Phosphorylation of P20 is associated with the actions of insulin in rat skeletal and smooth muscle

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Although a large number of protein kinases/phosphatases involved in insulin's actions have been characterized recently, relatively few of the downstream phosphoproteins have been identified. We have employed two-dimensional gel electrophoresis-based proteome analysis to investigate the insulin-evoked phosphorylation cascade in rat soleus muscle. Insulin reproducibly increased phosphorylation of a 20-kDa protein with a pI value of 6.0, which was identified subsequently as a phospho-isoform of P20, a small heat-shock-related protein. The adenylyl cyclase activator, forskolin, decreased phosphorylation of this P20 isoform and increased phosphorylation of another two P20 isoforms, with pI values of 5.9 and 5.6. Two-dimensional peptide mapping revealed that the phospho-peptides of these

three P20 isoforms are different. In contrast to its action in soleus muscle, insulin decreased phosphorylation of the P20 isoform with pI 6.0 and increased phosphorylation of the two isoforms with pI values of 5.9 and 5.6 in vascular smooth muscle. This effect is similar to that induced by vasodilatory stimuli, suggesting that insulin could exert its vasodilatory action by affecting phosphorylation of P20. In summary, these results demonstrate that insulin differently modulates phosphorylation of P20 in skeletal and smooth muscle, and suggest that P20 could be a potential modulator of insulin's functions in these tissues.

Key words: adenylyl cyclase, aorta, phosphoprotein, proteomics, signal transduction.

INTRODUCTION

Insulin is the principal hormone controlling glucose homeostasis and directing energy storage in mammals. It acts by stimulating glucose uptake in muscle and adipocytes, and suppressing hepatic glucose production [1,2]. In addition, insulin modifies the expression or activity of a variety of enzymes and transport systems in a wide range of cells [3]. The impairment of insulin's action (insulin resistance) in skeletal muscle, its major target tissue, is a common phenomenon in type-II diabetes as well as other human diseases such as hypertension, hyperlipidaemia and obesity [4].

Although considerable progress has been made in recent years, the detailed molecular mechanisms underlying insulin's biological functions and the cause of insulin resistance are still far from clear. Insulin exerts its actions by binding with the α -subunits of its receptor on the plasma membrane of the target tissue and activating the intrinsic tyrosine kinase activity of the β -subunit [3,5]. Activation of the receptor by autophosphorylation triggers tyrosine phosphorylation of its substrates, insulin receptor substrates, which in turn create binding sites that allow the recruitment and activation of multiple signal generators such as phosphatidylinositol 3-kinase and regulators of small GTPases such as Ras. Subsequently, a variety of protein kinases, such as mitogen-activated protein kinase, phosphoinositide-dependent protein kinase, protein kinase B and p70 S6 kinase, are activated to transmit and amplify the signals of insulin. The Ras/mitogen-activated protein kinase pathway plays a central role in cell-fate decisions, whereas the downstream signalling events of phosphatidylinositol 3-kinase are responsible for insulin's regulation of fuel metabolism such as glucose transport and glycogen synthesis [6]. In addition to activation of these serine/threonine kinase cascades, insulin also elicits its biological functions by

stimulating the activity of protein phosphatases such as protein phosphatase 1 (PP-1), which in turn dephosphorylates some intracellular enzymes and proteins [7].

Although a great number of protein kinases/phosphatases involved in insulin's actions has been identified, relatively few of their downstream substrates have been found and the links between these enzymes and the endpoint responses of insulin are still missing. In this study, we have used the technique of two-dimensional gel electrophoresis (2-DE) combined with sensitive metabolic labelling to analyse the insulin-evoked protein phosphorylation cascade in rat muscle tissues and thus to identify the potential proteins whose phosphorylation is affected by this hormone. Here, we describe that insulin modulates phosphorylation of P20, a small heat-shock-related protein that was reported to be functionally linked to smooth-muscle contraction and relaxation [8–11]. In soleus muscle, phosphorylation of a P20 isoform with pI 6.0 is increased by insulin. Interestingly, insulin's effect on phosphorylation of P20 in vascular smooth muscle is opposite to that in soleus muscle. The separate effects on phosphorylation of P20 in these two different muscle tissues could be associated with insulin's functions to stimulate glucose uptake in skeletal muscle and vasodilation in smooth muscle.

