Insulin regulation of hepatic insulin-like growth factor-binding protein-1 (IGFBP-1) gene expression and mammalian target of rapamycin (mTOR) signalling is impaired by the presence of hydrogen peroxide

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Hepatic expression of insulin-like growth factor-binding protein-1 (IGFBP-1) is rapidly and completely inhibited by insulin. The signalling pathway that mediates this effect of insulin requires the activation of phosphoinositide 3-kinase (PI 3-kinase). Many of the cellular actions of insulin, including activation of PI 3-kinase, can be 'mimicked' by oxidative stresses, such as H_2O_2. In the present study, we demonstrate that H_2O_2 does not 'mimic' but rather antagonizes insulin repression of IGFBP-1 gene expression in H4IIE cells. This effect is accompanied by a decrease in the insulin-induced activation of mammalian target of rapamycin (mTOR)-dependent signalling. However, insulin-induced phosphorylation and regulation of protein kinase B, glycogen synthase kinase-3 and FKHR (forkhead in rhabdomyosarcoma) are not affected by H_2O_2 in the same cells. In addition, H_2O_2 strongly activates the p42/p44 mitogen-activated protein kinases, but the presence of PD184352 (an inhibitor of this pathway) does not block the effect of H_2O_2 on IGFBP-1 gene expression. Our results support the view that the insulin-mediated repression of IGFBP-1 gene expression is partly mTOR-dependent, and demonstrate that H_2O_2 selectively antagonizes mTOR-dependent insulin action. The implications for the use of H_2O_2-generating agents as therapeutics for the treatment of insulin resistance, as well as the role of oxidative stress in the development of insulin resistance, are discussed.

Key words: diabetes, gene transcription, phosphorylation, protein kinase B, signalling.

INTRODUCTION

Insulin regulates the expression of over 100 gene products in multiple cell types [1]. Defects in the regulation of gene expression may underlie or contribute to the development of insulin-resistant states, such as Type II diabetes mellitus. The identification of agents that can mimic key actions of insulin, thereby overriding insulin resistance, may aid the development of treatments for such disorders. Phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6Pase) and insulin-like growth factor (IGF)-binding protein-1 (IGFBP-1) are three insulin-regulated metabolic genes expressed in the liver. PEPCK and G6Pase are rate-controlling enzymes of hepatic gluconeogenesis, whereas IGFBP-1 binds to and inhibits IGFI and IGFII with high affinity. The expression of all three genes is induced by glucocorticoids and inhibited by insulin in a dominant manner [2]. Insulin mediates its actions by the regulation of signalling pathways, including the activation of phosphoinositide 3-kinase (PI 3-kinase) activity, to generate 3-phosphoinositides that in turn stimulate the activity of protein kinases, such as 3-phosphoinositide-dependent kinase (PDK1) and protein kinase B (PKB). In addition, insulin stimulates the small G-protein Ras, leading to activation of a protein kinase cascade consisting of Raf-1, mitogen-activated protein kinase (MAPK) kinase-1, p42/p44 MAPK and p90 ribosomal S6 kinase (p90RSK). The repression of PEPCK, G6Pase and IGFBP-1 by insulin is dependent on PI 3-kinase activity, but does not require activation of the p42/p44 MAPK cascade [3–7]. However, the identity of the molecules linking PI 3-kinase to these gene promoters remains unclear. We have demonstrated previously [8] that inhibitors of the protein kinase glycogen synthase kinase-3 (GSK3), whose activity is reduced by insulin in a PI 3-kinase- and PKB-dependent fashion [9], can mimic the effect of insulin on the expression of PEPCK and G6Pase. Indeed, overexpression of PKB can regulate the thymine-rich insulin-response element (tIRE) that mediates insulin repression of the IGFBP-1 gene promoter [10–12]. Taken together, these data suggest that the effect of PKB overexpression on the tIRE may be due to inhibition of GSK3. However, PKB also phosphorylates and inhibits the transcription factor FKHR (forkhead in rhabdomyosarcoma; for review see [12]). This protein can bind to the tIRE *in vitro* and induces the activity of tIRE-containing promoters. This indicates a potential role for FKHR in the insulin regulation of tIRE-containing promoters. However, insulin regulation of endogenous IGFBP-1 (but not G6Pase and PEPCK) gene expression is antagonized by rapamycin, a selective inhibitor of mTOR (mammalian target of rapamycin) [4,13], another protein kinase thought to lie downstream of PI 3-kinase. Rapamycin blocks insulin regulation of the IGFBP-1 tIRE, but does not antagonize insulin regulation of PKB, FKHR or GSK3 [13]. Similarly, insulin regulation of mTOR activity and IGFBP-1 expression both require the presence of amino acids [13]. Amino acids do not affect insulin regulation of PKB, FKHR or GSK3. Therefore insulin regulates the expression of IGFBP-1, at least in part, by activation of an mTOR-dependent signalling pathway. Meanwhile, agents that strongly activate the MAPK cascade (e.g. phorbol esters), reduce

