Alteration in Phosphorylation of P20 Is Associated With Insulin Resistance

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We have recently identified a small phosphoprotein, P20, as a common intracellular target for insulin and several of its antagonists, including amylin, epinephrine, and calcitonin gene-related peptide. These hormones elicit phosphorylation of P20 at its different sites, producing three phosphorylated isoforms: S1 with an isoelectric point (pI) value of 6.0, S2 with a pI value of 5.9, and S3 with a pI value of 5.6 (FEBS Letters 457:149–152 and 462:25–30, 1999). In the current study, we showed that P20 is one of the most abundant phosphoproteins in rat extensor digitorum longus (EDL) muscle. Insulin and amylin antagonize each other’s actions in the phosphorylation of this protein in rat EDL muscle. Insulin inhibits amylin-evoked phosphorylation of S2 and S3, whereas amylin decreases insulin-induced phosphorylation of S1. In rats made insulin resistant by dexamethasone treatment, levels of the phosphoisoforms S2 and S3, which were barely detectable in healthy rats in the absence of hormone stimulation, were significantly increased. Moreover, the ability of insulin to inhibit amylin-evoked phosphorylation of these two isoforms was greatly attenuated. These results suggested that alterations in the phosphorylation of P20 might be associated with insulin resistance and that P20 could serve as a useful marker to dissect the cellular mechanisms of this disease. Diabetes 50: 1821–1827, 2001

Insulin resistance is characterized by diminished insulin sensitivity of target tissues, including liver, skeletal muscle, and adipocytes (1). It is a key factor in the pathogenesis of type 2 diabetes and is also associated with other pathological states, such as obesity, dyslipidemia, hyperinsulinemia, hypertension, and cardiovascular disease. These clustering metabolic defects have been termed “syndrome X” or “the insulin-resistance syndrome” (2).

The molecular basis of insulin resistance is extremely complex and multifactorial. Defects in several steps of insulin action, such as the activation of insulin receptors, postreceptor signal transduction, and the glucose transport effector system, have been implicated in this disease (3,4). Defective insulin receptor kinase activity, reduced insulin receptor substrate-1 tyrosine phosphorylation, and decreased phosphatidylinositol (PI)-3 kinase phosphorylation, and decreased phosphatidylinositol (PI)-3 kinase activity were observed in both human type 2 diabetic patients as well as animal models, such as ob/ob mice (5,6).

In addition to the intrinsic defects of the insulin receptor and postreceptor signaling components, other circulating factors, such as tumor necrosis factor-α, leptin, free fatty acids, and amylin, may also contribute to the pathogenesis of insulin resistance (7–11). For example, amylin, a hormone co-secreted with insulin from pancreatic islet β-cells, has been shown to antagonize insulin’s metabolic actions both in vivo and in vitro (12–16). It can inhibit insulin-stimulated glucose uptake and glycogen synthesis. In vivo administration of amylin results in hyperglycemia and induced insulin resistance similar to that observed in type 2 diabetes. Although some earlier studies have suggested that amylin’s biological effects on fuel metabolism were only of pharmacological interest, more recent in vivo studies with an amylin-selective antagonist have strongly supported its physiological relevance (17). Moreover, amylin-deficient mice have shown increased insulin responsiveness and more rapid blood glucose elimination after glucose loading, further confirming the role of amylin in causing insulin resistance (18). Indeed, elevated levels of circulating amylin (hyperamylinemia) and an increased ratio of amylin to insulin have been observed in patients with type 2 diabetes and other diseases associated with insulin resistance, such as obesity and glucose intolerance (19).