EXPERIMENTAL

Isolation and metabolic labelling of skeletal-muscle strips and aortic rings

All protocols were approved by the Institutional Animal Ethics Committee. Animals were kept in a temperature-controlled animal house in a 12:12-h light:dark cycle, with access to food and water *ad libitum*. Male Wistar rats (≈ 250 g) were anaesthetized with pentobarbital (5–7 mg/100 g of body weight, intra-

Abbreviations used: 2-DE, two-dimensional gel electrophoresis; CBB, Coomassie Brilliant Blue R250; HSP, heat-shock protein; PP-1, protein phosphatase 1.

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peritoneally) and soleus-muscle strips were prepared as described previously [12]. The thoracic aorta was excised, cleaned of adhering connective tissues and fat, and cut into rings of 2–3 mm in length. Muscle strips or aortic rings were pre-incubated in a shaking incubator at 30 °C for 1 h in 5 ml of Dulbecco's modified Eagle's medium without sodium phosphate to deplete the metabolic pools. All incubation media were gassed with a mixture of 95 % O₂/5 % CO₂. These samples were then radiolabelled with [³²P]orthophosphate (0.25 mCi/ml; ICN) for 4 h and treated with 70 nM human insulin (Actrapid; Novo Nordisk) or 10 μ M forskolin (Sigma) for 30 min. Reactions were terminated by freezing muscle strips in liquid nitrogen.

Muscle extraction and 2-DE

³²P-Radiolabelled muscle strips or aortic rings were solubilized in a sample buffer containing 9 M urea, 4 % w/v CHAPS, 2 % (v/v) pharmlate, pH 3–10, 200 mM dithiothreitol and 8 mM PMSF. The lysates were sonicated briefly, incubated on ice for 20 min and microcentrifuged at 13 500 g for 10 min to remove debris. Protein concentrations were determined by the Bradford method and radioactivity was measured by liquid scintillation counting. ³²P-Labelled lysates with equivalent amounts of radioactivity were isoelectrically focused on IPG Drystrip pH 4–7 Linear gels (Pharmacia) and subsequently separated by second-dimensional SDS/PAGE using a multiphor RII electrophoresis system (Pharmacia). The separated proteins were visualized by PhosphorImaging, autoradiography or Coomassie Brilliant Blue R250 (CBB) staining. The radioactivity of each protein spot was quantified by either Imagemaster™ 2D (Pharmacia) or MacBAS software (Fujifilm). All the results presented are based on at least three independent experiments.

Protein microcharacterization

Protein spots of interest were excised and pooled from CBB-stained preparative gels. Gel pieces were subjected to in-gel trypsin digestion as described by Rosenfeld et al. [13]. The extracted peptide mixture was fractionated by reversed-phase HPLC on a Jupiter 5 μ C₁₈ column (Phenomenex). One well-resolved tryptic peptide was subjected to amino acid sequencing using the Edman degradation method with a Perkin-Elmer (Procise, Model 492) protein sequencer.

Western blotting

Proteins from the muscle tissues were separated by 2-DE and transferred subsequently to nitrocellulose membranes using a Multiphor II Novablot electrophoretic transfer unit (Pharmacia). The membranes were then incubated with rabbit anti-P20 polyclonal antibody (1:1000) [14] or mouse anti-heat-shock protein (HSP) 27 monoclonal antibody (1:5000) [15] overnight at 4 °C and the bound antibodies were detected by either chemiluminescence (Amersham) or by detecting horseradish peroxidase activity with 4-chloronaphthol as substrate.

