Stimulation of glycogen synthesis by heat shock in L6 skeletal-muscle cells: regulatory role of site-specific phosphorylation of glycogen-associated protein phosphatase 1

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Recent evidence suggests that glycogen-associated protein phosphatase 1 (PP-1_G) is essential for basal and exercise-induced glycogen synthesis, which is mediated in part by dephosphorylation and activation of glycogen synthase (GS). In the present study, we examined the potential role of site-specific phosphorylation of PP-1_G in heat-shock-induced glycogen synthesis. L6 rat skeletal-muscle cells were stably transfected with wild-type PP-1_G or with PP-1_G mutants in which site-1 (S1) Ser⁴⁸ and site-2 (S2) Ser⁶⁷ residues were substituted with Ala. Cells expressing wild-type and PP-1_G mutants, S1, S2 and S1/S2, were examined for potential alterations in glycogen synthesis after a 60 min heat shock at 45 °C, followed by analysis of [¹⁴C]glucose incorporation into glycogen at 37 °C. PP-1_G S1 mutation caused a 90 % increase in glycogen synthesis on heat-shock treatment, whereas the PP-1_G S2 mutant was not sensitive to heat stress. The S1/S2 double mutant was comparable with wild-type, which showed a 30 % increase over basal. Heat-shock-induced glycogen synthesis was accompanied by increased PP-1 and GS activities. The highest activation was observed in S1 mutant. Heat shock also resulted

in a rapid and sustained Akt/ glycogen synthase kinase 3β (GSK-3β) phosphorylation. Wortmannin blocked heat-shock-induced Akt/GSK-3β phosphorylation, prevented 2-deoxyglucose uptake and abolished the heat-shock-induced glycogen synthesis. Muscle glycogen levels regulate GS activity and glycogen synthesis and were found to be markedly depleted in S1 mutant on heat-shock treatment, suggesting that PP-1_G S1 Ser phosphorylation may inhibit glycogen degradation during thermal stimulation, as S1 mutation resulted in excessive glycogen synthesis on heat-shock treatment. In contrast, PP-1_G S2 Ser phosphorylation may promote glycogen breakdown under stressful conditions. Heat-shock-induced glycogenesis appears to be mediated via phosphoinositide 3-kinase/Akt-dependent GSK-3β inactivation as well as phosphoinositide 3-kinase-independent PP-1 activation.

Key words: cellular stress, contraction, glycogen synthase, protein phosphatase 1.

INTRODUCTION

The mammalian regulatory subunit of glycogen-associated protein phosphatase 1 (PP-1_G/R_{GL}) protein has been recently shown to be essential for the regulation of glycogen metabolism under basal conditions and in response to contractile activity [1,2]. PP-1_G dephosphorylates the regulatory sites on glycogen synthase (GS), glycogen phosphorylase (Gp) *a* and phosphorylase kinase [3–5], all of which results in the activation of GS and simultaneous inactivation of Gp *a*, leading to increased glycogen synthesis. PP-1 activity was initially supposed to be hormonally regulated by phosphorylation of Ser⁴⁸ (site 1; S1) and Ser⁶⁷ (S2) on the PP-1_G subunit by mitogen-activated protein kinase (MAPK) 1, also known as p90 S6 kinase, and cAMP-activated protein kinase A respectively [4]. Also, insulin was found to increase the phosphorylation of PP-1_G at S1 and S2 in Chinese-hamster ovary cells expressing insulin receptor (CHO-IR) transfected with PP-1_G, which resulted in the dissociation of PP-1 catalytic subunit from PP-1_G [6]. However, studies by Yamamoto-

Honda et al. [7] using CHO-IR cells transfected with various PP-1_G site-specific mutants reported no dissociation of PP-1 from PP-1_G on insulin or forskolin treatment. In addition, using site- and phosphorylation-specific antibodies, it was recently shown that insulin does not affect the *in vivo* phosphorylation of PP-1_G in rat or mouse skeletal muscle [1,2], but adrenaline does increase phosphorylation of PP-1_G at S1/S2 [8], supporting the view that protein kinase A-mediated regulation of the PP-1_G complex may play a role in the adrenergic control of glycogen metabolism. Studies on PP-1_G-null mice showing normal activation of GS by insulin indicated that although PP-1_G may not be required for insulin to activate GS [2], it was essential for contraction and the exercise-induced increase in GS activation and glycogen synthesis, since PP-1_G-null mice exhibited low basal glycogen levels, decreased GS activity and impaired response to exercise-induced increase in GS activity [1].

Skeletal-muscle contraction during exercise produces heat and increases muscle temperature [9], in addition to ischaemia [10], protein degradation [11], hypoxia [12], acidosis [13], decreased

Abbreviations used: AMPK, AMP kinase; CHO-IR, Chinese-hamster ovary cells expressing insulin receptor; ERK, extracellular-signal-regulated protein kinase; Glc6P, glucose 6-phosphate; Gp, glycogen phosphorylase; GS, glycogen synthase; GSK-3, glycogen synthase kinase 3; HSF, heat-shock transcription factor; HSP, heat-shock protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; OA, okadaic acid; PI 3-kinase, phosphoinositide 3-kinase; PP-1, protein phosphatase 1; PP-1_G, glycogen-associated PP-1; S1 and S2, site 1 and site 2 respectively; SAPK, stress-activated protein kinase.

