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Insulin-induced phosphorylation of a 38 kDa DNA-binding protein in ventricular cardiomyocytes: possible implication of nuclear protein phosphatase activity

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Abstract

Ventricular cardiomyocytes isolated from adult rat heart were used to analyze the effect of insulin on the phosphorylation of DNA-binding nuclear proteins and to elucidate the potential involvement of protein phosphatase-1 (PP-1) and PP-2A in this hormonal action. Cells were labelled with [^{33}P]orthophosphate, stimulated with insulin (1.7×10^{-7} M) and processed for the isolation of nuclei and extraction of DNA-binding proteins. Insulin was found to induce a rapid and constant increase in the serine/threonine phosphorylation of a 38 kDa DNA-binding protein, reaching 150% of control after 15 min and 180% after 150 min. Immunoprecipitation and Western blotting experiments revealed the presence of phosphorylated numatrin in the nuclear extract, however, insulin did not modify its phosphorylation state. Treatment of cardiomyocytes with okadaic acid (1 μM) resulted in a large increase (246 $\pm$ 30%) in the phosphorylation of the 38 kDa protein. Using ^{32}P-labelled phosphorylase as a substrate, we observed a significant inhibition of nuclear PP-1 activity to 38.5 $\pm$ 7% ($n = 3$) of control after incubation of cardiomyocytes with insulin for 15 min. PP-2A, which corresponds to about 25% of total phosphatase activity, was also inhibited to the same extent. These data show the presence of an insulin-responsive 38 kDa DNA-binding phosphoprotein in the nucleus of cardiomyocytes, which is at least partly regulated by nuclear phosphatase activity. It is suggested that inhibition of nuclear PP-1 and PP-2A represents a possible mechanism of insulin signalling to the nucleus of target cells.

Keywords: Cardiomyocytes; Nucleus; Protein phosphatase; Insulin

1. Introduction

Insulin exerts a pleiotropic pattern of hormonal actions ranging from acute rapid regulations of a variety of metabolic processes up to long-term effects on cell growth and differentiation (Kahn, 1994; O'Brien and Granner, 1991). It is now widely accepted that the transduction of the insulin signal from its cell surface receptors to the cytoplasm involves the tyrosine kinase activity of the receptor and a complex cascade of serine/threonine protein kinases and phosphatases (White and Kahn, 1994). In order to perform transcriptional control of gene expression the insulin signal must be extended to the nucleus. This process, however, remains largely unknown.

It is reasonable to believe that protein phosphorylation/dephosphorylation may also represent a major pathway for insulin signalling at the nuclear level. This assumption is supported by the observation that (a) components of the cytosolic insulin signalling machinery like mitogen activated protein kinase and PP-1 can be detected in the nucleus (Cohen, 1989; Chen et al., 1992; Sanghera et al., 1992), and (b) that insulin stimulates the phosphorylation of several structural nuclear DNA-binding proteins like lamins (Friedman and Ken, 1988; Csermely and Kahn, 1992), numatrin (Feuerstein and Randazzo, 1991) and nucleolin (Csermely et al.,

Abbreviations: PP-1, protein phosphatase-1; PP-2A, protein phosphatase-2A; OA, okadaic acid; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; dsDNA, double-stranded DNA.

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1993). Furthermore, very recently insulin was also shown to affect the phosphorylation of several AP-1 transcription factors like c-Jun, c-Fos, and Fos-related proteins (Kim and Kahn, 1994) and the cAMP response element-binding protein (CREB) (Reusch et al., 1994, 1995). This fits to a number of observations showing that the activity of transcription factors may be modulated post-translationally by their phosphorylation state (Prywes et al., 1988; Yamamoto et al., 1988; Boyle et al., 1991).

Both kinases and phosphatases may control the phosphorylation state of nuclear proteins and very limited information is presently available concerning this aspect of nuclear insulin action. Interestingly, Kim and Kahn, 1993 reported the insulin-stimulated accumulation of insulin receptors in nuclei of 3T3-F442A cells and a tenfold increase in nuclear tyrosine kinase activity in response to insulin. On the other hand, Reusch et al., 1994 reported that insulin exerts a strong inhibitory effect on PP-2A in nuclei from isolated rat adipocytes. This paralleled a 40% increase in the phosphorylation of CREB in response to insulin and suggested a potential role of phosphoprotein phosphatases for the transmission of the insulin signal at the nuclear level. In a very recent study the importance of the carboxy-terminal domain of the insulin receptor for this hormonal effect could be demonstrated (Reusch et al., 1995).