Immunoprecipitation and co-migration experiments

³²P-Labelled muscle tissues were solubilized by homogenizing in lysis buffer [2 % (w/v) CHAPS/50 mM Hepes, pH 7.5/100 mM NaCl/10 % (w/v) glycerol/5 mM EDTA/2 mM PMSF/10 units/ml apyrase] and incubated on ice for 30 min with shaking. The debris were removed by microcentrifugation and immune complexes formed by shaking the clarified muscle lysate with antisera overnight at 4 °C and recovered following addition of Protein A–Sepharose for 1 h. ³²P-Labelled immunoprecipitated proteins

were eluted from the beads by incubating with 2-DE lysis buffer at 60 °C for 30 min and mixed with unlabelled samples. The mixed samples were separated by 2-DE as above.

Two-dimensional thin-layer phosphopeptide mapping

The procedure of Boyle and co-workers [16] was followed with some modifications. ³²P-Radiolabelled phosphoproteins excised from the CBB gels were subjected to V8 protease (50 μ g/ml; Sigma) digestion. The digested peptide mixtures were extracted, lyophilized and solubilized in 10 μ l of electrophoresis buffer (1 % pyridine/10 % acetic acid, pH 3.5). Individual or mixed-sample aliquots were spotted in the middle of the TLC plate (20 cm $\times$ 20 cm; Sigma) 4 cm from the bottom along with a trace of Basic Fuchsin dye. Electrophoresis was carried out at 350 V until the Basic Fuchsin dye had migrated 2.5 cm towards the cathode. The plates were air-dried and subjected to vertical chromatography in pyridine/butanol/acetic acid/water (10:15:3:12, by vol.) until the solvent front reached the top of the plate. Plates were then air-dried and detected by autoradiography.

RESULTS

Phosphorylation of several proteins was modified by insulin in soleus muscle

Autoradiography of the 2-DE-separated ³²P-labelled soleus-muscle lysates revealed around 150 phosphoproteins (Figure 1). This phosphoprotein pattern was stable at 4 h after radio-

Figure 1 Phosphorylation of several proteins in rat soleus muscle is altered by insulin

Soleus-muscle strips were radiolabelled with ³²P for 4 h and treated subsequently with or without 70 nM insulin for 30 min. Aliquots of radiolabelled proteins, with equivalent amounts of radioactivity, were separated by 2-DE and visualized by autoradiography. The arrows and letter symbols indicate phosphoproteins affected by insulin as described in text.

Figure 2 Separation and characterization of insulin-responsive phosphoprotein A1 in soleus muscle

^{32}P -Labelled proteins (≈ 2 mg) from insulin-stimulated soleus muscles were separated by 2-DE and visualized by autoradiography and CBB staining. The position of phosphoprotein A1 in the CBB-stained gel was located by matching the gel with the corresponding autoradiogram. 2-DE-separated proteins from insulin-stimulated soleus muscle were analysed by Western blotting using anti-P20 polyclonal antibody. Two unphosphorylated isoforms of P20 are marked as a and b.

labelling, and did not vary significantly for up to 4 h further incubation in the absence of hormone (results not shown). Following treatment with 70 nM insulin, changes in phosphorylation of several groups of proteins were observed reproducibly. Quantitative analysis using PhosphorImaging revealed that phosphorylation of proteins A1 and B1 were increased by 2.3- and 2.6-fold, respectively, whereas phosphorylation of protein D1 was decreased by 2.2-fold following insulin treatment. In insulin-stimulated samples, radioactivities of a string of phosphoproteins (C1) with similar molecular masses but different pI values were 2- to 4-fold higher than in control samples.