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glucose availability [14], oxyradical formation [15] and increased intracellular Ca^{2+} [16]. These cellular processes take place throughout concentric exercise and are capable of initiating a heat-shock response, which results in increased heat-shock protein (HSP) expression in skeletal muscle in human subjects as well as rats [9,17–22]. Studies by Febbraio et al. [23] have also shown that in humans exercising under a cooler environment, muscle glycogenolysis was decreased during exercise. Further studies are needed to differentiate clearly the effects of high temperature and other exercise-induced cellular events on HSP gene expression. HSP expression may activate phosphatases as reported in an *in vitro* study [24]. Furthermore, recent evidence suggests that decreased availability of glycogen is associated with an elevation in HSP72 in contracting human skeletal muscle [25]. More importantly, a correlation between decreased HSP72 expression and insulin resistance in the skeletal muscle of Type II diabetics has been reported [26]. These observations led us to hypothesize that PP-1_G may be a component of the heat-shock-induced signalling pathway serving to dephosphorylate and inactivate Gp *a* to prevent glycogen breakdown as well as to activate GS, in addition to dephosphorylation of heat-shock transcription factor 1 (HSF-1), leading to its activation, and the associated expression of HSP70 and other proteins involved in cell survival.

Under normal conditions, HSF is known to exist as a monomer and does not bind DNA, probably through its interaction with HSPs: HSP70, HSP90 or the other isoform, HSP72 [27]. On heat-shock treatment, the increased level of thermally denatured or malformed proteins reacts with the available HSPs, setting free the bound HSF [27,28]. The HSF released then forms a trimer and binds to heat-shock element. In addition, HSF-1 transcriptional activity is regulated by inter- and intramolecular interactions as well as by covalent modification [29]. HSF-1 is moderately phosphorylated under normal conditions and becomes hyperphosphorylated on heat-shock treatment [29]. The hyperphosphorylation of HSF-1 is involved in enhancing human HSF-1 transcriptional activity as well as its return to the inactive state after heat shock. Thus protein kinases and phosphatases may interact with HSF and regulate its activity. Contrary to the notion that hyperphosphorylation of HSF-1 may increase its transcriptional activity, studies in *Saccharomyces cerevisiae* indicate that the regulatory subunit of PP-1, Gac1, interacts with the HSF-repressing region and increases HSF transcriptional activity [29], whereas GS kinase 3 (GSK-3) and MAPKs [extracellular-signal-regulated protein kinases (ERKs)] cause repression of HSF transcriptional activity [30].

Heat shock might be a useful *in vitro* model for studying intracellular signalling mechanisms, since physical exercise generates heat and cellular stress, resulting in the induction of HSPs [9,31,32]. Since exercise responses are largely dependent on glycogen availability and since PP-1_G regulates glycogen synthesis, in the present study we investigated the role of the site-specific phosphorylation of PP-1_G in heat-shock-induced stimulation of glycogen synthesis and examined the signalling components that participate in heat shock response.

MATERIALS AND METHODS

Materials

Cell culture reagents, antibiotics, foetal bovine serum, Lipofectamine plusTM, Geneticin (also termed G418), Gp *b* and phosphorylase kinase were purchased from Life Technologies (Grand Island, NY, U.S.A.). [γ -³²P]ATP (specific activity >3000 Ci/mmol), D-[U-¹⁴C]glucose, [¹⁴C]glucose-1 phosphate, [2-³H]deoxy-D-glucose and [U-¹⁴C]UDP-glucose were obtained from DuPont/

New England Nuclear (Boston, MA, U.S.A.); the mammalian expression vector pcDNA3 was from Invitrogen (Carlsbad, CA, U.S.A.); the Chameleon site-specific mutagenesis kit was from Stratagene (La Jolla, CA, U.S.A.); okadaic acid (OA) was from Moana Bioproducts (Honolulu, Hawaii); restriction endonucleases were from Boehringer Mannheim (Indianapolis, IN, U.S.A.); electrophoresis and Bradford protein assay reagents were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); bicinechonic acid protein assay reagent was from Pierce (Rockford, IL, U.S.A.); anti-PP-1C β antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Phospho-Akt, Akt, phospho-GSK-3 β , phospho-MAPK, phospho-p38MAPK, pJNK, pAMPK (where JNK stands for c-Jun N-terminal kinase and AMPK for AMP kinase) were from Cell Signaling (Beverly, MA, U.S.A.). All other reagents were from Sigma (St. Louis, MO, U.S.A.). Porcine insulin was a gift from the Eli Lilly Co (Indianapolis, IN, U.S.A.). Antibody against PP-1_G subunit was generated and affinity-purified as described in our previous papers [33].

Site-directed mutagenesis of PP-1_G cDNA

The PP-1_G cDNA cloned from the rabbit skeletal-muscle cDNA library is described in [33]. Substitution of Ser⁴⁸ and Ser⁶⁷ at sites 1 and 2 of rabbit PP-1_G, separately and in combination, was performed with the Chameleon site-specific mutagenesis kit. The mutations were confirmed by restriction digestion and nucleotide sequence analyses.

Construction of expression vectors carrying wild-type and mutant PP-1_G

The mammalian expression vector pcDNA3 containing the recombinant wild-type or mutant PP-1_G constructs was prepared by standard molecular biology techniques. Briefly, the wild-type and mutant forms of PP-1_G constructs were cleaved from the Bluescript plasmid using *Eco*RI and subcloned into pcDNA3. In addition, the cytomegalovirus promoter from pcDNA3 was replaced by muscle creatine kinase promoter to enhance the expression of the transgene.

Transfection and selection of stable cell lines expressing recombinant wild-type or mutant PP-1_G subunits

The spontaneously fusing rat skeletal-muscle cell line, L6, was a gift from Dr Amira Klip (The Hospital for Sick Children, Toronto, ON, Canada). L6 myoblasts at the second passage were transfected with the expression vectors carrying the wild-type or mutant PP-1_G subunit cDNA as described in [34] using Lipofectamine plusTM reagent according to the manufacturer's instructions. After 72 h, cells were passaged (1:5) into the medium containing 2 mg/ml of G418 for selection. A general pool of G418-resistant cells was screened for the expression of PP-1_G. Screening was performed by immunoblot analysis of cell extracts prepared from myotubes (9–10 days in culture) using the anti-PP-1_G antibody described in the Materials subsection. Control cells (referred to as neo control throughout the text) were transfected with the empty expression vector. Transfection by itself did not affect the extent of differentiation of L6 cells, as monitored by analysis of myogenin protein and cell morphology. To avoid clonal variability, a pool of G418-resistant cells expressing comparable levels of recombinant wild-type PP-1_G or mutant PP-1_G (PP-1_{GS48A}, PP-1_{GS67A}, PP-1_{GS48/S67A}) was used in all the heat-shock experiments described below.