In contrast to adipose tissue, the insulin-regulated phosphorylation of nuclear proteins in muscle has not been investigated until now. This issue, however, needs specific attention since muscle is a major target of insulin action and may represent a primary site of insulin resistance (Kahn, 1994). In the present investigation we have taken advantage of our well characterized preparation of adult ventricular cardiomyocytes (Eckel et al., 1990; Kolter et al., 1992; Russ and Eckel, 1995), which represents a valuable tool for studies of insulin action on muscle tissue. Using this approach we aimed at (a) detecting insulin-regulated DNA-binding phosphoproteins in cardiac nuclei, (b) identifying putative candidate phosphoproteins, and (c) elucidating insulin action on nuclear protein phosphatase activity. The data support a model which implicates PP-1 and PP-2A in insulin-mediated regulation of a 38 kDa DNA-binding phosphoprotein and represent the first report on an inhibition of PP-1 by insulin in a target cell.

2. Materials and methods

2.1. Chemicals

Collagenase (EC 3.4.24.3) was purchased from Serva (Heidelberg, Germany). Bovine serum albumin (Fraction V, fatty acid free) was obtained from Boehringer (Mannheim, Germany). Insulin (porcine monocomponent) was a product of Novo BioLabs (Bagsvaerd, Denmark). [^{33}P]Orthophosphate (6000 Ci/mmol), [γ-^{32}P]ATP (6000 Ci/mmol) and [^{125}I]-labelled protein A (30 mCi/mg) were from Amersham (Braunschweig, Germany). Phosphorylase b, phosphorylase kinase, cellulose and the dsDNA-cellulose were purchased from Sigma (München, Germany). Protein A acrylamide beads were from Pierce (Rockford, USA) and protein G sepharose from Pharmacia (Freiburg, Germany). Okadaic acid was obtained from Calbiochem (Bad Soden, Germany). The anti-numatrin antibody (mouse, monoclonal) was a kind gift of Dr. P.K. Chan (Baylor College of Medicine, Houston, USA). Anti-phosphotyrosine antibody (monoclonal) was purchased from UBI (Lake Placid, NY). All other chemicals were of the highest grade commercially available.

2.2. Isolation of cardiac myocytes

For all experiments, male Wistar rats fed ad libitum and weighing 280–340 g were used. Calcium-tolerant cardiac myocytes were isolated by perfusion of the heart with collagenase as described earlier (Eckel et al., 1983), except that the Hepes buffer did not contain any KH_2PO_4. The final cell suspension was washed three times with Hepes buffer (NaCl 130 mM, KCl 4.8 mM, Hepes 25 mM, glucose 5 mM, bovine serum albumin 20 g/l, pH 7.4, equilibrated with O_2) and incubated in silicone-treated Erlenmeyer flasks in a rotating water-bath shaker at 37°C. After 20 min, $CaCl_2$ and $MgSO_4$ (final concentration 1 mM) were added and incubation was continued for 10 min. The cells were then either labelled with 25 μCi/ml [^{33}P]orthophosphate for 2.5 h while incubation at 37°C was continued or incubated at 37°C for 2.5 h without labelling. If not otherwise specified, the cells were treated with 1.7×10^{-7} M insulin during the last 15 min of the [^{33}P]orthophosphate labelling, washed twice with Hepes buffer at 4°C, frozen in liquid nitrogen and stored at −70°C. A mean of 5×10^6 cells per heart was obtained, with a mean viability (defined as the ratio of viable, rod-shaped cells to total number of cells) of 87%.