Insulin-responsive phosphoprotein A1 is P20

To characterize further these insulin-responsive phosphoproteins, ≈ 100 μg of ^{32}P -labelled proteins and 2 mg of unlabelled proteins from insulin-treated soleus-muscle strips were mixed and separated by preparative 2-DE. The separated proteins were visualized by CBB staining and autoradiography. The positions of the insulin-responsive phosphoproteins were located by matching the gel with the corresponding autoradiogram. The result showed that only phosphoprotein A1 was abundant enough to be visualized by CBB staining (Figure 2). Phosphoproteins B1, C1 and D1 were not detected at all by either CBB staining or

Figure 3 The effect of forskolin on phosphorylation of P20 in skeletal muscle

^{32}P -Pre-labelled soleus-muscle strips were treated without (left-hand panels) or with 10 μM forskolin (right-hand panels) and separated by 2-DE. The separated proteins were then visualized by autoradiography (A), CBB staining (B) and Western blotting using anti-P20 (C), as described in Figure 2. The three phosphorylated P20 isoforms are denoted A1, A2 and A3. The two unphosphorylated P20 isoforms are marked as a and b.

silver staining (results not shown), suggesting that the abundance of these proteins is extremely low.

To characterize phosphoprotein A1 further, it was excised from CBB-stained gels, digested by trypsin and the tryptic peptide mixture separated by reversed-phase HPLC. One well-resolved tryptic peptide was chosen for amino acid sequencing, which yielded the N-terminal sequence VVGHDHVEVHA. Database comparison (available on the worldwide web at <http://expasy.hcuge.ch>) revealed that this sequence is exclusively and completely assigned to P20, a small heat-shock-related protein that was initially co-purified with HSP27/HSP28 from rat and human skeletal muscle [14]. The result of immunoblotting using rabbit polyclonal antibody against P20 further confirmed the identity of phosphoprotein A1 as P20 (Figure 2). In addition, this analysis detected two unphosphorylated isoforms of P20 with pI values of 6.4 and 6.5 respectively.

Forskolin stimulation produces two distinctive P20 phospho-isoforms with pI values of 5.9 and 5.6 in soleus muscle

Forskolin, a naturally occurring diterpene, can allosterically stimulate adenylyl cyclase and has been used extensively to increase cAMP and to elicit cAMP-dependent physiological responses [17]. Previous studies have shown that there are three phosphorylated isoforms of P20, with pI values of 6.0, 5.9 and 5.7, in smooth muscle [10]. Forskolin can increase phosphorylation of the latter two isoforms. Thus we also investigated whether forskolin influences the phosphorylation of P20 and thus evokes production of additional phospho-isoforms of this protein in soleus muscle. Stimulation of soleus muscle with forskolin evoked phosphorylation of two 20-kDa protein species with pI values of 5.9 and 5.6, both of which were immunoreactive with anti-P20 antibody (Figure 3). In contrast, phosphorylation of the P20 isoform with pI 6.0 was decreased 2.2-fold following forskolin treatment. This result indicates that phosphorylation

Figure 4 The effect of insulin and forskolin on phosphorylation of P20 in vascular smooth muscle

Rat aortic rings were radiolabelled with ^{32}P for 4 h and stimulated subsequently without (A) or with 10 μM forskolin (B) or 70 nM insulin (C). A portion of each sample was subjected to immunoprecipitation using anti-P20 polyclonal antibody. The muscle lysates (left-hand panels) and the immunoprecipitated complexes (right-hand panels) were separated by 2-DE and visualized by autoradiography. The three phosphorylated isoforms of P20 are denoted A1, A2 and A3. The circled proteins are two phospho-isoforms of HSP27 that were co-precipitated by the anti-P20 antibody. The histogram (D) represents the increased/decreased radioactivities of the three P20 isoforms relative to those in control samples.

of P20 responds distinctively to insulin and forskolin, which produce different phospho-isoforms of this protein in soleus muscle.