Despite these advances, the detailed cellular mechanisms of insulin resistance are far from clear. Recent studies that have used genetic approaches to identify specific genes that account for the genetic predisposition to this disease have been generally unrewarding (4,20–22). We recently used comparative proteomic analysis to systematically investigate the phosphorylation cascades evoked by insulin and its antagonists in rat skeletal muscle and identified a novel phosphoprotein, P20, as the common intracellular target of these hormones (23,24). Insulin and its antagonistic hormones amylin, epinephrine, and calcitonin gene-related peptide (CGRP), through distinctive signaling pathways, phosphorylate P20 at different serine residues to produce multiple phosphoisoforms of this protein. In this study, we demonstrated that P20 in skeletal muscle from insulin-resistant diabetic rats has an abnormal phosphorylation pattern, although the expression level of this protein is not changed. Moreover, the responsiveness of

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CGRP, calcitonin gene-related peptide; 2-DE, two-dimensional gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; ECL, enhanced chemiluminescence; EDL, extensor digitorum longus; GLUT4, myc-tagged GLUT4; HSP, heat shock protein; KHB, Krebs-Henseleit buffer; OD, optical density; pI, isoelectric point; PSL, photostimulated luminescence; VOL, a feature’s volume.
P20 to insulin and amylin is also altered in insulin-resistant animals. We propose that P20 may serve as a useful marker for investigating the mechanisms of insulin resistance.

RESEARCH DESIGN AND METHODS

Materials. Male Wistar rats were fed standard rat diet (NRM Diet 58, Tegal, Auckland, New Zealand) with water ad libitum. [3H]orthophosphate and α-[3H]D-glucose (specific activity 1067 Ci/mmol) was obtained from DuPont-NEN and [3-14C]glucose (specific activity 16.0 Ci/mmol) was obtained from Amersham Pharmacia. Human insulin (Actrapid) was obtained from Novo Nordisk. Rat amylin and CGRP were purchased from Bachem (Torrance, CA); epinephrine was from Pfizer (New York, NY); and dexamethasone was from Sigma.

Establishment of the dexamethasone- or high fat–induced rat models of insulin resistance. All experimental protocols were approved by the Institutional Animal Ethics Committee. Male Wistar rats were injected with dexamethasone (3.1 mg·kg⁻¹·day⁻¹, i.p.) for 7 days. The weight of rats in both control and dexamethasone-treated groups were monitored daily. By the end of the treatment period, the mean weight of the control group had increased by 12 ± 1%, whereas that of the glucocorticoid-treated group had sharply decreased, by 16 ± 2% (n = 3 experiments, each with three rats per group). Rats were fasted for 18 h before each experiment and were killed by cervical dislocation. Blood was obtained by cardiac puncture from anesthetized animals. The mean glucose concentration was 5 ± 0.2 and 10.8 ± 0.6 mmol/l in control and dexamethasone-treated rats, respectively, as measured by the hexokinase method.

Western blot analysis. Total cellular RNA was isolated from EDL muscle of 18 h–fasted control and dexamethasone-treated rats using TRIzol reagent (Life Technology). Then, 15 µg of RNA from each sample were separated by 1.5% agarose gel electrophoresis and subsequently transferred to Hybond-N+ nylon membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) by capillary blotting at 20°C in sodium chloride–sodium citrate. The P20 cDNA probe was labeled with [32P]dCTP using a random primer labeling system. The membranes were preincubated with hybridization buffer (0.5 mol/l Na,HPO₄ [pH 7.2], 10 mmol/l EDTA, 7% SDS) for 3 h at 65°C and subsequently incubated with fresh buffer containing the labeled probe for 18 h. Membranes were then washed, analyzed using a phosphorimager, and quantitated by MacBAS v2.5 software (Fuji Machine Manufacturing, Chiryu, Aichi, Japan). For comparison, RNA samples from EDL muscle strips treated with or without 50 nmol/l amylin were also analyzed in parallel.

Phosphoprotein P20 and Insulin Resistance

Measures. Male Wistar rats were separated by SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C and incubated with the corresponding antibody (1:1,000) for 2 h at room temperature. After incubation with streptavidin-biotinylated horseradish peroxidase–conjugated secondary antibody for another hour at room temperature, the proteins immunoreactive to the primary antibody were visualized by ECL detection according to the manufacturer’s instructions.