2.3. Isolation of nuclei

All buffers used contained the phosphatase inhibitors 10 mM tetra-sodium pyrophosphate and 0.3 mM vanadate, and the following protease inhibitors: 2.5 μg/ml aprotinin, 10 mM benzamidine, 2.5 μg/ml leupeptin, 2.5 μg/ml pepstatin and 0.2 mM PMSF. Nuclei were isolated according to Jackowski and Liew, 1980 with minor modifications (Petersen et al., 1995). Briefly, the cells were disrupted in a hypotonic buffer (10 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 10 mM NaCl, 5 mM $CaCl_2$, 2.8 mM DTT) by 30 min swelling, 2 × 2 min

sonification and 25 strokes in a Dounce homogenizer in the presence of 0.1% Triton X-100. The nuclei were then separated by 10 min centrifugation at 800 × *g* and the pellet was layered on a cushion of sucrose buffer (10 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 2.8 mM DTT, 2.2 M sucrose) followed by 1 h centrifugation at 118 000 × *g*. An initial characterization of these nuclei has been reported previously (Petersen et al., 1993). Protein was determined using a modification of the Bio-Rad protein assay with BSA as a standard.

2.4. Purification of nuclear dsDNA-binding protein

The dsDNA-binding proteins were extracted from aliquots of nuclei containing 1 mg of protein by adding 0.4 M NaCl and 0.5% Triton X-100. Samples were rotated for 2 h at 4°C, centrifuged at 10 000 × *g* for 5 min and the supernatant diluted 4 times with a buffer containing 10 mM Hepes, 0.1% Triton X-100 and 10% glycerol (pH 7.4). Samples were then pre-cleared by a 2 h rotation at 4°C in the presence of 20 mg cellulose and subsequent centrifugation at 5000 × *g* for 1 min, 20 mg of dsDNA-cellulose were added to the supernatant, samples were again rotated for 2 h at 4°C and centrifuged at 5000 × *g* for 1 min and the pellet washed twice with the same buffer. Thirty microliters of Laemmli sample buffer with 100 mM DTT was added and samples were analyzed with 10% SDS-PAGE and autoradiography. In addition, gels were visualized on a FUJIX BAS 1000 bio-imaging analyzer (Fuji, Japan). Quantification was performed on a SPARCstation (Sun Microsystems, USA) using image analysis software. Significance of reported differences was evaluated using the null hypothesis and *t*-statistics for unpaired data.

2.5. Alkaline hydrolysis of phosphoproteins on Immobilon

The dsDNA-binding nuclear proteins, resolved in a 10% SDS-PAGE, were blotted to an Immobilon membrane in a semidry blotting apparatus. After being air-dried, the blot was incubated in methanol for 1 min and then in 50 mM Tris–HCl, 150 mM NaCl, pH 7.4 for 10 min. The hydrolysis was performed in 1 N KOH at 55°C for 2 h. For the neutralization the blot was rinsed once with PBS for 1 min, once with 1 M Tris–HCl, pH 7.0 for 5 min and 2 × 5 min with water. The blot was finally air-dried and analyzed by autoradiography.

2.6. Immunoprecipitation

Proteins were immunoprecipitated from aliquots of ^{33}P-labelled nuclei by the addition of 100 units of DNase I, 0.4 M NaCl, 0.5% Triton X-100, 1% deoxycholate, rotation for 2 h at 4°C and centrifugation at 2000 × *g* for 1 min. The supernatant was taken and precleared with 30 μl of protein A-beads and 30 μl of protein G sepharose, rotated for 2 h at 4°C and centrifuged at 5000 × *g* for 1 min. An aliquot of the supernatant containing 2 mg of protein was taken. For immunoprecipitation with the anti-numatrin antibody, the aliquot was heated at 70°C for 30 min, 5 μl of the anti-numatrin antibody or 3 μl of the other antibodies were added and samples were rotated for 2 h at room temperature. Immunocomplexes were adsorbed to 30 μl of protein A-beads and 30 μl of protein G Sepharose by rotation for 2 h at 4°C. The immunoprecipitates were washed twice with a buffer containing 10 mM Hepes, 0.1% Triton X-100, 10% glycerol (pH 7.4). Thirty microliters of Laemmli sample buffer containing 100 mM DTT was added to the pellet and the samples were analyzed by 10% SDS-PAGE and visualized on a Fujix BAS 1000 bio-imaging analyzer and quantified as outlined above.