Insulin and forskolin exert similar effects on phosphorylation of P20 in rat smooth muscle

Recent studies suggest that phosphorylation of P20 is involved in the regulation of smooth-muscle contraction/relaxation [8–10]. Insulin has also been proposed as a potential vasodilator controlling vascular tone [18,19]. Therefore, we investigated the phosphorylation of P20 and its responsiveness to insulin and forskolin in smooth muscle. To do this, ^{32}P -labelled samples from control, insulin- or forskolin-stimulated aortic rings were analysed by 2-DE as above and the spots corresponding to P20 phospho-isoforms were located by immunoprecipitation (Figure 4). All three phospho-isoforms can be detected in control smooth muscle (Figure 4A). Remarkably, insulin's action on phosphorylation of P20 in smooth muscle is opposite to that observed in soleus muscle and is similar to that evoked by forskolin (Figures 4B and 4C). Both insulin and forskolin significantly

Figure 5 Two-dimensional mapping of phosphopeptides derived from the three phospho-isoforms of P20 in soleus muscle

Extracts from forskolin-stimulated soleus muscle labelled with ^{32}P were separated by 2-DE. The three phospho-isoforms of P20 (A1 with pI 6.0, A2 with pI 5.9 and A3 with pI 5.6) were excised from the gel and subjected to endopeptidase V8 digestion. The resulting peptides or their respective mixtures were analysed by two-dimensional mapping as described in the Experimental section.

increased the phosphorylation of P20 isoforms with pI values of 5.9 and 5.7 and decreased the phosphorylation of the isoform with a pI of 6.0. In addition to the three phospho-isoforms of P20, two phosphoproteins with slightly larger molecular masses were also co-immunoprecipitated by the anti-P20 antibody. Both of these phosphoproteins were identified as isoforms of HSP27 by immunoprecipitation and by Western blotting using the anti-HSP27 antibody (results not shown). This result is consistent with previous reports that P20 is associated with HSP27 [14,20].

The three phospho-isoforms of P20 are phosphorylated at different sites

To elucidate the relationship between the three phosphorylated isoforms of P20, the corresponding ^{32}P -labelled phosphoproteins (A1 with pI 6.0, A2 with pI 5.9 and A3 with pI 5.6) in 2-DE gels generated from forskolin-stimulated soleus muscles were excised individually and digested with V8 protease. The resulting peptide mixtures were then analysed by two-dimensional phosphopeptide mapping (Figure 5). This revealed that there was only a single ^{32}P -labelled phosphopeptide in the peptides derived from each of the p20 isoforms A1 and A2. The two phosphopeptides from A1 and A2 migrated to different positions on the plates, suggesting that the phosphorylation of A1 and A2 occurred at different sites in P20. Three ^{32}P -labelled phosphopeptides were detected in the

V8-digested mixture of A3, one of which co-migrated with the phosphopeptide derived from A2. The mobilities of the other two phosphopeptides were different from those derived from either A1 or A2. These findings demonstrate that the existence of three phosphorylated isoforms of P20 is due to phosphorylation of the parent molecule at distinct sites.

DISCUSSION

Skeletal muscle is the major tissue of insulin-stimulated glucose disposal, as it accounts for over 80 % of whole-body glucose disposal [21]. To gain insight into the molecular mechanisms underlying insulin's metabolic actions, we have investigated the insulin-evoked phosphorylation cascades in rat skeletal muscle using 2-DE-based proteome analysis. Among hundreds of resolved phosphoproteins, phosphorylation of four of these was found to be detectably modulated by insulin (Figure 1). One of the insulin-responsive proteins with high abundance was identified as a phospho-isoform of P20, with a pI value of 6.0. The protein-kinase-A-pathway activator forskolin decreased phosphorylation of this P20 isoform and stimulated phosphorylation of another two isoforms of the same protein, with pI values of 5.9 and 5.7 (Figure 3). Two-dimensional mapping of V8-digested peptide mixtures indicated that these three isoforms of P20 were phosphorylated at different sites (Figure 5).