Abbreviations used: 4E-BP1, eIF4E-binding protein 1; FKHR, forkhead in rhabdomyosarcoma; FKHR-L1, FKHR-like 1; GSK3, glycogen synthase kinase-3; G6Pase, glucose 6-phosphatase; IGF, insulin-like growth factor; IGFBP-1, IGF-binding protein-1; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; p90RSK, p90 ribosomal S6 kinase; PDK1, 3-phosphoinositide-dependent kinase; PEPCK, phosphoenolpyruvate carboxykinase; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; S6K, ribosomal S6 protein kinase; tIRE, thymine-rich insulin-response element.

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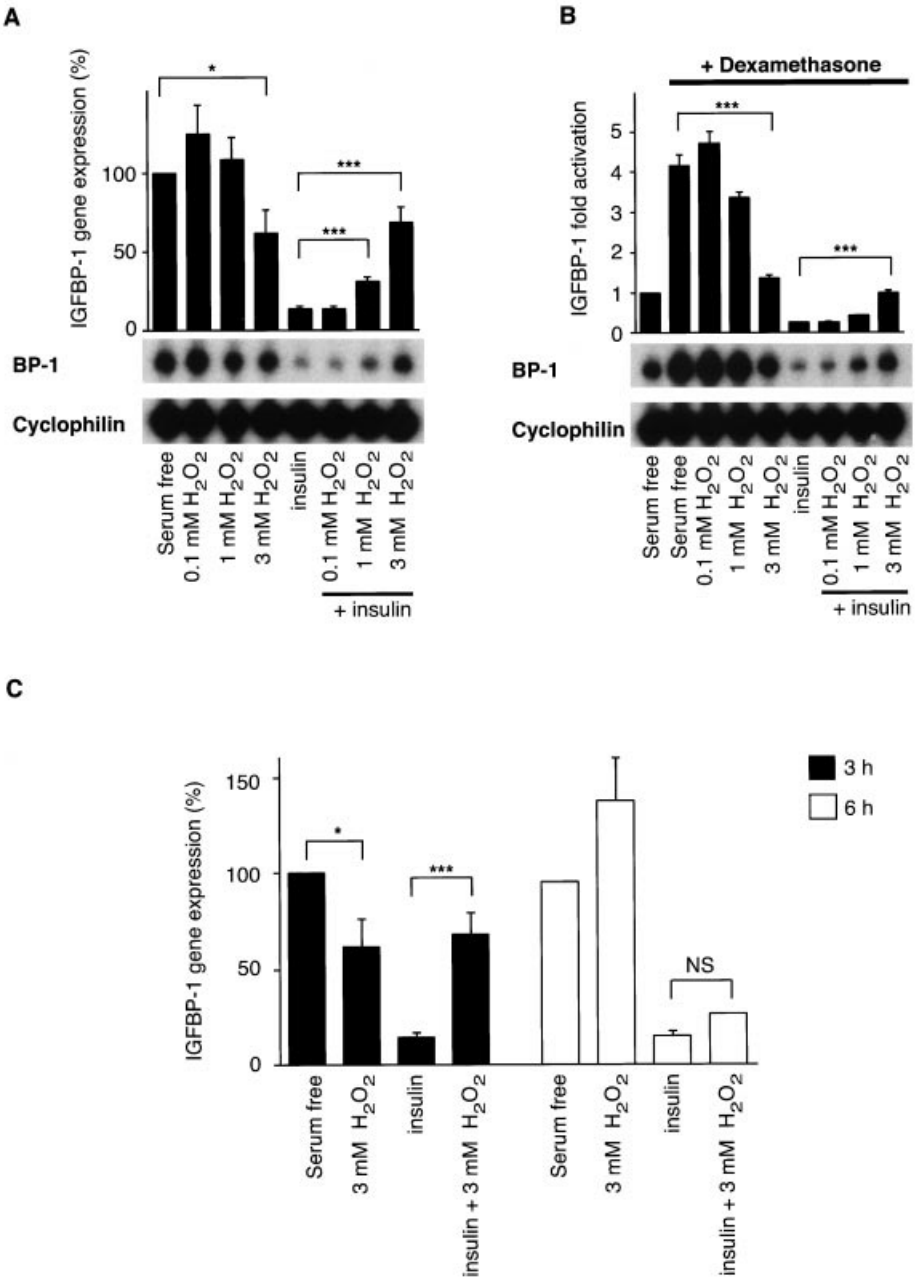