2.7. Assay of protein phosphatase activity

Nuclei were prepared from control or insulin treated cardiomyocytes as outlined above. High-salt extracts were then obtained from 8 × 10^5 nuclei by addition of 0.4 M NaCl and 0.5% Triton X-100. After 2 h at 4°C the samples were centrifuged, the supernatant was diluted with phosphatase assay buffer (50 mM Tris–HCl, pH 7.0, 0.1 mM EGTA, 0.1% mercaptoethanol) and concentrated by ultrafiltration. Protein phosphatase activity was then determined according to the method of Cohen et al. (1989) using ^{32}P-labelled phosphorylase as a substrate. Nuclear extracts were pre-incubated in the absence or presence of okadaic acid (10 nM and 1 μM) for 10 min at 30°C. The reaction was started by addition of ^{32}P-labelled phosphorylase in assay buffer containing 15 mM caffeine and conducted at 30°C in a final volume of 30 μl. After 10 min the reaction was stopped by adding 100 μl 20% trichloroacetic acid and 100 μl BSA (6 mg/ml). The mixture was centrifuged and 200 μl aliquots of the supernatant were counted to determine the amount of radioactivity released. PP-1 was defined as the phosphatase activity measured in the presence of 10 nM okadaic acid; PP-2A was calculated as the difference between total phosphatase activity and PP-1 (Cohen et al., 1989). All data were corrected for non-specific activity determined in the presence of 1 μM okadaic acid. This value was about 5% of total activity.

^{32}P-labelled phosphorylase a was obtained by phosphorylation of phosphorylase b with phosphorylase kinase in the presence of [γ-^{32}P]ATP according to the method of Cohen, 1983. The reaction was terminated by ammonium sulfate precipitation and excess ^{32}P-ATP was finally removed by repeated ultrafiltration steps.

3. Results

3.1. Insulin-induced phosphorylation of a 38 kDa nuclear DNA-binding protein

Purified nuclear DNA-binding proteins obtained from ^{33}P-labelled cardiomyocytes exhibited a highly reproducible pattern of three phosphoproteins with molecular masses of 38, 53 and 64 kDa (Fig. 1, upper panel). From these phosphoproteins only the 38 kDa species was significantly affected by insulin with a maximal action between 15 and 30 min (Fig. 1, upper panel). It should be noted that this phosphoprotein is already present in the nuclei under basal conditions, thus most probably representing a constitutive component of cardiac nuclei. Silver staining of DNA-binding nuclear proteins was performed in parallel and indicated the presence of at least fifteen protein bands with molecular masses between 15 and 170 kDa which were not affected by insulin (Fig. 1, lower panel). They comprise three main bands at 23, 35 and 74 kDa and four minor bands at 18, 23, 44 and 54 kDa. No significant protein band could be detected at 38 kDa both in the absence or presence of insulin. The quantification of insulin action revealed a rapid and constant increase in the phosphorylation of the 38 kDa protein reaching 149% and 182% at 15 min and 150 min, respectively (Fig. 2). When the ^{33}P-labelled nuclear DNA-binding proteins were blotted to an Immobilon membrane and then subjected to alkaline hydrolysis, the band corresponding to the 38 kDa phosphoprotein disappeared completely indicating that the 38 kDa phosphoprotein is phosporylated on serine and/or threonine. This applies also to the 53 and 64 kDa insulin-insensitive phosphoproteins (data not shown).