Cell culture and heat-shock treatment

Transfected cell lines expressing PP-1_G wild-type or mutant PP-1_{GS48A} (S1) PP-1_{GS67A} (S2) and PP-1_{GS47A/S67A} subunits (S1/S2) were grown in α -minimum essential medium containing 2% (v/v) foetal bovine serum, 400 μ g/ml of G418 and 1% antibiotic/antimycotic mixture in an atmosphere of 5% CO₂ at 37 °C as described previously [33,34]. Unless otherwise stated, differentiated cells were used in all experiments after a 12 h starvation in serum-free α -minimum essential medium containing 5 mM glucose. For heat-shock treatment, dishes were heated at 45 °C for 0–1 h followed by recovery at 37 °C, as indicated in the Figure legends. Control cells were left at 37 °C.

Extraction and assay of PP-1

Serum-starved cells were fed with Hanks balanced salt solution and subjected to heat-shock treatment at 45 °C for 1 h. A second batch of cells was left at 37 °C for 1 h. At the end of the incubation period, the medium was removed and the cells rinsed three times with ice-cold PBS, followed by extraction with PP-1 extraction buffer as described in our previous papers [35,36]. PP-1 assay was performed using ³²P-labelled Gp *a* as a substrate [33,36]. OA (1 nM) was included in the assay to inhibit PP-2A. At this concentration, OA inhibits only PP-2A activity and the remaining activity represents PP-1 [36,37].

Assay of GS activity

Cells were subjected to heat-shock treatment for 1 h followed by a recovery period of 1 h at 37 °C and were then extracted with GS extraction buffer [33,34] and assayed for GS activity in the presence of low (0.1 mM) and high (20 mM) concentrations of glucose 6-phosphate (Glc6P), using 0.7 mM [U-¹⁴C]UDP-glucose as a substrate as described in [34]. GS activity {pmol of [U-¹⁴C]UDP-glucose incorporated into glycogen · min⁻¹ · (mg of protein)⁻¹} was expressed as the percentage fractional activity measured in the presence of low Glc6P divided by the activity measured in the presence of high Glc6P.

Assay of Gp *a* activity

Gp *a* was assayed by monitoring the conversion of [¹⁴C]glucose 1-phosphate (Glc1P) into glycogen by the method as described previously [34]. Extracts from GS assay were used for the measurement of Gp *a* activity. The final assay mixture contained 75 mM Glc1P, 125 mM NaF, 0.6% glycogen and labelled Glc1P at 0.08 μ Ci/assay. Gp *a* activity was determined in the presence and absence of 5 mM AMP. Caffeine (1 mM) was added to the assay mixture in tubes without AMP to inhibit Gp *b* activity.

Glucose incorporation into glycogen

Glucose incorporation into glycogen was measured using D-[U-¹⁴C]glucose as described previously [33,34]. To examine the effect of heat shock on glycogen synthesis, cells were heated at 45 °C for 1 h, then shifted to 37 °C, and [U-¹⁴C]glucose was added followed by incubation at 37 °C for 90 min.

Measurement of glycogen content in L6 cells

Glycogen content in L6 cell lines was measured by KOH hydrolysis of cells after heat-shock treatment followed by ethanol precipitation and aminoglucosidase digestion as described in [2]. The resulting glucosyl residues were determined using a hexokinase/Glc6P dehydrogenase-phenazine methosulphate-based

colorimetric assay using a glucose assay kit from Sigma Diagnostics (St. Louis, MO, U.S.A.). Data are expressed as μ g of glucose formed/mg of protein.

Immunoblot analysis

Cells were subjected to heat-shock treatment and washed four times with ice-cold PBS, followed by the addition of 200 μ l of cell lysis buffer containing 50 mM Tris/HCl (pH 7.6), 2.0 mM EDTA, 2.0 mM EGTA, 1.0% SDS, 1.0 mM benzamide, 2.0 mM PMSF and 10 μ g/ml each of leupeptin, aprotinin, antipain, soya bean trypsin inhibitor and pepstatin A. Where indicated, phosphatase inhibitors were added to prevent dephosphorylation [38]. The cells were scraped and the cell lysate was sonicated and centrifuged at 2000 *g* for 5 min. Extraction resulted in the complete recovery of proteins in the supernatant. Typically, 20 μ g of protein was mixed with Laemmli sample buffer containing 0.1% Bromophenol Blue, 1.0 M NaH₂PO₄ (pH 7.0), 50% (v/v) glycerol and 10% (w/v) SDS, boiled for 5 min and loaded on to a 7.5% SDS/PAGE gel. The proteins were transferred to PVDF membrane and the membranes were probed with various antibodies as described in the Figure legends. This was followed by incubation with horseradish peroxidase-conjugated goat IgG followed by ECL[®] and autoradiography. The intensity of the signal was quantified by densitometric analysis of the autoradiograms [38].

Protein assay

The protein content of the cell extracts was determined with either bicinchoninic acid [39] or the Bradford reagent [40].

Statistics

The Student's *t* test or ANOVA was used to evaluate the significance of the effect of heat shock on PP-1 activity, GS activity and glycogen synthesis. Data are expressed as means $\pm$ S.E.M. for 4–5 different experiments, each performed in triplicate.