Figure 1 Effects of H_2O_2 on IGFBP-1 gene expression

H4IIE cells were serum-starved overnight prior to incubation for 3 h (**A**, **B** and **C**) or 6 h (**C**) with 500 nM dexamethasone, 10 nM insulin and various concentrations of H_2O_2 as indicated. (**A**–**C**) Total cellular RNA was isolated and an RNase-protection assay was performed to assess IGFBP-1 gene expression, as described in the Experimental section. Representative experiments showing the levels of IGFBP-1 (BP-1) and cyclophilin mRNA (**A** and **B**, middle and bottom panels respectively) as well as quantification of two such experiments performed in duplicate are shown (**A** and **B**, upper panels). Results are the means ± S.E.M., presented either as percentage gene expression relative to control (**A** and **C**) or fold induction over control (**B**). $^{*}P < 0.05$; $^{***}P < 0.001$; and NS, not significant.

the sensitivity of the IGFBP-1, but not the G6Pase and PEPCK, promoters to insulin [14]. All of these data suggest that distinct mechanisms are used by insulin to regulate the expression of these three genes, despite the presence of a related tIRE in all three promoters.

There is a great deal of evidence that H_2O_2 treatment of cells can mimic many of the cellular actions of insulin. Indeed, H_2O_2 has been hypothesized to be a potential 'second messenger' for insulin, since nanomolar amounts of H_2O_2 are generated in adipocytes upon stimulation with insulin [15,16]. These insulin-like actions include the stimulation of glucose transport [17–19], glycogen synthesis [20], lipogenesis [17], and the reduction of PEPCK [21], G6Pase (P. A. Lochhead and C. Sutherland, unpublished work), cytochrome P450 1A1 ('CYP1A1') and cytochrome P450 1A2 ('CYP1A2') [22] gene expression. These effects of H_2O_2 are linked to the regulation of the insulin

signalling pathways described above. H_2O_2 treatment of cells leads to tyrosine phosphorylation of the insulin receptor [23,24], the insulin receptor substrate 1 ('IRS-1') [25,26] and stimulation of PI 3-kinase [27] and PKB [28,29]. Recent studies demonstrated that H_2O_2 treatment of Swiss 3T3 fibroblasts generates PtdIns(3,4)P_2 via the activation of a PtdIns 4-kinase [30]. This lipid binds to and activates PDK1 as well as PKB, thereby explaining some of the 'insulinomimetic' effects observed with H_2O_2. The question arises as to whether agents that generate H_2O_2 in cells mimic enough actions of insulin to alleviate the complications associated with insulin resistance.

In the present study, we report that H_2O_2 antagonizes insulin inhibition of IGFBP-1 gene expression in H4IIE cells. In addition, we demonstrate that the effect of H_2O_2 is linked to a transient inhibition of mTOR signalling, without any reduction in insulin regulation of PI 3-kinase, PKB or PKB substrates.

EXPERIMENTAL

Materials

Radioisotopes were obtained from Amersham, Little Chalfont, Bucks., U.K. ([γ-^{32}P]ATP) and ICN, Thame, Oxon., U.K. ([α-^{32}P]UTP). Insulin was purchased from Novo Nordisk (Crawley, West Sussex, U.K.), H_2O_2 was from Sigma (Poole, Dorset, U.K.), and the RNase Protection Assay Kit II was from AMS Biotech/Ambion (Austin, TX, U.S.A.). Peptide substrates for protein kinase assays were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). All other chemicals were of the highest grade available.

Cell culture

The rat hepatoma cell line H4IIE was maintained in Dulbecco's modified Eagle's medium containing 1000 mg/l glucose and 5% (v/v) foetal calf serum, as described previously [31]. Cells were incubated with the agents at 37 °C for the times and at the concentrations indicated in the Figure legends.

RNA isolation and RNase-protection assay

H4IIE cells were serum-starved overnight and treated with agent/inhibitor for the times and at the concentrations indicated in the Figure legends. Total cellular RNA was isolated using TriReagent™ (Sigma) following the manufacturer's instructions. An RNase-protection assay was performed to determine the relative amounts of IGFBP-1 and cyclophilin mRNA in each sample [14]. Band intensity was quantified on a Phosphorimager (Fuji, Straubenhardt, Germany) and data calculated as a ratio of IGFBP-1/cyclophilin mRNA and presented as fold activation (for induced samples), where the intensity of control samples was set at 1, or as percentage gene expression (for non-induced samples), where the level of gene expression in untreated cells was set at 100%.