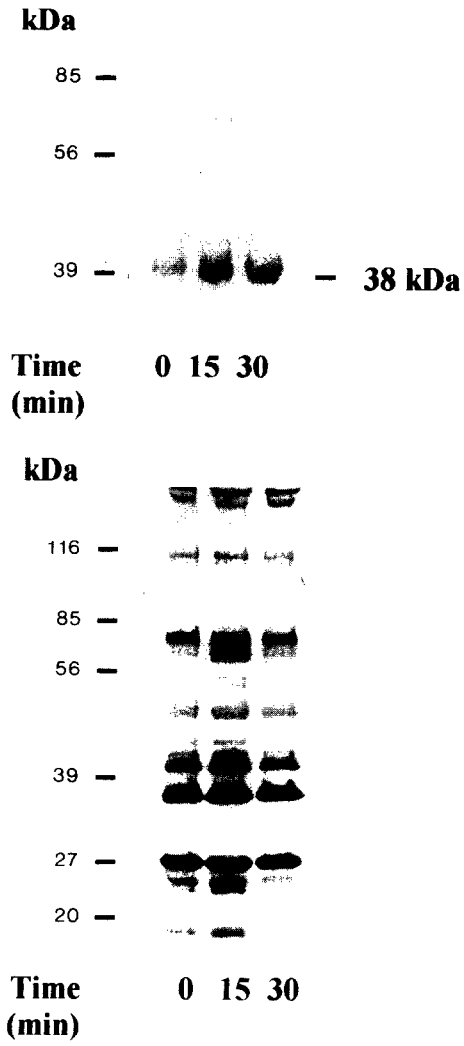

Fig. 1. Effect of insulin on the phosphorylation of nuclear DNA-binding proteins. Ventricular cardiomyocytes were labelled with ^{33}P-orthophosphate (25 μCi/ml) for 150 min and treated with insulin (1.7×10^{-7} M) for the indicated times. Nuclei were then isolated and dsDNA-binding proteins were extracted from aliquots of nuclei containing 1 mg of protein, as described in detail in Section 2. (Upper panel) The samples were subjected to SDS-PAGE and analyzed by autoradiography. A representative experiment is shown. (Lower panel) Nuclei were processed as outlined above, subjected to SDS-PAGE, and proteins were visualized by silver staining.

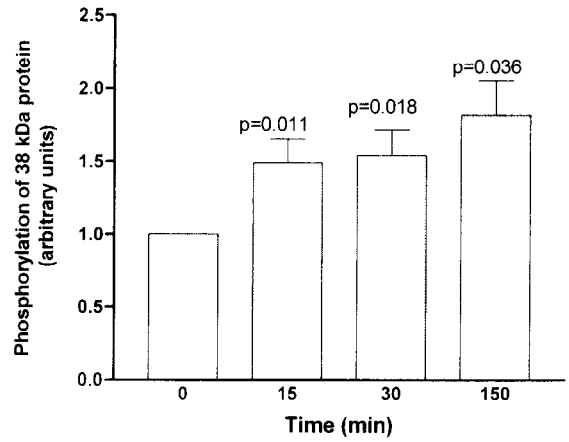

Fig. 2. Quantification of insulin-induced phosphorylation of 38 kDa DNA-binding protein. Nuclear DNA-binding phosphoproteins were detected by autoradiography as outlined in Fig. 1. Quantification of the 38 kDa signal was then performed by image analysis software, as described in the Materials and methods section. Intensity of the bands is given in arbitrary units relative to the control signal set as 1.0. Data reported represent mean values ± S.E.M. of 3–7 separate experiments.

In an attempt to identify the 38 kDa phosphoprotein, the high-salt nuclear extracts were subjected to immunoprecipitation with an anti-numatrin antibody. Numatrin is a 40 kDa DNA-binding phosphoprotein that is associated to the nuclear matrix and exhibits increased phosphorylation after insulin-treatment (Feuerstein, 1991). Numatrin could be detected, but its phosphorylation was not significantly and consistently affected by treatment with 1.7×10^{-7} M insulin for different lengths of time (Fig. 3). At 30 min an appar-

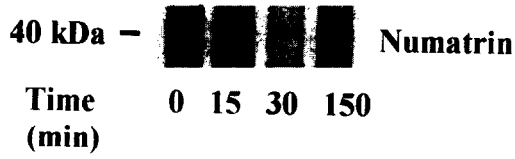

Fig. 3. Detection of ^{33}P-labelled numatrin in nuclear extracts from cardiomyocytes. ^{33}P-labelled cardiac nuclei obtained from control or insulin (1.7×10^{-7} M) treated cells were subjected to high-salt extraction as outlined in Section 2. Two milligrams of this protein extract were used for immunoprecipitation with an anti-numatrin antibody. The immunoprecipitates were washed and analyzed by SDS-PAGE and bio-imaging analysis. A representative experiment out of a total of 3–4 is shown.

ent, insignificant ($P > 0.05$) decrease of numatrin phosphorylation to 96 ± 27% of control ($n = 3$) could be quantified, reaching 105 ± 8% of control at 150 min ($P > 0.05$). This contrasts with an increase in the phosphorylation of the 38 kDa protein to 182 ± 23% at 150 min under the same experimental conditions (see Fig. 2). Thus, the 38 kDa DNA-binding protein is most probably different from numatrin. It should also be noted that we were unable to immunoprecipitate any tyrosine phosphorylated protein from the nuclear extracts.

