*C*).

Insulin Leads to Ser$^{485/491}$ Phosphorylation of the AMPK α1/α2-Subunits and Reduces Subsequent Thr^{172} Phosphorylation during Ischemia—Under normoxic conditions, perfusion of hearts with insulin caused a 5-fold activation of PKB, which persisted for at least 15 min (controls, 0.34 ± 0.1 milliunit/mg of protein; insulin, 1.55 ± 0.6 milliunit/mg of protein, means ± S.D. for three separate experiments). This priming of the hearts with insulin was previously shown to decrease the extent of phosphorylation of α1/α2 Thr^{172} and the activation of AMPK brought about by ischemia or anoxia (14). We tested whether the effect of insulin to decrease the phosphorylation state of Thr^{172} resulted from a hierarchical mechanism whereby Ser$^{485/491}$ phosphorylation would prevent subsequent phosphorylation of Thr^{172} and hence AMPK activation. Immunoblotting with anti-phospho-Ser$^{485/491}$ α1/α2 AMPK antibodies was first undertaken to assess their extent of phosphorylation in insulin-perfused hearts. Insulin increased Ser^{485} α1 AMPK phosphorylation within 5 min of treatment, and this phosphorylation was maximal after 10 min (Fig. 3*A*, *upper panel*). Moreover, 10 min of ischemia also increased Ser^{485} α1 AMPK phosphorylation (Fig. 3*A*, *lower panel*), in agreement with the *in vitro* phosphorylation studies (Fig. 2) indicating that Ser^{485} can be autophosphorylated as a consequence of AMPK activation as well as being a site for PKB. The phosphorylation states of Ser$^{485/491}$ and Thr^{172} in the AMPK α-subunits were then compared in ischemic hearts pretreated with or without insulin (100 nM) for 5 min (Fig. 3*B*). In the absence of insulin, ischemia led to Thr^{172} phosphorylation within 5 min ($p = 0.02$, Student's *t* test), as expected (Fig. 3*B*, *left-hand panel*). After 10 min of ischemia, there was a tendency for total AMPK content to decrease, but this effect was not systematically observed and, for example, was not apparent in Fig. 3*A*. Insulin pretreatment antagonized the increase in Thr^{172} phosphorylation brought about by ischemia (Fig. 3*B*, *left-hand panel*). Moreover, in this condition, the phosphorylation state of Ser^{485}/Ser^{491} was already increased at the beginning of ischemia ($p = 0.0003$, Student's *t* test) and did not further increase (Fig. 3*B*, *right-hand panel*). Taken together, the results suggest that insulin-induced hierarchical phosphorylation is involved in the decrease in AMPK activation in response to ischemia.

In Vitro Phosphorylation of Ser^{485} by PKB Reduces Subsequent Activation of α1β1γ1 by LKB1—The *in vitro* phosphorylation studies (Fig. 2) indicated that PKB phosphorylated Ser^{485} of the AMPK α1-subunits but was unable to phosphorylate Thr^{172}. As expected, treatment with LKB1 led to AMPK activation whereas treatment with PKB did not (Fig. 4*A*). We tested whether prior *in vitro* phosphorylation of α1β1γ1 AMPK by PKB would antagonize AMPK activation by LKB1. Indeed, pretreatment with PKB reduced LKB1-induced AMPK activation by about 30% (Fig. 4*B*). As expected, a S485A mutant was refractory to the inhibition of LKB1-induced AMPK activation by PKB (Fig. 4*B*). Moreover, activation of a preparation of S485D α1β1γ1 AMPK mutant by LKB1 was decreased by about 30% compared with activation of the wild type (Fig. 4*C*). The inhibitory effects of PKB phosphorylation and mutagenesis of S485 to Asp on LKB1-induced AMPK activation are comparable with the inhibition of AMPK activation seen during ischemia (a maximum of about 40%) reported previously (14).