RESULTS

Differential effects of PP-1_G site-specific phosphorylations on heat-shock-induced glycogen synthesis

Acute and concentric exercise and associated muscle contractions are known to increase skeletal-muscle temperature and other cellular events, such as ischaemia, protein degradation, hypoxia, acidosis, decreased glucose availability, oxyradical formation and increased intracellular Ca²⁺ [9], which produce a cellular stress response [9,17–22]. Recent studies indicate that PP-1_G is essential for basal as well as exercise- and contraction-induced increases in glycogen synthesis [1]. Therefore we examined the effect of heat shock on glycogen synthesis in L6 cells expressing comparable levels of wild-type PP-1_G and its site-specific mutants (S1, S2 and S1/S2). Serum-starved L6 cell lines were exposed to heat shock at 45 °C for 1 h, and the incorporation of [¹⁴C]glucose into glycogen was measured at the end of 90 min at 37 °C. As shown in Figure 1(A), heat shock caused a 24% and 30% increase in glycogen synthesis in L6 neo controls and cells expressing wild-type PP-1_G respectively when compared with corresponding basal values at 37 °C (*P* < 0.05). In contrast, the PP-1_G S1 mutation resulted in a 90% increase in glycogen synthesis in response to heat shock (*P* < 0.001 versus all the other groups; Figure 1A). Heat shock did not affect glycogen synthesis in cells expressing PP-1_G with the S2 mutation, whereas the responsiveness of PP-1_G site-1/site-2 double mutant (S1/S2) was comparable with wild-

Figure 1 (A) Effect of heat shock on glycogen synthesis in L6 cells expressing various PP-1_G mutants, and (B) PP-1_G S1 mutation results in glycogen depletion during heat shock

(A) L6 cells expressing empty expression vector (neo control), wild-type PP-1_G (Wt), S1 mutant (S48A), S2 mutant (S67A) and S1/S2 double mutant (S48A/S67A) were serum-starved and exposed to heat shock at 45 °C for 1 h. Plates were returned to 37 °C and [¹⁴C]glucose incorporation into glycogen was measured after 90 min incubation at 37 °C. Results are means ± S.E.M. for five different experiments performed on duplicate dishes and expressed as d.p.m. incorporated into glycogen · min⁻¹ · (mg of protein⁻¹) (4.0e+4 = 4 × 10⁴ etc.) **P* < 0.05 versus basal L6 neo control, ***P* < 0.05 versus basal wild-type, ****P* < 0.05 versus heat shock (HS)-treated neo control and Wt, *****P* < 0.05 versus S1, #*P* < 0.05 versus S1. (B) L6 cells expressing empty expression vector (neo control), wild-type PP-1_G (Wt), S1 mutant (S48A), S2 mutant (S67A) and S1/S2 double mutant (S48A/S67A) were grown in 60 mm plates, serum-starved, exposed to heat shock at 45 °C for 1 h and tested for glycogen levels after digestion with KOH followed by glycogen precipitation. Glucosyl residues released after digestion with the aminoglycosidase were measured using glucose enzymic assay kit as described in the Materials and methods section. Results are expressed as the percentage decrease in glycogen content after heat-shock treatment. Basal glycogen levels were: neo control, 18 ± 2.5; Wt, 24 ± 1.8; S48, 21 ± 2; S2, 27 ± 3; S1/S2, 22 ± 2.5 μg of glucose formed/mg of protein. Results are the means ± S.E.M. for four independent experiments performed in duplicate. **P* < 0.05 versus neo control and Wt, ***P* < 0.05 versus S1, S1/S2 and Wt.

type (Figure 1A). Expression of PP-1_G and its mutants caused a small increase of 8% (wild-type PP-1_G), 14% (S1), 18.6% (S2) and 14% (S1/S2) respectively in basal glycogen synthesis when compared with L6 neo control (Figure 1A). Substitution of Ser with Asp at site 1, which imparts negative charge and mimics phosphorylation [41,42], abolished heat-shock-induced increase in glycogen synthesis (results not shown), suggesting that Ser phosphorylation at site 1 may protect glycogen from breakdown during heat-shock stress. Alternatively, it can be interpreted as

Figure 2 (A) Differential effect of heat shock on PP-1 enzymic activity in S1 mutant, and (B) heat shock does not affect the contents of PP-1_G and PP-1_C

(A) Serum-starved cells were subjected to heat-shock treatment for 1 h at 45 °C. PP-1 assay was performed on cells incubated at 37 °C (basal activity) and compared with cells subjected to heat shock. Results are the means ± S.E.M. for four independent experiments. **P* < 0.05 versus HS-treated neo control and Wt, ***P* < 0.05 versus S1, ****P* < 0.05 versus S1. Basal PP-1 activities were: L6 neo control, 1.42 ± 0.04; Wt, 1.6 ± 0.06; S1, 1.3 ± 0.04; S2, 1.65 ± 0.028 nmol of P_i released · mg of protein⁻¹ · min⁻¹. (B) Cell lysate proteins (10 μg) were subjected to SDS/PAGE followed by Western-blot analysis with antibodies against PP-1_G and PP-1_{Cβ}. A representative blot is shown. Similar results were obtained from three independent experiments. HS, heat shock.

S1 phosphorylation negatively modulating glycogen synthesis in response to heat shock. Addition of insulin during heat shock as well as during the 90 min incubation at 37 °C did not further increase glycogen synthesis (results not shown). In agreement with our results, studies by Zachayus and Plas [42] have reported increased glycogen synthesis in cultured foetal hepatocytes after heat-shock treatment. However, no information exists on the role of PP-1_G in mediating the effect of thermal stimulation. Analysis of cellular glycogen levels immediately after heat shock revealed a 66% decrease in glycogen levels over the basal values in S1 mutant, whereas S2 mutant exhibited a minimal reduction of 12% in glycogen levels after heat-shock treatment (Figure 1B), which coincides with the observed increase in glycogen synthesis in S1 mutant and a lack of heat-shock effect in S2 mutant. The responsiveness of S1/S2 double mutant was comparable with the wild-type, which exhibited 30% and 36% decreases in glycogen levels respectively, after heat-shock treatment (Figure 1B).