3.2. Effect of insulin on nuclear phosphatase activity

The serine/threonine phosphatases PP-1 and PP-2A, which exhibit an insulin-regulated activity (Chan et al., 1988; Begum et al., 1993) and have been detected in the nucleus (DeFranco et al., 1991; Fernandez et al., 1992), represent newly recognized candidates for mediating the insulin signal at the nuclear level (Reusch et al., 1994; Reusch et al., 1995). In order to evaluate the possible relationship between these phosphatases and the 38 kDa phosphoprotein, [^{33}P]orthophosphate-labelled cardiac myocytes were incubated with 1 μM okadaic acid for 30 min. At this concentration okadaic acid is a specific inhibitor of both PP-1 and PP-2A. As shown in Fig. 4, okadaic acid alone leads to a two-fold increase in the phosphorylation of the 38 kDa protein when

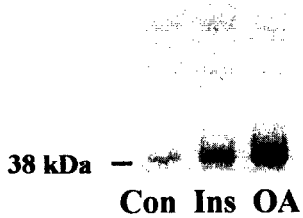

Fig. 4. Effect of okadaic acid on the phosphorylation of the 38 kDa DNA-binding protein. ^{33}P-labelled cardiomyocytes were treated with insulin (1.7×10^{-7} M, 15 min) or okadaic acid (1 μM, 40 min) and processed for the extraction and analysis of nuclear DNA-binding phosphoproteins, as described in Fig. 1. A representative example of three experiments is presented.

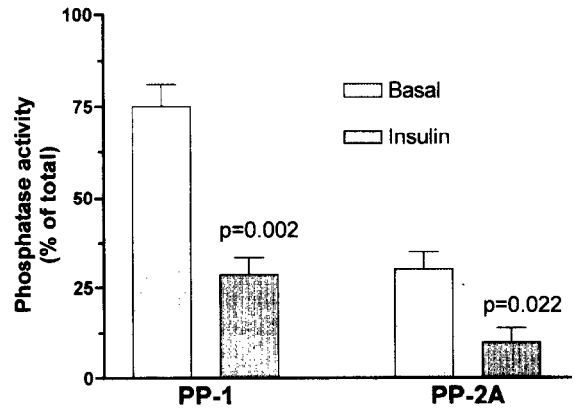

Fig. 5. Effect of insulin on nuclear phosphatase activity. Cardiomyocytes were incubated with insulin (1.7×10^{-7} M) for 15 min and nuclear extracts were prepared as described in Section 2. Phosphatase activity was then determined using ^{32}P-labelled phosphorylase as a substrate. For quantification of PP-1 the extracts were preincubated with 10 nM okadaic acid at 30°C, and PP-2A was calculated as the difference between total phosphatase activity and PP-1. All data were corrected for non-specific phosphatase activity determined in the presence of 1 μM okadaic acid and represent mean values ± S.E.M. of 3–4 separate experiments.

compared to the control, with no effect on the other phosphoproteins. This finding suggests that the 38 kDa phosphoprotein represents a potential substrate for PP-1 and PP-2A and that any modulation of these phosphatases must lead to an altered phosphorylation state of the 38 kDa DNA-binding protein.

In light of these observations we evaluated the effect of insulin on the activity of nuclear PP-1 and PP-2A using ^{32}P-labelled phosphorylase as a substrate. Total nuclear phosphatase activity was found to vary from 0.1 to 0.3 nmol P_i released/mg protein/min. As presented in Fig. 5, at least 75% of total phosphatase activity could be attributed to PP-1. Cardiomyocytes were then incubated with insulin (1.7×10^{-7} M) for 15 min, a condition leading to a substantial increase in the phosphorylation of the 38 kDa protein (see Fig. 1). Nuclear extracts obtained from these cells exhibited a largely reduced phosphatase activity (30–40% of basal). As can be seen from the data, the activities of both PP-1 (38% of basal) and PP-2A (32% of basal) were reduced to the same extent in response to insulin (Fig. 5).