Preparation of cell extract for kinase assays and Western blotting

H4IIE cells were incubated in serum-free medium with the agents and inhibitors for the times and at the concentrations indicated in the Figure legends. Cells were then scraped into ice-cold lysis buffer [25 mM Tris/HCl (pH 7.4), 50 mM NaF, 100 mM NaCl, 1 mM sodium vanadate, 5 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, 1 mM benzamidine, 0.1 mM PMSF, 0.27 M sucrose, 2 μM microcystin and 0.1% (v/v) 2-mercaptoethanol]. Cell debris was removed by centrifugation at 13000 ***g*** for 5 min and the protein concentration determined, using BSA as an internal standard, by the method of Bradford [32].

Immunoprecipitation and assay of protein kinases

Cell extract (0.1 mg) was incubated for 1 h on a shaking platform with Protein G–Sepharose conjugated to the appropriate antibody. The immunocomplexes were pelleted and washed twice with 1.0 ml of buffer A [50 mM Tris/HCl (pH 7.5), 50 mM NaF, 500 mM NaCl, 1 mM sodium vanadate, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 5 mM sodium pyrophosphate, 0.27 M sucrose and 0.1% (v/v) 2-mercaptoethanol], and twice with 1.0 ml of buffer B [50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA and 0.1% (v/v) 2-mercaptoethanol]. The immunoprecipitated kinase activities were assayed at 30 °C, in a total volume of 50 μl containing 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 2.5 μM protein kinase A-inhibitor peptide, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (2×10^6 c.p.m./nmol) and 30 μM crosstide (for assay of PKB and p90RSK) [9] or 30 μM long S6 peptide [for assay of ribosomal S6 protein kinase (S6K)] [33]. One unit of kinase activity was defined as the amount which catalyses the phosphorylation of 1 nmol of substrate in 1 min.

Antibodies for immunoprecipitation and Western-blot analysis

Antibodies to p90RSK, anti-phospho ribosomal protein S6 (Ser-235), phospho-specific Thr-32 FKHR-L1 (FKHR-like 1), total S6K, PH-domain-specific PKB and GSK3β were purchased from Upstate Biotechnology, and phospho-specific Ser-9/Ser-21 GSK3, Thr-308 PKB, Ser-473 PKB, Thr-389 S6K1, Thr-183/Tyr-185 p42/p44 MAPK antibodies were purchased from New England Biolabs (Hitchin, Herts., U.K.). The phospho-specific and control eIF4E-binding protein 1 (4E-BP1) antibodies were a gift from Professor C. G. Proud (Department of Anatomy and Physiology, University of Dundee, Dundee, U.K.). H4IIE cell lysates were prepared following incubation with the agents, as described in Figure legends, and analysed by Western-blot analysis [31]. A similar procedure was performed for 4E-BP1, except an Immobilion™-P membrane (Millipore, Watford, Herts., U.K.) replaced nitrocellulose and proteins were fixed with glutaraldehyde prior to incubation with primary and secondary antibodies.

PI 3-kinase assay

PI 3-kinase was immunoprecipitated from H4IIE lysate using 1 μg of anti-phosphotyrosine antibody (PY20; Affinity Research, Mamhead, Exeter, Devon, U.K.) and assayed as described previously [30].

Lipid extraction

H4IIE cells (60 mm dishes) were serum-starved overnight prior to incubation for 10 min at 37 °C with the agents, as indicated in the Figure legends. Lipids were harvested by addition of 1 ml of ice-cold 10% (v/v) tricholoroacetic acid and centrifugation at 14000 ***g*** for 5 min at 4 °C. Pellets were washed with 0.5 ml of ice-cold 5% (v/v) tricholoroacetic acid /1 mM EDTA and extracted with 0.75 ml of chloroform/methanol/HCl (20:40:1, by vol.). After standing on ice for 20 min, 0.25 ml of chloroform and 0.45 ml of 0.1 M HCl were added, the samples were vortexed and centrifuged at 12000 ***g*** for 2 min at 4 °C. The lower organic phase was transferred to a clean screw-cap Eppendorf tube, the upper aqueous phase was re-extracted with 0.5 ml of chloroform/methanol/HCl (1:1:0.9, by vol.) and the organic phases pooled. Extracted lipids were dried and alkaline hydrolysed. The concentration of the released Ins(1,3,4,5)P_4 [the polar