Heat shock increases PP-1 activity

Given the knowledge that glycogen synthesis is regulated by the simultaneous activation of GS and inactivation of Gp *a* by PP-1, we assayed cellular PP-1 enzymic activity after 1 h heat-shock treatment of L6 cells expressing PP-1_G or its mutants. When compared with L6 neo control, wild-type PP-1_G, S2 and S1/S2 mutants exhibited 14, 16 and 12.7% increases in basal PP-1

Figure 3 Effect of heat shock on GS fractional activity

GS enzymic activity was assayed on cell lysates prepared from cells left at 37 °C (basal) and heat-shock (HS)-treated cells (45 °C, 1 h) followed by 1 h recovery at 37 °C. GS enzymic activity was assayed at 30 °C using [³H]UDP-glucose as substrate in the presence of low (0.1 mM) and high (10 mM) concentrations of Glc6P as described in the Materials and methods section. Results are the means ± S.E.M. for four independent experiments and are expressed as the percentage GS fractional activity. **P* < 0.05 versus neo control and Wt basal activity; ***P* < 0.05 versus basal L6 neo control; ****P* < 0.05 versus basal wild-type (Wt); *****P* < 0.05 versus HS-treated neo control and Wt; #*P* < 0.05 versus S1; ##*P* < 0.05 versus S1.

Figure 4 Heat shock increases Akt phosphorylation

Serum-starved cells were subjected to heat shock (HS) for 0, 15, 30 and 60 min followed by extraction in lysis buffer containing phosphatase and protease inhibitors. Equal amounts of cell lysate proteins (50 µg) in duplicate were subjected to SDS/PAGE followed by immunoblot analysis with phospho-Akt (pAkt) and Akt antibodies respectively. (A) A representative blot is shown. Similar results were obtained from four other experiments. (B) Quantitative analysis of heat-shock-induced Akt phosphorylation in wild-type L6 cells. Linear ECL[®] signals from four independent experiments were quantified by densitometric analysis and normalized for Akt proteins by dividing the intensity of pAkt signals with the Akt signal. Results are the means ± S.E.M. for four experiments and are expressed as pAkt/Akt ratio. **P* < 0.05 versus basal (0 min) Akt phosphorylation.

Figure 5 Heat shock (HS) increases phospho-JNK (pJNK) and pAMPK levels

Equal amounts of protein lysates in duplicate from the experiment described in the legend of Figure 4 were subjected to SDS/PAGE followed by immunoblot analysis with phospho-JNK/SAPK, pAMPK, JNK/SAPK and AMPK antibodies. (A) A representative blot is shown. (B) Signal intensities from four independent experiments were quantified by densitometric analysis, corrected for variations in proteins by dividing the intensity of phospho-JNK/SAPK and pAMPK signals with that of their respective protein signals. Results are the means ± S.E.M. for four independent experiments and are expressed as fold increase in JNK/AMPK phosphorylation over basal values. **P* < 0.05 versus basal (0 min) JNK/AMPK phosphorylation.

activity. In contrast, S1 mutation resulted in a small decrease (15%) in basal PP-1 activity at 37 °C when compared with wild-type and other mutants. Heat-shock treatment increased PP-1 activity by 58% and 70% over the basal values in neo control and wild-type PP-1_G-expressing cells respectively (Figure 2A). S1 mutation exhibited a 118% increase in PP-1 activity on heat-shock treatment, whereas S2 and S1/S2 mutants exhibited 41% and 48% increases in PP-1 activity respectively, which was less than the percentage increase in heat-shock-induced PP-1 activity observed in L6 neo controls (Figure 2A). Western-blot analysis revealed a 2- to 3-fold increase in PP-1_G and PP-2C protein content respectively in cells expressing wild-type and PP-1_G mutants when compared with L6 neo controls. Heat shock did not alter cellular PP-1_G and PP-1C contents (Figure 2B). Moreover, immunoprecipitation studies indicated that the association between PP-1C and PP-1_G was not altered by site-specific mutation or heat shock (results not shown). Thus the increase observed in PP-1 enzymic activity after heat-shock treatment was not due to changes in PP-1_G and PP-1C protein levels or their association.

Effect of heat shock on GS and Gp a fractional activities

The effect of heat shock on GS and Gp a fractional activities was examined in an attempt to correlate the differential effects of PP-1_G mutants on glycogen synthesis. In the basal state, S1 mutant exhibited the lowest GS fractional activity when compared with

Figure 6 Wortmannin abolishes basal and heat-shock-induced Akt phosphorylation

Cells were treated with 100 nM wortmannin for 30 min before exposure to heat shock (HS) for 1 h at 45 °C. Equal amounts of proteins were examined for Akt phosphorylation by Western-blot analysis using phospho-Akt (pAkt) and Akt antibodies. A representative blot from four independent experiments is shown.

Figure 7 Wortmannin blocks glucose uptake and glycogen synthesis

Cells were exposed to wortmannin as described in the legend of Figure 6, followed by heat shock. 2-Deoxyglucose (DOG) uptake was assessed by using [3 H]DOG (A). Results are the means $\pm$ S.E.M. for three independent experiments performed in duplicate. * P < 0.05 versus respective untreated groups in the basal state; ** P < 0.05 versus respective HS-treated groups. (B) Wortmannin-treated cells were subjected to heat shock and glycogen synthesis was assessed as described in the legend of Figure 1(A). Results are the means $\pm$ S.E.M. for 3–4 independent experiments performed in duplicate. * P < 0.05 versus basal L6 neo control; ** P < 0.05 versus basal Wt; *** P < 0.05 versus HS-treated neo control and Wt; **** P < 0.05 versus S1; # P < 0.001 versus HS-treated S1; ## P < 0.05 versus respective HS-treated groups.

wild-type, S2 and the S1/S2 double mutant (Figure 3). However, this mutant exhibited maximal elevations in GS fractional activity measured after 1 h heat shock (Figure 3). The increase in GS fractional activity observed on heat-shock treatment correlated very well with the increase in glycogen synthesis observed in this mutant. S2 mutant did not exhibit increased GS fractional activity after heat-shock treatment, which coincides with the lack of thermal response on glycogen synthesis in this mutant. GS fractional activity in S1/S2 double mutant was comparable with neo control, which showed a significant increase of 34 % in GS activity on heat-shock treatment (P < 0.05).