DISCUSSION

Our data suggest that phosphorylation of AMPK α-subunits at Ser$^{485/491}$ by PKB in response to insulin in the heart decreases Thr^{172} phosphorylation by LKB1 and hence AMPK activation during ischemia. The involvement of PKB in the *in vivo* effect of insulin on AMPK activity is supported by our *in vitro* data showing that prior phosphorylation of α1β1γ1 AMPK by PKB at Ser^{485} decreased subsequent phosphorylation and activation by LKB1. Although Ser^{485} in α1β1γ1 AMPK was phosphorylated *in vitro* following treatment with LKB1, this was clearly an autophosphorylation event, since LKB1 did not phosphorylate Ser^{485} in

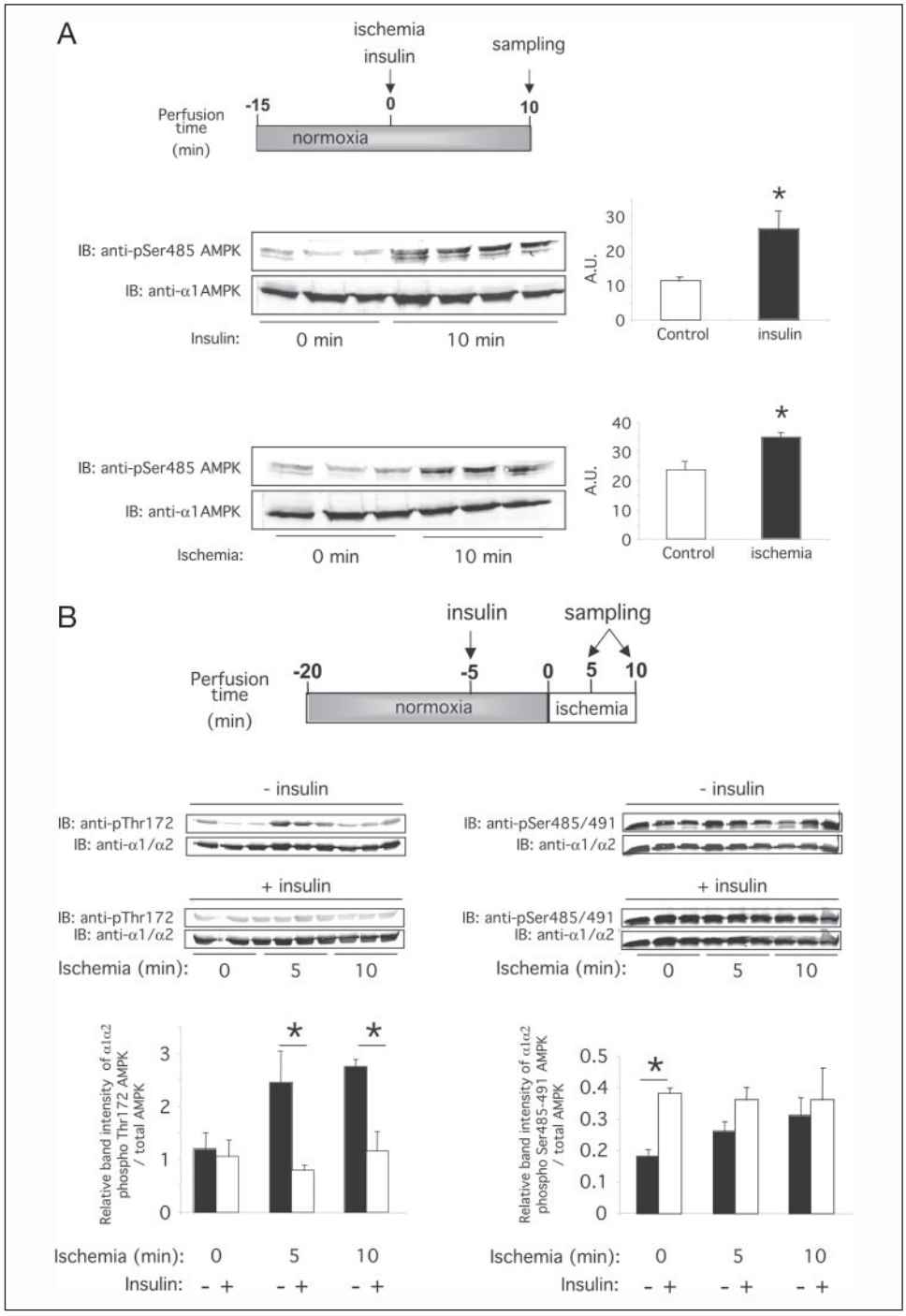

FIGURE 3. **Effect of insulin pretreatment on the phosphorylation state of Ser$^{485/491}$ and Thr^{172} after 5 and 10 min of ischemia.** A scheme of the experimental protocol is given at the *top* of the figure. After 15-min equilibration, hearts were perfused for 10 min with insulin (100 nM) or submitted for 10 min to ischemia (*A*), or hearts were perfused for 5 min with insulin before ischemia was started (*B*). The phosphorylation state of Ser^{485}/Ser^{491} and Thr^{172} in the $\alpha1/\alpha2$-subunits was analyzed by immunoblotting with phospho-specific antibodies. As a loading control, nonspecific $\alpha1/\alpha2$-subunit antibodies that do not distinguish the phosphorylated proteins were used. The results are expressed as the ratio of phosphorylated $\alpha1/\alpha2$ AMPK subunits to total AMPK. *Open bars* correspond to hearts preincubated with insulin, whereas *filled bars* correspond to hearts incubated without insulin. Values are the means ± S.D. for three separate perfusions in each condition. * indicates a statistically significant difference (Student's *t* test and two-way ANOVA, $p < 0.05$). Statistical analysis by two-way ANOVA indicated that the effect of time on ischemia-induced Thr^{172} AMPK α-subunit phosphorylation was dependent on whether insulin was present or not, and there was a statistically significant interaction between time and insulin treatment ($p = 0.006$). Phosphorylation of Ser$^{485/491}$ was significant with respect to insulin treatment but not with respect to time.