4. Discussion

Insulin represents an important regulator of gene expression, cell growth and differentiation in a variety of cells (Granner and Andreone, 1985; O'Brien and Granner, 1991). Increasing evidence has now accumulated indicating that phosphorylation/dephosphorylation of nuclear proteins may represent a major pathway

for insulin mediated signalling at the nuclear level (Friedman and Ken, 1988; Feuerstein and Randazzo, 1991; Csermely and Kahn, 1992; Csermely et al., 1993; Kim and Kahn, 1994; Reusch et al., 1994; Reusch et al., 1995). The aim of the present work was to analyze this relationship in muscle tissue using nuclei obtained from ventricular cardiomyocytes, and to evaluate potential mechanisms of nuclear insulin signal transduction. The data show the existence of an insulin-sensitive DNA-binding phosphoprotein in cardiac nuclei and suggest that nuclear phosphatase activity is involved in the regulatory action of the hormone.

A major finding of the present investigation consists in the observation of a rapid and constant phosphorylation of a 38 kDa nuclear DNA-binding protein in response to insulin. Silver staining of the gels showed an essentially unaltered amount of proteins under all conditions and makes it most unlikely that the enhanced phosphorylation of the 38 kDa protein results from altered affinity of the protein to DNA or increased protein abundance. Interestingly, only a total of three phosphoproteins could be detected in the material eluted from the dsDNA-cellulose affinity matrix. This differs considerably from the data of Csermely and Kahn, 1992 who detected at least seven insulin-reponsive nuclear DNA-binding phosphoproteins ranging from 34 to 72 kDa using comparable experimental conditions in 3T3-F442A adipocytes. This most likely reflects the difference between this cell line and our primary muscle cell and indicates that nuclear insulin signalling represents a highly complex process which may adapt to the specific needs of a given tissue or cell. This view is further supported by our inability to immunoprecipitate phosphorylated lamins (Csermely and Eckel, unpublished observation) from nuclear extracts of cardiomyocytes, whereas in 3T3 cells (Csermely and Kahn, 1992) and in BHK-21 cells (Friedman and Ken, 1988) the DNA-binding proteins lamin A and C with molecular masses of 62–64 kDa were found to exhibit a 4–6 fold increase in phosphorylation in response to insulin. In contrast to lamins, we were able to detect phosphorylated numatrin in cardiac nuclei. Numatrin is a 40 kDa nuclear matrix protein which is thought to be involved in mitogenesis in a number of normal and malignant cells (Feuerstein and Mond, 1987) and exhibits an insulin-dependent phosphorylation in NIH 3T3-HIR cells (Feuerstein and Randazzo, 1991). However, at least up to 150 min insulin treatment of cardiocytes did not affect the phosphorylation state of numatrin, in agreement with the data of Feuerstein and Randazzo, 1991, who did not observe a significant insulin effect before a 4 h treatment. This clearly indicates that the 38 kDa insulin-sensitive cardiac phosphoprotein must be different from numatrin.