Analysis of Gp α fractional activity revealed small reductions in S1 and S2 mutants after heat-shock treatment when compared with basal levels, which were not significantly different between wild-type and PP-1_G mutants (results not shown).

Heat shock increases Akt phosphorylation

The phosphoinositide 3-kinase (PI 3-kinase)/Akt signalling pathway is known to regulate most of the metabolic effects of insulin, including glucose uptake and glycogen synthesis [43]. Akt phosphorylation/activation increases phosphorylation of GSK-3, leading to its inactivation, which in turn may prevent GS phosphorylation and, thereby, increase GS activation in addition to PP-1-mediated activation of GS. To determine whether the observed increases in heat-shock-induced glycogen synthesis, GS fractional activity and PP-1 activity are due to the activation of Akt, one of the survival promoters, we examined the phosphorylation status of Akt after exposure to heat shock. As shown in Figure 4(A), exposure to heat shock for 15 min increased Akt phosphorylation by 4-fold in wild-type L6 cells. A maximal 10-fold increase in Akt phosphorylation was observed after 30 min of heat shock, which remained elevated by 6-fold over the basal value for the entire 1 h period (Figure 4B) as well as during the recovery phase (results not shown). S1 mutant exhibited a small increase in basal Akt phosphorylation, which was further increased after heat shock (results not shown).

In contrast with Akt, p38MAPK was phosphorylated in serum-starved cells in the basal state and after 15 min of heat shock, with a progressive decrease in p38MAPK phosphorylation after 30 and 60 min of exposure to elevated temperatures (results not shown). ERK was also phosphorylated in the basal state and heat shock did not further increase ERK phosphorylation at any time point between 0 and 1 h (results not shown). p45JNK/stress-activated protein kinase (SAPK) was considerably phosphorylated in the basal state when compared with the p54 isoform. Phosphorylation of p45JNK/SAPK isoform increased by 3-fold over the basal value after 30 min of heat-shock treatment, with a maximum effect seen at 60 min after heat shock (Figures 5A and 5B). In addition, AMPK, the SAPK which is known to be activated on exercise, was phosphorylated after 30 and 60 min of heat-shock treatment (Figure 5B). A 2-fold increase in AMPK phosphorylation was observed after 15 and 30 min of heat-shock treatment, which further increased after 60 min of heat-shock treatment. S2 mutant exhibited elevated basal pAMPK levels which decreased after heat shock when compared with neo control, wild-type PP-1_G and the other PP-1_G mutants (results not shown).

Wortmannin blocks heat-shock-induced Akt phosphorylation, glucose uptake and glycogen synthesis without affecting PP-1 activity

To test if heat-shock-induced glycogen synthesis was mediated by PI 3-kinase signalling via Akt, cells expressing the wild-type

Figure 8 Wortmannin blocks basal and heat-shock-induced GSK-3 β phosphorylation

Cell lysate proteins from the experiment described in the legend of Figure 6 were examined for GSK-3 β phosphorylation by immunoblot analysis using phospho-GSK-3 β (pGSK-3 β) antibody. (A) ECL[®] signal from a representative blot is shown. (B) Quantitative analysis of heat-shock (HS)-induced GSK-3 β phosphorylation by densitometry. Signal intensities from four independent experiments were quantified by densitometric scanning, and phospho-GSK-3 signals were normalized for GSK-3 β proteins and expressed as pGSK-3 β /GSK-3 ratio. Results are the means $\pm$ S.E.M. for four independent experiments. * P < 0.05 versus basal; ** P < 0.05 versus basal and heat-shock-induced GSK-3 phosphorylation and after 1 h recovery from heat shock.

and mutant versions of PP-1_G were pretreated with 100 nM wortmannin for 30 min at 37 °C followed by exposure to heat shock at 45 °C for 1 h and examined for Akt phosphorylation. Wortmannin not only inhibited basal Akt phosphorylation but completely abolished heat-shock-induced increase in Akt phosphorylation (Figure 6). Inhibition of p38MAPK by SB203580 did not affect heat-shock-induced Akt phosphorylation, nor did PD98059, an MAPK/ERK inhibitor, abolish heat-shock-induced phosphorylation of Akt (results not shown). Thus Akt activation was not related to p38MAPK or ERK activation. In contrast with Akt, basal p38MAPK phosphorylation was increased by more than 2-fold by wortmannin (results not shown). Taken together, these results suggest that heat-shock-induced activation of Akt is completely dependent on the activity of PI 3-kinase and is independent of the activation of p38MAPK and ERK1/ERK2.