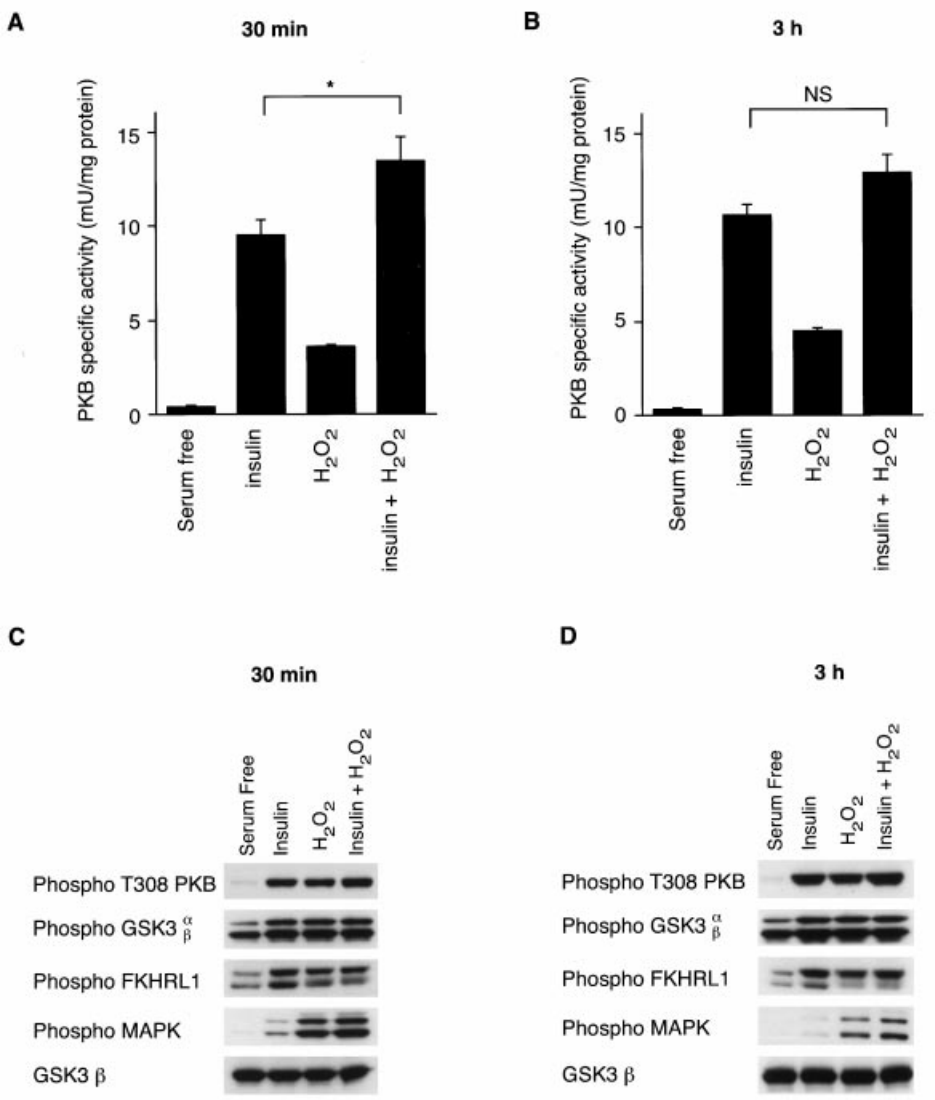

Figure 2 Insulin regulation of PKB, GSK3, FKHR-L1 and MAPK is not reduced by H_2O_2

H4IIE cells were serum-starved overnight prior to incubation with 10 nM insulin and/or 3 mM H_2O_2 for 30 min (**A** and **C**) or 3 h (**B** and **D**). (**A** and **B**) Cells were lysed, PKB was immunoprecipitated and assayed as described in the Experimental section. Results are the means ± S.E.M. from two experiments performed in triplicate. *$P < 0.05$; and NS, not significant. (**C** and **D**) Cells were lysed, the lysates subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with antibodies as labelled (phospho, phospho-specific antibody). Similar results were obtained from two experiments carried out in duplicate.

headgroup of PtdIns(3,4,5)P_3] was measured using a radioligand displacement assay as described previously [30]. Briefly, displacement of [3-^{33}P]Ins(1,3,4,5)P_4 from recombinant GAP1^{IP4BP} (a Ras GTPase-activating protein) by sample-derived Ins(1,3,4,5)P_4 [or authentic Ins(1,3,4,5)P_4 for calibration] was measured by scintillation counting.