RESULTS AND DISCUSSION

P20 is the major insulin-responsive phosphoprotein in rat skeletal muscle detected by 2-DE. P20 was initially isolated from rat skeletal muscle as a byproduct during the purification of the small heat shock proteins (HSPs) HSP27/28 and α-crystallin (25). Under normal physiological conditions, it exists as large aggregates. P20 has been thought to be a heat shock–related protein, given that it...
has significant amino acid sequence similarity with αB-crystallin (47%) and HSP27/28 (35%) (25,28). However, unlike other small HSPs, heat treatment or chemical stress does not induce the expression of P20. Several recent studies have suggested that P20 may be an actin-binding protein that is involved in cyclic nucleotide-mediated vasodilation and relaxation of rat smooth muscle or histamine- and phorbol ester–induced contraction of bovine carotid artery smooth muscle (29–31). Interestingly, this protein is also present at high concentrations in circulating whole blood in patients with vascular diseases. It can strongly suppress platelet aggregation in vitro and ex vivo, possibly by inhibiting receptor-mediated calcium influx in platelets (32). However, the precise physiological functions of P20 are still uncertain.

Analysis of the protein content of P20 by Western blot showed that this protein is mainly expressed in rat soleus muscle, EDL muscle, and heart muscle tissues, which account for 35.1 ± 3.2, 29.6 ± 2.7, and 23.3 ± 2.5% of the total P20 in all the tested tissues, respectively (n = 3; means ± SD) (Fig. 1). A small amount of this protein was also detected in smooth muscle (4.9 ± 0.6%), adipose tissue (1.9 ± 0.3%), and blood (5.2 ± 0.6%). 2-DE analysis of 32P-labeled rat EDL muscle revealed ~150 phosphoproteins after insulin stimulation (Fig. 2). Quantitative analysis by Melanie II software (Bio-Rad) revealed that P20 is the second most abundant phosphoprotein in insulin-stimulated rat EDL muscle, representing >2% of the total VOL for all features detected. Moreover, P20 is the only detected phosphoprotein that is responsive to both insulin and its antagonists, as analyzed by the proteome approach.

**Interplay between insulin and amylin on phosphorylation of P20.** Our previous studies demonstrated that insulin and its antagonists, epinephrine, amylin, and CGRP, elicit differential phosphorylation on different sites of P20, thus producing three phosphorylated isoelectric variants of P20 (termed S1, with an isoelectric point [pI] value of 6.0; S2, with a pI value of 5.9; and S3, with a pI value of 5.6) (23,24). Phosphorylation of S1 occurs at serine 157 of P20, and insulin can increase its phosphorylation through a PI-3 kinase–mediated pathway. Amylin, CGRP, and epinephrine evoke phosphorylation at Ser16 of P20 through a cAMP-mediated pathway, leading to the production of the phosphoisof orm S2. In addition, these catabolic hormones also induce the phosphorylation of P20 at another two unidentified sites to produce the phosphoisof orm S3.

Here, we further investigated the effect of the interplay between insulin and several of its antagonists on the phosphorylation of P20. Interestingly, we found that insulin and amylin can antagonize each other’s actions in the phosphorylation of this protein (Fig. 3). On the one hand, insulin-induced phosphorylation of S1 was significantly decreased in the presence of amylin. Phosphorylation of S1 in samples treated with 50 nmol/l insulin + 50 nmol/l amylin was 49% lower than that in samples stimulated with 50 nmol/l insulin alone. On the other hand, insulin blocked amylin-evoked phosphorylation of S2 and S3. In the presence of insulin, phosphorylation of S2 and S3 was decreased by ~72 and ~74%, respectively, relative to that in muscles treated with amylin alone. However, insulin had no effect on the phosphorylation of S2 and S3 induced by the other two catabolic hormones, epinephrine and CGRP, and vice versa. This result indicated that cross-talk occurs between only the insulin- and amylin-evoked signaling pathways, although all three catabolic hormones are thought to act through G-protein–coupled receptors and to have similar metabolic effects. Amylin inhibited the insulin-evoked PI-3 kinase cascade–mediated phosphorylation of S1. Conversely, insulin suppressed the amylin-evoked cAMP pathway–mediated phosphorylation of S2 and S3. Such an inhibitory effect of insulin on amylin’s biological actions could provide a reasonable explanation as to why administration of exogenous amylin in physiological quantities did not induce hyperglycemia and insulin resistance in some experimental systems.