Inhibition of Akt phosphorylation by wortmannin was accompanied by a 50 % decrease in glucose uptake measured under basal as well as heat-shock-treated conditions (Figure 7A). Either PP-1_G mutants or heat shock alone did not significantly affect glucose uptake (Figure 7A). In addition, wortmannin decreased basal glycogen synthesis by 50 % in all cell types, which was proportional to the decrease observed in basal glucose uptake as shown in Figure 7(A). Furthermore, heat-shock-induced glycogen synthesis was also completely prevented by wortmannin in neo controls, wild-type and PP-1_G mutants (Figure 7B). In contrast, SB203580 did not abolish heat-shock-induced glycogen

Figure 9 Heat shock (HS) increases HSF-1 protein expression which is sustained during the recovery period

Protein lysates (100 μ g) were immunoprecipitated (IP) with HSF-1 antibody (Ab) followed by immunoblot analysis of HSF-1 in the immunoprecipitates. A representative autoradiogram is shown. (B) Quantification of HSF-1 expression by densitometric analysis. Results are the means $\pm$ S.E.M. for three independent experiments. * P < 0.05 versus Wt and S1; ** P < 0.05 versus respective basal values; *** P < 0.05 versus HS-treated Wt, S1 and S2; **** P < 0.05 versus heat-shock-induced S1 and S2; # P < 0.05 versus 1 h recovery S1, S2; ## P < 0.05 versus respective 1 h recovery groups.

synthesis as well as PP-1 activity (results not shown). More importantly, heat-shock-induced increase in PP-1 activity was also not affected significantly by wortmannin, although S2 mutant and S1/S2 double mutant exhibited a small decrease in basal and heat-shock-induced PP-1 activity after wortmannin treatment (results not shown).

Wortmannin blocks heat-shock-induced GSK-3 β phosphorylation

GSK-3 is a major regulatory enzyme of GS activation which is inactivated by Akt-mediated phosphorylation, thereby relieving the inhibition of GS. To examine further whether heat-shock-induced increase in glycogen synthesis is due to inactivation of GSK-3, we measured GSK-3 β phosphorylation after heat-shock treatment. As shown in Figures 8(A) and 8(B), GSK-3 is phosphorylated to a considerable extent at 37 °C in cell lines expressing wild-type PP-1_G as well as PP-1_G mutants (results not shown). Heat shock at 45 °C for 1 h resulted in a 2-fold increase in GSK-3 β phosphorylation in wild-type (Figure 8B) and mutant cell lines. GSK-3 β phosphorylation was sustained after 1 h recovery at 37 °C. Pretreatment with wortmannin completely blocked basal and heat-shock-induced GSK-3 β phosphorylation (Figures 8A and 8B), thereby resulting in GSK-3 β activation in all of the PP-1_G mutants examined. Thus a combination of inhibition of glucose uptake and inactivation of GS via GSK-3 may account for wortmannin-mediated inhibition of heat-shock-induced glycogen synthesis.

Effect of PP-1_G mutants on HSF-1 and HSP70 expression during heat shock and recovery

A small amount of HSF-1 protein was present under basal conditions at 37 °C. Heat shock caused a corresponding 2-fold

increase in HSF-1 protein content in cells expressing wild-type PP-1_G and its mutants, which was further increased after 1 h recovery at 37 °C in S1 and S2 mutants. In contrast, S1/S2 double mutant, which exhibited elevated basal and heat-shock-induced HSF-1 protein expression, revealed gradual reductions in HSF-1 protein expression during the 2 h recovery period. Overall, PP-1_G mutants exhibited increased HSF-1 expression after heat shock and during recovery when compared with cells expressing wild-type PP-1_G (Figures 9A and 9B). In contrast, HSP70 expression was very high under basal conditions and was not decreased by heat shock or during recovery from heat shock (results not shown).

DISCUSSION

The present study demonstrates a new role for site-specific phosphorylations of PP-1_G in heat-shock-induced glycogen synthesis. The results indicate that substitution of site-1 Ser with Ala in PP-1_G caused induction of glycogen synthesis during the recovery period from heat shock, whereas mutation of PP-1_G site-2 Ser→Ala did not affect glycogen synthesis after heat-shock treatment. The 2-fold increase in glycogen synthesis after heat-shock treatment observed in cells expressing PP-1_G site-1 mutant was due to a combination of an increase in GS fractional activity and decreased Gp *a* due to a 2-fold increase in the enzymic activity of PP-1, which dephosphorylates these two regulatory enzymes, causing activation of GS and simultaneous inactivation of Gp. The increase observed in glycogen synthesis in S1 mutant was preceded by a marked depletion of cellular glycogen levels after heat-shock treatment, in contrast with S2 mutant which exhibited minimal reductions in cellular glycogen levels. Because muscle glycogen content is known to play a pivotal role in the regulation of GS activity and glycogen synthesis [44], one of the simplest interpretations of these complex results is that PP-1_G site-1 Ser → Ala mutation causes excessive glycogen degradation during heat shock which stimulates glycogen synthesis via GS activation to replenish glycogen stores. From the physiological point of view, this would mean that PP-1_G site-1 Ser phosphorylation may protect skeletal-muscle cells from the catabolic effects of heat shock by preventing excessive glycogen breakdown via PP-1 activation and, thereby, may protect cells from the adverse effects of heat generated during exercise. Site-2 Ser phosphorylation of PP-1_G, on the other hand, may promote glycogen degradation during cellular heat stress. Alternatively, based on the biochemical findings, it can be argued that site-1 phosphorylation of PP-1_G modulates negatively PP-1 and GS activity under heat stress, whereas site-2 phosphorylation may affect this in a positive way. We favour our first interpretation based on the fact that muscle glycogen synthesis is dependent on glycogen levels in addition to GS activity ratio. Recently, *in vivo* studies in rats by Walker et al. [45] and *in vitro* studies in CHO-IR cells by Liu and Brautigan [6] have shown that β -adrenergic stimuli and cAMP agonists increase site-2 phosphorylation of PP-1_G, which results in GS inactivation and inhibition of glycogen synthesis.