RESULTS AND DISCUSSION

H_2O_2 regulation of IGFBP-1 gene expression

Oxidative stress (e.g. H_2O_2) has long been known to modulate cellular metabolism and regulate the expression of various genes (for review see [34]). In numerous studies, H_2O_2 has been shown to enhance and even mimic the cellular actions of insulin. The regulation of basal and glucocorticoid-induced IGFBP-1 gene expression is dominantly blocked by insulin in H4IIE cells. Treatment of these cells with H_2O_2 caused a small reduction (approx. 40 %) in basal expression of IGFBP-1 (Figure 1A). In contrast, the glucocorticoid-induced expression of IGFBP-1 was much more markedly reduced by the presence of an equivalent concentration of H_2O_2 (Figure 1B). It has been reported previously [35] that H_2O_2 reduces glucocorticoid receptor activity, and this may underlie the reduction of glucocorticoid-induced IGFBP-1 gene expression. In this respect, this is an insulin-like effect of H_2O_2. However, most interestingly, insulin repression of basal (or glucocorticoid-induced) IGFBP-1 expression was actually blocked by the presence of H_2O_2 (Figures 1A–1C). This is in contrast with the insulin 'mimetic' effects of H_2O_2 on PEPCK [21] or G6Pase (P. A. Lochhead and C. Sutherland, unpublished work) gene expression in the same cell line. The antagonistic effects of H_2O_2 on this insulin action were observed in cells incubated for up to 3 h, but were lost at longer incubation times

Table 1 H_2O_2 induces the generation of PtdIns(3,4,5)P_3 but does not antagonize insulin induction of PtdIns(3,4,5)P_3 and PI 3-kinase activity in H4IIE cells

H4IIE cells were serum-starved overnight before incubation with 10 nM insulin and/or 3 mM H_2O_2 for 10 min. Cellular lipids were extracted and PtdIns(3,4,5)P_3 levels were determined as described in the Experimental section. Results are means ± S.E.M. from two experiments performed in triplicate and are expressed as accumulation of PtdIns(3,4,5)P_3 per 10^6 cells. *$P < 0.05$ between serum-free and H_2O_2-treated cells. No significant difference was observed between insulin- and insulin + H_2O_2-treated cells. Alternatively, cells were lysed, PI 3-kinase immunoprecipitated and assayed as described in the Experimental section. Results are means ± S.E.M. from two experiments performed in duplicate and presented as percentage stimulation in relation to insulin (100%). No significant difference was observed between untreated cells and cells treated with H_2O_2.

	PtdIns(3,4,5)P_3 (pmol/10^6 cells)	PI 3-kinase activity (%)
Serum-free	1.563 ± 0.472*	5.35 ± 0.88
Insulin	3.113 ± 0.223	100 ± 12.64
H_2O_2	2.461 ± 0.315	6.01 ± 0.84
Insulin + H_2O_2	3.333 ± 0.481	134 ± 6.37

(Figure 1C). Further additions of H_2O_2 did not overcome the transient nature of this H_2O_2 effect (results not shown). We have proceeded to characterize in detail the signalling mechanisms that mediate the H_2O_2 antagonism of insulin regulation of basal IGFBP-1 gene expression.

H_2O_2 regulation of PI 3-kinase and PKB

Within the liver, insulin-induced tyrosine phosphorylation of insulin receptor substrate 1 and activation of PI 3-kinase can be mimicked by the administration of H_2O_2 [27]. In agreement with this, we observed an increase in the accumulation of the product of the PI 3-kinase reaction [PtdIns(3,4,5)P_3] in H_2O_2-treated H4IIE cells (Table 1), although we could not detect a significant stimulation of PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates (Table 1). This may reflect the difference in sensitivity of these two techniques. Importantly, H_2O_2 did not inhibit insulin-induced PI 3-kinase activity (Table 1) or insulin stimulated production of PtdIns(3,4,5)P_3 (Table 1). Next, we examined the regulation of PKB and two of its substrates, GSK3 and FKHR-L1. H_2O_2 and insulin potently activated PKB in H4IIE cells, whereas simultaneous treatment resulted in an additive induction of PKB activity (Figures 2A and 2B). This activation was accompanied by an equivalent level of phosphorylation of PKB (Thr-308), GSK3α (Ser-21), GSK3β (Ser-9) and FKHR-L1 (Thr-32) (Figures 2C and 2D). Similar results were obtained when H4IIE cells were treated for 30 min or 3 h (Figure 2). Phosphorylation of Thr-308 correlates with the activation of PKB, whereas phosphorylation of GSK3β on Ser-9, GSK3α on Ser-21 and FKHR-L1 on Thr-32 is indicative of inhibition of these PKB substrates. The inhibition of GSK3 by H_2O_2 may well account for the inhibition of PEPCK [21] and G6Pase (P. A. Lochhead and C. Sutherland, unpublished work) expression following H_2O_2 treatment of liver cells, since selective inhibitors of GSK3 also reduce PEPCK and G6Pase gene expression [8]. However, our data suggest that the mechanism by which H_2O_2 attenuates insulin regulation of IGFBP-1 gene expression is not due to inhibition of PI 3-kinase, the PKB/GSK3 or PKB/FKHR-L1 pathways. This is consistent with previous data [4,13] demonstrating that insulin regulates IGFBP-1 in an mTOR-dependent fashion, as opposed to the mechanism that has been proposed for certain tIRE-containing promoters (a PI 3-kinase- and PKB-dependent inhibition of the transcription factor FKHR [36]).