The fact that insulin has separate effects on the inhibition of biological actions of amylin and CGRP further excludes the possibility that amylin acts solely through a CGRP receptor, although the two peptide hormones are members of the calcitonin-related polypeptide family (33). The amylin-specific receptor still remains to be identified.
However, several recent studies have suggested that the identity of an amylin-selective receptor may be determined in part by receptor activity-modifying proteins (34).

Alteration in phosphorylation of P20, but not its expression, is associated with insulin resistance. We next investigated the phosphorylation patterns of P20 and the effect of insulin and amylin on this protein in dexamethasone-induced diabetic rats with insulin resistance. The diabetic state of these rats was confirmed by the demonstrated loss of body weight, hyperglycemia, and decrease in insulin-stimulated incorporation of glucose into glycogen (results not shown). In dexamethasone-treated rats, the fasted basal plasma concentrations of both insulin (789 ± 694 vs. 203 ± 628 pmol/l in control rats) and amylin (144 ± 617 vs. 22.7 ± 5.9 pmol/l in control rats) were significantly increased (P < 0.05 in each case).

EDL muscle strips from these rats were radiolabeled with 32P and treated with or without insulin and amylin. Analysis of P20 revealed that the mRNA level and protein abundance of P20 was not changed in either the diabetic rats or the amylin-treated muscle strips (Fig. 6). These results indicated that the increased phosphorylation of S2 and S3 was not attributable to the increased expression of P20, but rather to a phosphorimaging (Fig. 4). Under the incubation conditions without hormone stimulation, phosphorylation of S2 and S3 was hardly detected in the nondiabetic control rats (Fig. 4A). By contrast, these two phosphoisoforms were clearly visualized in muscle samples from the insulin-resistant rats (Fig. 4B). Quantitative analysis by phosphorimager and MacBAS software showed that the signals associated with both S2 and S3 in dexamethasone-treated rats were about fivefold higher (Table 1). This phenomenon was also observed in a high-fat-induced insulin-resistant rat model (Fig. 5), suggesting that the increased phosphorylation of two isoforms of P20 (S2 and S3) may be associated with insulin-resistant states in general. Analysis of P20 expression revealed that the mRNA level and protein abundance of P20 was not changed in either the diabetic rats or the amylin-treated muscle strips (Fig. 6). These results indicated that the increased phosphorylation of S2 and S3 was not attributable to the increased expression of P20, but rather to a
The absence of hormone (basal state), in the presence of insulin (50 nmol/l), amylin (50 nmol/l), or both hormones. 32P-labeled isoforms of P20 from an intracellular pool to the plasma membrane, al-

Another major alteration in insulin-resistant rats is insulin’s ability to inhibit amylin-evoked phosphorylation of S2 and S3. In normal rats, 50 nmol/l insulin decreased phosphorylation of S2 and S3 by 71.6 and 73%, respectively, compared with that in samples treated with 50 nmol/l amylin alone (Figs. 4E and G). In diabetic rats, on the other hand, amylin-evoked phosphorylation of S2 and S3 was little affected by insulin (Figs. 4F and H). Under this condition, the radioactivity of both S2 and S3 was ∼3.3-fold higher than that of nondiabetic control rats (Table 1).