Although the mechanism by which site-1 PP-1_G phosphorylation may regulate glycogen synthesis is not clear at present, heat shock did result in the induction of the survival promoter Akt, which was rapidly phosphorylated in a PI 3-kinase-dependent manner after heat-shock treatment, and this phosphorylation was sustained during the entire recovery period. Furthermore, heat-shock-induced Akt phosphorylation was accompanied by increased phosphorylation and inactivation of GSK-3 β , which may further contribute to the observed increase in GS fractional activity and glycogen synthesis. Treatment with wort-

mannin prevented heat-shock-induced GSK-3 β phosphorylation, resulting in GSK-3 β activation, and abolished heat-shock-induced increase in glycogen synthesis. These results suggest that the PI 3-kinase pathway mediates heat-shock-induced increase in Akt phosphorylation and subsequently inactivates GSK-3 β by phosphorylation, further contributing to the observed increase in glycogen synthesis. The observed inhibition of heat-shock-induced glycogen synthesis by wortmannin in the absence of inhibition of PP-1 activity appears to be due to marked inhibition of glucose uptake coupled with activation of GSK-3 β . Furthermore, GSK-3 β is a well-known negative regulator of both DNA binding and transcriptional activation of HSF-1 via constitutive phosphorylation of the regulatory domain of HSF-1 [30]. Our results demonstrating the increased accumulation of HSF-1 protein during heat shock and recovery suggest that GSK-3 β inactivation may increase the promoter activity of HSF-1, contributing to increased accumulation of this transcription factor which may lead to increased transcription of cytoprotective HSPs. However, we did not observe alterations in the protein contents of HSP70 after heat-shock treatment and during the recovery phase. It should be noted that HSP70 expression was high under basal conditions in all mutants. It is plausible that increased PP-1 activity may limit the increase in HSP70 expression after heat-shock treatment. Joyeux et al. [46] demonstrated that OA, a PP-1 inhibitor, increased the expression of HSP70. Alternatively, HSF-1 may be inducing the expression of the other HSPs such as HSP72. Furthermore, HSP90 is known to bind to Akt and modulate its kinase activity in BALB/3T3 and HEK-293 cells [47]. Inhibition of Akt–HSP90 binding resulted in Akt dephosphorylation in these cell lines [47]. In our system, Akt phosphorylation is modulated by heat-shock-induced PI 3-kinase signalling and, therefore, HSP90 may not play a major role in modulating Akt activation status.

It is worth noting that heat-shock-induced increase in PP-1 activity is not dependent on the PI 3-kinase signalling pathway, as wortmannin did not affect PP-1 activity in cells expressing wild-type and PP-1_G mutants. Therefore it is plausible that elevations in PP-1 activity after heat-shock treatment may be mediated via other signalling mediators which are known to be affected during cellular heat stress. Jenkins et al. [48] have reported acute activation of *de novo* sphingolipid biosynthesis in Molt-4 cells after heat-shock treatment, causing accumulation of ceramide- and heat-shock-induced dephosphorylation of sarcoplasmic reticulum proteins probably via elevations in PP-1 activity. In this context, it is interesting to note that PP-1_G and sarcoplasmic reticulum-associated PP-1-regulatory subunits are similar. Therefore it is plausible that heat-shock-induced elevations in ceramide may differentially activate the PP-1 catalytic subunit associated with S1 mutant as opposed to wild-type and the other PP-1_G mutants. Further studies are needed to document the role of ceramide in PP-1 activation by heat shock. Other signalling pathways such as p38MAPK, which are commonly elevated during heat shock and other forms of cellular stress, do not appear to contribute to the observed elevations in heat-shock-induced glycogen synthesis, since SB203580 did not block the heat-shock effect on Akt/GSK-3 β phosphorylation, PP-1 activation and glycogen synthesis.

Differential sensitivities of PP-1_G mutants to heat shock in terms of glycogen depletion, glycogen synthesis, GS activation and Gp inactivation underscore the importance of PP-1_G site-specific phosphorylation in the control of glycogen synthesis under stressful conditions such as exercise, which requires regulated glycogen breakdown as well as a rapid build-up of depleted glycogen stores after the cessation of exercise. In this context, studies by Aschenbach et al. [1] have shown that the

absence of PP-1_G in PP-1_G-null mice resulted in lack of exercise-induced increases in GS fractional activity and glycogen levels along with significantly decreased exercise capacity without an effect on exercise-induced increase in glucose uptake when compared with mice overexpressing PP-1_G. Our results not only confirm the observations of the study mentioned above regarding the importance of PP-1_G in the regulation of basal and exercise-induced glycogen synthesis, but extend them further by documenting the importance of site-specific phosphorylations of PP-1_G in the regulation of glycogen metabolism during cellular stress caused by hyperthermia, commonly observed during exercise. The role of PP-1_G site-2 phosphorylation in adrenaline-induced glycogen breakdown *in vivo* has been well-documented [3,4]. The present study demonstrates that although PP-1_G site-2 phosphorylation may be responsible for glycogen breakdown, PP-1_G site-1 phosphorylation may protect cells from excessive breakdown during heat stress. In confirmation with this observation, we noticed that substitution of Ser with Asp, which is known to mimic phosphorylation, prevented glycogen breakdown during heat shock and abolished heat-shock-induced increase in glycogen synthesis.

Unlike that in *S. cerevisiae* [29], mammalian PP-1_G does not appear to interact with HSF-1 to cause gene transcription, since immunoprecipitation of heat-shock-treated cell extracts with HSF-1 antibody followed by immunoblot analysis of the immunoprecipitates with PP-1_G and vice versa did not document association of these two proteins. However, we cannot exclude the possibility that PP-1_G may cause dephosphorylation of HSF-1 without protein-protein interaction.

In summary, the results presented indicate that site-specific phosphorylations of PP-1_G may control glycogen breakdown during heat stress by regulating the activation status of GS and Gp. In addition, PI 3-kinase-dependent Akt/GSK-3 β phosphorylation appears to play a predominant role in heat-shock-induced glycogen synthesis in addition to elevated PP-1 activity which may be regulated independent of PI 3-kinase activation.

This work was supported in part by a research award from the American Diabetes Association and by Medical Education Funds from Winthrop University Hospital.

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Received 21 October 2002/3 January 2003; accepted 23 January 2003

Published as BJ Immediate Publication 23 January 2003, DOI 10.1042/BJ20021644