the kinase-inactive $\alpha1\beta1\gamma1$ AMPK mutant (Fig. 2). Therefore, phosphorylation of AMPK by LKB1 leads first to phosphorylation of Thr^{172}, which is required for AMPK activation, and then to phosphorylation of Ser^{485} in the $\alpha1$-subunit. On the other hand, only Ser^{485} in $\alpha1\beta1\gamma1$ AMPK was phosphorylated by treatment with PKB (Fig. 2). Phosphorylation of the $\alpha2$-subunits, the predominant isoform in heart, was less than that of the $\alpha1$-subunits (Fig. 1). However in perfused hearts, insulin decreased the extent of Thr^{172} phosphorylation of both $\alpha1$ AMPK and $\alpha2$ AMPK in response to ischemia in a PI3K-dependent manner (14). We cannot exclude that insulin-stimulated protein kinases other than PKB and downstream of PI3K might participate in this inhibitory effect. Inspection of the sequences surrounding Ser^{485} and Ser^{491} revealed a consensus with arginine at the −3 position (with respect to the phosphorylated Ser residue) and a hydrophobic residue at +1. This sequence corresponds to the minimal consensus for phosphorylation by PKB (24).

Several other studies support our hypothesis that insulin-induced PKB activation inhibits the AMPK signaling pathway. Activation of PKB in heart *in vivo* was sufficient to decrease Thr^{172} phosphorylation, suggesting that the insulin-induced down-regulation of AMPK activation is mediated by a PKB-dependent pathway (15). In 3T3-L1 adipocytes, insulin was shown to antagonize isoproterenol-induced lipolysis by reducing Thr^{172} AMPK phosphorylation via a PI3K-dependent pathway (26). A similar circuit has also been suggested in *C. elegans*, where insulin-like signals feedback on the AMPK α-subunit, AAK-2 (27). Insulin