The protein phosphatases PP-1 and PP-2A are widely distributed in eukaryotic cells and are involved in such diverse processes as glycogen metabolism, intracellular transport, muscle contraction and protein synthesis (Bollen and Stalmans, 1992). PP-1 and PP-2A have both been detected in the nucleus, however, PP-1 seems to be the more abundant form in the majority of the cells studied (Kuret et al., 1986; Jessus et al., 1989; Beullens et al., 1992). It is well established that PP-1 responds to insulin with a rapid stimulation of the PP-1 activity, usually reaching a maximum after 5–10 min in a number of different cell types (Chan et al., 1988; Begum et al., 1993; Srinivasan and Begum, 1994; Begum, 1995), whereas PP-2A exhibits a corresponding inhibition by the hormone (Begum, 1995). On the other hand, as shown very recently in isolated adipocytes, nuclear PP-1 was not affected by insulin whereas the hormone exerts a strong inhibitory action on nuclear PP-2A (Reusch et al., 1994, 1995). The results of the present investigation clearly demonstrate that in cardiac muscle insulin is able to inhibit nuclear PP-1 and PP-2A activities to a large extent. Based on this observation we suggest that the increased phosphorylation of the 38 kDa DNA-binding protein at least partly results from a modulation of nuclear phosphatase activity by insulin. Several lines of evidence support this assumption. First, okadaic acid which represents a specific inhibitor of both the cytosolic and the nuclear forms of PP-1 and PP-2A (Cohen et al., 1990; Wadzinski et al., 1993), was found to increase the phosphorylation state of the 38 kDa protein 2–3 fold. Second, the 38 kDa protein is present in cardiac nuclei in the absence of insulin and therefore, available for modification by nuclear phosphatases. Third, insulin strongly inhibits PP-1 and PP-2A under the same experimental conditions that lead to enhanced phosphorylation of the 38 kDa protein in response to the hormone. Certainly, additional pathways of insulin action on this protein like the participation of nuclear kinases (Pulverer et al., 1991; Chen et al., 1992; Sanghera et al., 1992), or the translocation of this protein to the nucleus cannot be excluded at the present stage. It should be noted, however, that we were unable to detect significant amounts of mitogen-activated protein kinase in nuclear extracts of both basal and insulin-treated cardiomyocytes (Von Holtey et al., 1995).

A major finding of the present investigation consists in the observation that insulin is able to inhibit the nuclear activity of PP-1. To the best of our knowledge this is the first report on an inhibition of this enzyme in response to insulin. In muscle tissue the majority of PP-1 activity is associated with glycogen (PP-1G), with the catalytic subunit being complexed to a glycogen binding regulatory subunit (Stralfors et al., 1985). Insulin has been shown to promote site-specific phosphorylation of PP-1G, an event that is thought to activate PP-1 (Dent et al., 1990) and possibly involves the p21ras/mitogen-activated protein kinase pathway (Be-

gum, 1995). Much less is presently known about the regulation of nuclear PP-1 activity. Interestingly, PP-1 modulator which is most abundant in the cytosol, has also been detected in the nucleus (Brautigan et al., 1990). It may be speculated that insulin mediates the inhibition of PP-1 by phosphorylation/dephosphorylation of this protein (Bollen and Stalmans, 1992). Alternatively, the hormone may also induce a redistribution of PP-1 between the nucleus and the cytosol, as shown for the cell-cycle dependent modulation of PP-1 activity (Fernandez et al., 1992). Further work will now be needed to dissect the mechanisms of insulin signalling to nuclear PP-1.

In contrast to PP-1, the cytosolic PP-2A is inhibited in response to growth factors including insulin (Chen et al., 1994) involving phosphorylation of this enzyme. We show here that insulin is able to inhibit nuclear PP-2A by about 70%. These findings agree well with the data of Reusch et al. (1994, 1995), who reported 80% inhibition of nuclear PP-2A activity in rat adipocytes. Obviously, this insulin action which has been shown to be related to the phosphorylation state of CREB and activating transcription factor (ATF-1) (Reusch et al., 1994), is not restricted to adipose tissue and could also play a role for cardiac gene expression. It should be noted that, in contrast to adipocytes, the nuclear activity of PP-2A in cardiomyocytes is considerably lower than the activity of PP-1. However, the relative contribution of PP-1 and PP-2A to the dephosphorylation of the CREB/ATF family of transcription factors has remained controversial (Hagiwara et al., 1992; Wadzinski et al., 1993) and it has been concluded (Reusch et al., 1994) that tissue specific differences are involved. Our data now show that insulin inhibits both nuclear phosphatases in cardiomyocytes making it most likely that this pathway contributes significantly to the insulin-dependent control of transcription factors.

In summary, this paper is the first demonstration of an insulin-sensitive DNA-binding phosphoprotein in the nucleus of ventricular cardiomyocytes, which is at least partly regulated by nuclear phosphatase activity. Inhibition of nuclear PP-1 and PP-2A may represent an essential component of insulin-mediated regulation of gene expression.

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