Insulin resistance is a well-known effect of glucocorticoid excess, but its mechanisms are still uncertain (35). Although muscle is quantitatively the most important tissue for glucose disposal in response to insulin, there are few studies on the effects of glucocorticoids in this tissue. Administration of dexamethasone did not affect the number or affinity of insulin receptors in skeletal muscle but decreased IRS-1 activation of PI-3 kinase, suggesting the existence of postreceptor defects (36). It has recently been reported that dexamethasone treatment significantly inhibited the insulin-stimulated translocation of GLUT4 from an intracellular pool to the plasma membrane, al-

![Healthy rats vs. High-fat rats](image)

**FIG. 5.** Enhanced phosphorylation of S2 and S3 is associated with insulin-resistant rats induced by high-fat feeding. Protein (100 μg) from muscle strips from healthy rats or high fat–induced diabetic rats was separated by 2-DE, and the three phosphoisoforms of P20 (S1, S2, and S3) were visualized by probing with anti-P20 antibody, as described in Fig. 1. The table in the lower panel represents the quantitative analysis for the abundance of each phosphoisoform of P20 in nondiabetic control rats and high fat–induced diabetic rats. The abundance of each isoform is expressed as the mean PSL value ± SD. *P < 0.01 vs. corresponding values in control rats (n = 4).

**TABLE 1**

<table>
<thead>
<tr>
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<th>Nondiabetic control rats</th>
<th>Dexamethasone-treated rats</th>
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<tbody>
<tr>
<td></td>
<td>S1</td>
<td>S2</td>
</tr>
<tr>
<td>Basal state</td>
<td>434 ± 13</td>
<td>21.6 ± 1.9</td>
</tr>
<tr>
<td>Insulin</td>
<td>831 ± 40</td>
<td>20.3 ± 3.4</td>
</tr>
<tr>
<td>Amylin</td>
<td>191 ± 9</td>
<td>289 ± 20</td>
</tr>
<tr>
<td>Insulin + amylin</td>
<td>417 ± 16</td>
<td>82 ± 4</td>
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Data are mean PSL values ± SD. Radiolabeled EDL muscle strips from control and dexamethasone-treated rats were incubated in the absence of hormone (basal state), in the presence of insulin (50 nmol/l), amylin (50 nmol/l), or both hormones. 32P-labeled isoforms of P20 (S1, S2, and S3) were separated, as in Fig. 4, detected using a phosphorimagery, and analyzed by MacBAS software. *P < 0.01 vs. corresponding values in control rats (n = 4).

Though expression of this transporter was paradoxically slightly increased (37).

Pieber et al. (38) observed that whenever diabetes occurred in dexamethasone-treated rats, the level of amylin and the ratio of amylin to insulin were significantly increased. The increase in the amylin-to-insulin ratio was associated with elevated content of proamylin mRNA relative to proinsulin mRNA. Those study results implied that amylin could also be an important contributing factor to the development of dexamethasone-induced insulin resistance. The results of our present study support such a role for amylin. The phosphoisoforms S2 and S3, which were hardly detected in healthy rats but could be induced by amylin, were clearly present in diabetic rats (Fig. 4B). This may have been because of the increased amylin level or the increased amylin-to-insulin ratio. It is interesting to note that in normal rats, insulin specifically suppresses amylin’s actions on the phosphorylation of P20 and elevation of cAMP levels, but has no detectable effect on the actions of two other catabolic hormones, epinephrine and CGRP (Fig. 3). Such an action of insulin was significantly

![Image of mRNA abundance and protein concentration of P20](image)

**FIG. 6.** mRNA abundance and protein concentration of P20 is not altered in rats treated with dexamethasone. I: Northern blot analysis. RNA was prepared from the EDL muscles of saline- (A) or dexamethasone-injected rats (B) or EDL muscle strips treated without (C) or with 50 nmol/l amylin (D) for 30 min in vitro, blotted, and probed with the labeled P20 cDNA. The negative image of the ethidium bromide–stained RNA loaded in each lane is also shown. Quantitative analysis was performed using a phosphorimagery. II: Western blot analysis of P20. Total protein (30 μg) from EDL muscles treated as for Northern blot analysis was separated by 12.5% SDS-PAGE and probed with anti-P20 antibody, as in Fig. 1. III: Table represents the increased/decreased fold in P20 mRNA and protein levels under the respective treatment, relative to saline-treated control rats. Results are means ± SD from three individual experiments.
could regulate glucose transport by modulating the phosphorylation states of P20.