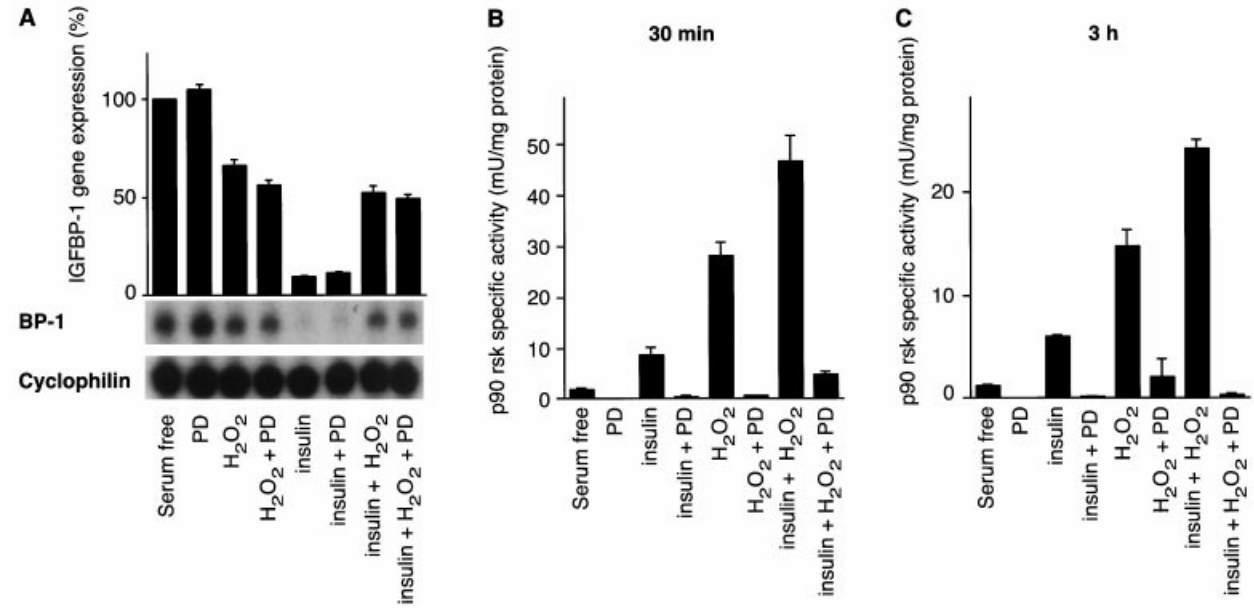

Figure 3 Strong activation of MAPK cascade does not mediate the effect of H_2O_2 on insulin regulation of IGFBP-1 gene expression

H4IIE cells were serum-starved overnight and pre-incubated with or without 2 μM PD184352 (PD) for 30 min, as indicated, prior to a 3 h incubation with 10 nM insulin and/or 3 mM H_2O_2 ± PD (2 μM). (**A**) Total cellular RNA was isolated and an RNase-protection assay performed to assess the IGFBP-1 gene expression. Results are presented as percentage gene expression relative to control (upper panel) and are means ± S.E.M. of two experiments performed in duplicate. A representative experiment showing the levels of IGFBP-1 (BP-1; middle panel) and cyclophilin mRNA (bottom panel) is also shown. Alternatively, after 30 min (**B**) or 3 h (**C**) of treatment, cells were lysed, p90RSK was immunoprecipitated and assayed as described in the Experimental section. Results are the means ± S.E.M. from two experiments performed in triplicate.