P20 was co-purified originally with the small heat-shock protein HSP27/HSP28 from rat and human skeletal muscle [14]. This protein is abundant in muscle tissue, especially in soleus muscle [22]. It has significant sequence similarities with α B crystallin (47 %) and HSP27 (35 %) and also shares some properties with these two proteins, such as self-aggregation and translocation after heat shock. However, P20 is not inducible by heat-shock stress [14]. Several recent studies on vascular smooth muscles suggested that phosphorylation of P20 might be involved in regulating muscle contractile activity [8–10]. The relaxation of several types of smooth muscle by the cAMP activator, forskolin, or the cGMP-activator, sodium nitroprusside, is associated with an increase in the extent of phosphorylation of two P20 isoforms, with pI values of 5.9 and 5.7 [23,24]. Conversely, stimuli that induce contraction of vascular smooth-muscle tissues elicit an increase in phosphorylation of another P20 isoform of pI 6.0 [25]. P20 was shown recently to be associated with actin in a phosphorylation-dependent manner, suggesting that this protein may regulate smooth-muscle relaxation/contraction via direct interaction with contractile elements [8].

In the current study, we showed that phosphorylation of the P20 isoform with a pI of 6.0 in rat soleus muscle, which is equivalent to that associated with contraction of smooth muscle, was increased significantly following insulin stimulation (Figure 1). Notably, both insulin and contraction play important roles in increasing muscle glucose uptake [26,27]. Although the signalling pathways employed by the two stimuli are different, many studies suggested that they have a convergent course. First, insulin and muscle contraction can enhance each other's sensitivity and thus synergistically stimulate glucose transport [26,28]. Secondly, both insulin- and contraction-stimulated glucose transport can be inhibited by wortmannin and calcium-channel blockers [27,29]. Muscle contractility was also decreased by high concentrations of wortmannin. Lastly, both these stimuli can induce a redistribution of the 'insulin-responsive' glucose-transporter isoform GLUT4 from an intracellular compartment to the plasma membrane [30]. Based on this evidence, we speculate that insulin- and contraction-stimuli-induced signalling cascades might converge on P20 and, by modulating phosphorylation of

this protein, synergistically increase glucose uptake in skeletal muscle.

Insulin and its physiological antagonists sometimes oppose each other's actions by competitively phosphorylating a common substrate at different sites. For example, protein kinase A inhibits the activity of PP-1 in skeletal muscle by phosphorylating both its glycogen-targeting subunit (G subunit) and inhibitor 1. In contrast, insulin activates PP-1 activity by phosphorylating the G subunit at a site different from that modified by protein kinase A [7]. Our results showed that the protein-kinase-A-pathway activator forskolin and insulin differentially influence phosphorylation of P20, suggesting that signalling cascades of insulin and its antagonists might also converge on this protein in skeletal muscle.

In addition to its metabolic functions, insulin is also a potent vasodilator involved in the regulation of vascular tone [19,31,32]. Insulin has been shown to exert direct vasodilatory actions in vascular smooth muscle from several vascular beds, including rat aorta, human pulmonary artery, canine femoral artery and rabbit efferent arteriole [33–35]. Moreover, via its effect to cause skeletal-muscle vasodilation, insulin can enhance its overall action to promote glucose uptake by increasing the delivery of both insulin and glucose to skeletal muscle [32,36]. Decreased insulin-mediated vasodilation is associated with resistance to insulin-induced glucose uptake, and may also account for the increased vascular tone and the development of hypertension [19,36,37]. Many studies indicate that insulin may stimulate vasodilation by increasing the production of nitric oxide and elevating cGMP and cAMP levels [34,38]. However, the exact molecular mechanisms underlying insulin's vasodilatory action are still unknown. Our results demonstrate that insulin decreases phosphorylation of the P20 isoform with pI 6.0 and increases the phosphorylation of the two P20 isoforms with pI 5.9 and pI 5.6 in smooth muscle (Figure 4). This effect is similar with that evoked by the cAMP activator forskolin. Therefore, P20 could potentially mediate insulin's vasodilatory action.

In summary, we have demonstrated that insulin distinctively modulates phosphorylation of P20 and produces different phospho-isoforms in smooth and skeletal muscle of rats. Further study is required to define the precise physiological relevance and the upstream signalling pathways for these actions.

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