To validate this hypothesis, we established stable transfectants of L6 cells that overexpress P20 (Fig. 7A). GLUT4myc was also coexpressed in these transfectants to increase insulin sensitivity (27). In the myotube cells overexpressing GLUT4myc alone, 50 nmol/l insulin increased 2-deoxyglucose uptake by 2.94 ± 0.31-fold over the basal level (Fig. 7B). This insulin-stimulated glucose uptake was decreased by 28% in the presence of 50 nmol/l amylin. However, in cells overexpressing both P20 and GLUT4myc, insulin-stimulated glucose uptake was significantly decreased by 41 ± 3% (n = 4; P < 0.05), whereas the inhibitory effect of amylin was significantly increased by 24 ± 2% (n = 4; P < 0.05). These results demonstrated that overexpression of P20 suppresses insulin-stimulated glucose uptake and enhances amylin's ability to inhibit insulin's action in L6 myotubes, thus suggesting a direct role of this protein in the regulation of glucose metabolism. The cellular mechanisms underlying such a regulatory role of P20 are currently under investigation in our laboratory.

In summary, our results demonstrated that insulin resistance in skeletal muscle is associated with the appearance of the two P20 phosphoisoforms, S2 and S3, and also with the inability of insulin to suppress the phosphorylation of these two isoforms. The role of P20 in the signal transduction pathways of insulin and amylin in skeletal muscle and the significance of the alterations in phosphorylation of this protein in insulin resistance remain to be clarified. Nevertheless, working backward toward receptors from phosphorylation of the three isoforms of P20 could serve as a method for elucidating postreceptor events of amylin and insulin and the functional interactions between these two hormones. Further studies on the intracellular defect that leads to the alteration of P20 phosphorylation in the insulin-resistant state could also help in understanding the pathogenesis of this disease.

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FIG. 7. The effect of P20 overexpression on glucose uptake in L6 myotubes. A: L6 cells were transfected with pCXN2-GLUT4myc or pCXN2-GLUT4myc and pcDNA.P20. Following selection with 400 μg/ml G418, clones expressing GLUT4myc alone or GLUT4myc + P20 were expanded and differentiated, as described in RESEARCH DESIGN AND METHODS. Cell lysates (30 μg) from L6 myotubes were separated by 10% SDS-PAGE. The levels of P20 and GLUT4myc expression were analyzed by Western blot using specific anti-p20 and anti-GLUT4 antibodies, respectively. B: The cell lines selected in A were differentiated in six-well plates and assayed for 2-deoxyglucose uptake in response to insulin or insulin plus amylin, as described in RESEARCH DESIGN AND METHODS (n = 4; means ± SD). Shown is the result of a typical experiment; similar results were also obtained from at least another two independent transfectants that express GLUT4myc (●) or GLUT4myc + P20 (■). *P < 0.01 vs. corresponding values in cells overexpressing GLUT4myc alone.

attenuated in dexamethasone-induced diabetic rats (Figs. 4F and H). Based on these results, it is tempting to speculate that under physiological conditions, amylin’s antagonism of insulin-stimulated glucose disposal is inhibited by insulin itself. The impairment of this action by insulin may lead to the enhanced catabolic action of amylin and thus partly cause insulin resistance in dexamethasone-induced diabetic rats.

P20 is involved in the regulation of the glucose uptake process in L6 myotube cells. Although the physiological role of P20 is uncertain, the high abundance of this protein and its diverse responsiveness to insulin and its antagonists suggest that it could be a mediator involved in the biological actions of these metabolic hormones. Notably, P20 has recently been shown to be an actin-binding protein (31). Both cytoskeletal actin filaments and actin-binding proteins have been suggested to play a role in directing traffic of glucose transporters to the cell membrane (39,40). Interestingly, two other proteins whose increased expression may contribute to insulin resistance in type 2 diabetes, Rad and PED/PEA-15, are also cytoskeleton-associated proteins involved in the regulation of glucose transport (41,42). Thus, it is intriguing to speculate that metabolic hormones, such as insulin and amylin,
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