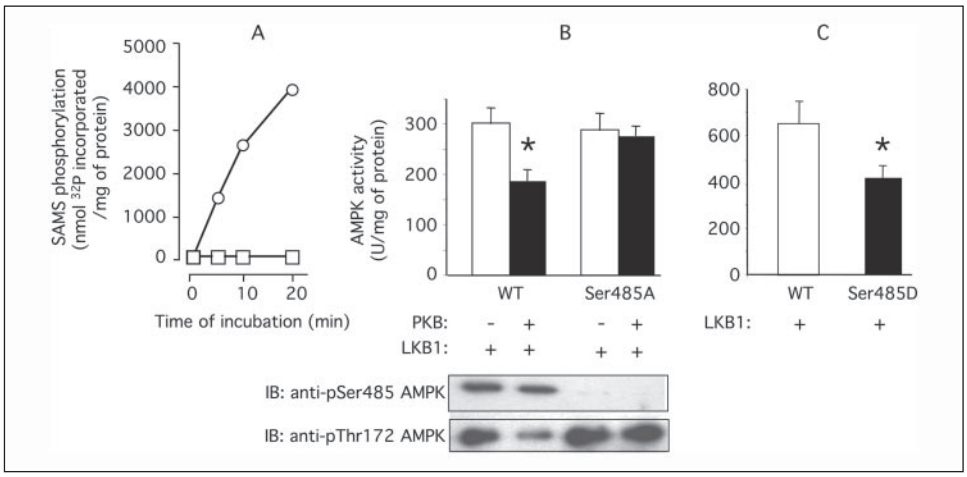

FIGURE 4. **Time-dependent changes in phosphorylation state and activity of $\alpha1\beta1\gamma1$ AMPK incubated with PKB and LKB1.** $\alpha1\beta1\gamma1$ AMPK (5 μg) was incubated with 0.1 mM [γ-^{32}P]MgATP and PKB or LKB1 in a final volume of 25 μl. At the indicated times, aliquots were removed for measurement of AMPK activitiy. *A*, AMPK activities after phosphorylation by PKB (□) and LKB1 (○) toward the SAMS peptide are indicated. *B*, $\alpha1\beta1\gamma1$ AMPK (wild-type or S485A mutant) (1 μg) was first phosphorylated with (*filled bars*) or without (*open bars*) PKB for 30 min. An aliquot (containing 0.2 μg of $\alpha1\beta1\gamma1$ protein) was taken and incubated for 30 min with LKB1 prior to removal of a second aliquot (0.02 μg of protein) for AMPK assay. *C*, $\alpha1\beta1\gamma1$ AMPK (wild-type or S485D mutant) was incubated for 10 min with LKB1 prior to AMPK assay. The band intensities of Coomassie Blue-stained gels were measured by scanning and processing with the program ImageJ (1.33 for Mac OS X) to correct for the amount of α1-subunits in the recombinant wild-type and S485A and S485D mutant preparations relative to known amounts of bovine serum albumin. In *B* and *C*, the values are the means ± S.D. for three separate experiments. * indicates a statistically significant difference ($p < 0.05$, Student's *t* test) with respect to the control.

has also been shown to decrease basal AMPK activity in Fao hepatoma cells (28) and in perfused heart (29). Interestingly, the effect of insulin to inhibit α2 AMPK activity in perfused heart was not observed in the presence of palmitate, which on its own activates AMPK (29).

While this work was in progress, it was shown that the activation of heart AMPK by ischemia was accompanied by an increase in AMPKK activity (30, 31) with no change in activity of LKB1 (30), suggesting the presence of another AMPKK(s) that activate AMPK in heart. The existence of multiple mammalian AMPKKs is not surprising, given that three AMPKKs are present in *S. cerevisiae* (8, 33). When LKB1 was immunoprecipitated from an extract of rat testis and incubated with PKB and ATP, no change in LKB1 activity was detected via AMPK activation and assay with the SAMS peptide (results not shown). Also, the effect of insulin to reduce AMPK activation in perfused hearts was not related to a change in AMP:ATP ratio (14).

In conclusion, although other studies have suggested that AMPK can regulate PKB expression and/or activity (34, 35), we propose a mechanism of hierarchical control by insulin for the reduction of AMPK activation in the ischemic heart via PKB-induced phosphorylation of Ser^{485}/Ser^{491}. The insulin-induced inhibition of AMPK could play a cardioprotective role by decreasing rates of fatty acid oxidation. Reduced AMPK activation would also be expected to favor protein synthesis (36, 37) and to reduce apoptosis (32).

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