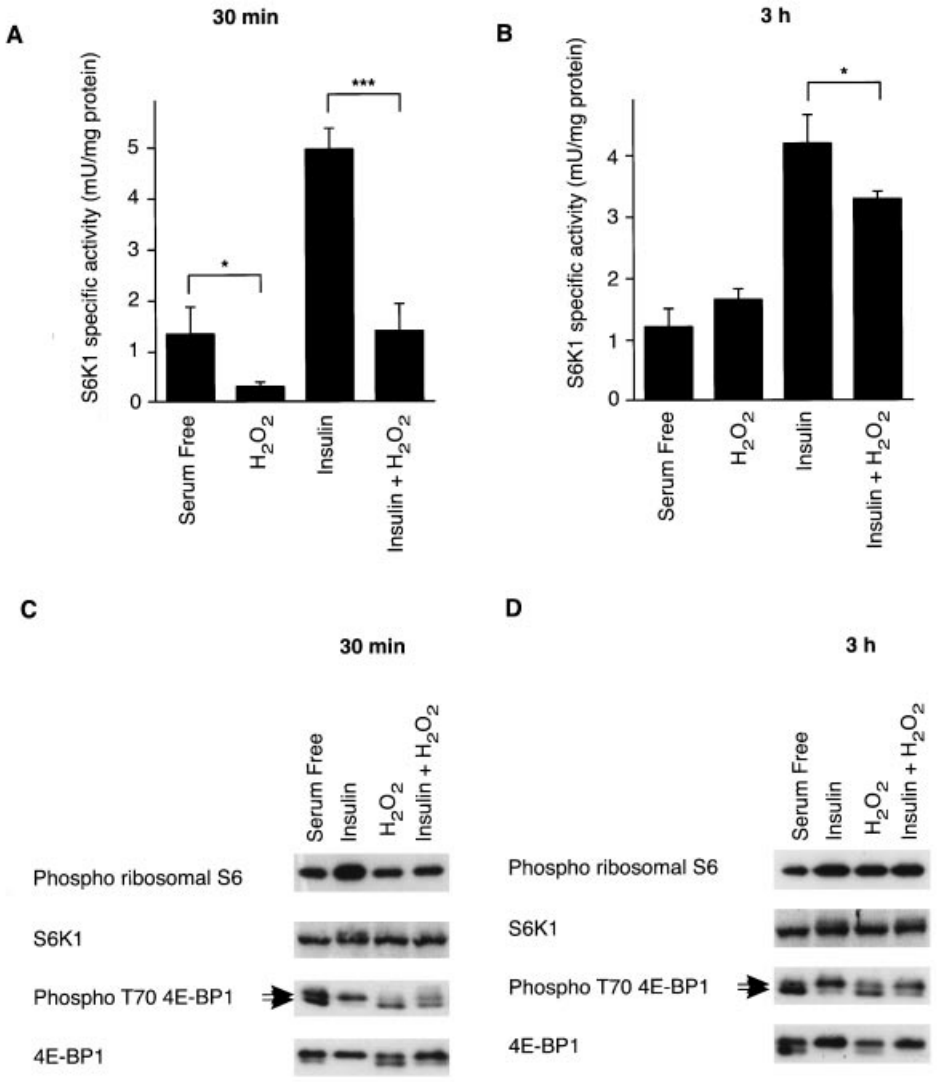

Figure 4 H_2O_2 antagonizes insulin activation of mTOR-regulated pathways

H4IIE cells were serum-starved overnight prior to incubation with 10 nM insulin and/or 3 mM H_2O_2 for 30 min (**A** and **C**) or 3 h (**B** and **D**). (**A** and **B**) Cells were lysed and S6K1 was immunoprecipitated and assayed as described in the Experimental section. Results are the means ± S.E.M. from two experiments performed in triplicate. (**C** and **D**) Cells were lysed and the lysates were subjected to SDS/PAGE, transferred to nitrocellulose or Immobilon^{TM}-P (4E-BP1) and immunoblotted with antibodies as labelled (phospho, phospho-specific antibody). Similar results were obtained from two experiments carried out in duplicate. $^{*}P < 0.05$ and $^{***}P < 0.001$

H_2O_2 regulation of p42/p44 MAPK

Insulin and H_2O_2 both activate the p42/p44 MAPK pathway; however, the activation by H_2O_2 was much stronger than that observed with insulin, as judged by the phosphorylation of Thr-183 and Tyr-185 of p42/p44 MAPK (Figures 2C and 2D). We have demonstrated previously [14] that phorbol ester treatment of H4IIE cells can block insulin regulation of IGFBP-1 gene expression and that part of this effect is mediated by a strong activation of the p42/p44 MAPK. Therefore we investigated whether the activation of this pathway by H_2O_2 underlies its antagonism of insulin regulation of the IGFBP-1 gene. However, in contrast with phorbol esters, the action of H_2O_2 on IGFBP-1 expression was not blocked by the presence of PD184352 (Figure 3A), at concentrations that fully inhibited insulin- or H_2O_2-induced activation of p42/p44 MAPK and its substrate p90RSK (Figures 3B and 3C). Thus we conclude that this pathway is not required for H_2O_2 antagonism of insulin regulation of the IGFBP-1 gene.

H_2O_2 regulation of mTOR signalling

Insulin regulates protein translation, in part, through the activation of S6K and the resultant phosphorylation of ribosomal S6 protein, but also through the phosphorylation and regulation of 4E-BP1 [37]. These processes are dependent on mTOR activity, as they are antagonized by rapamycin and require the presence

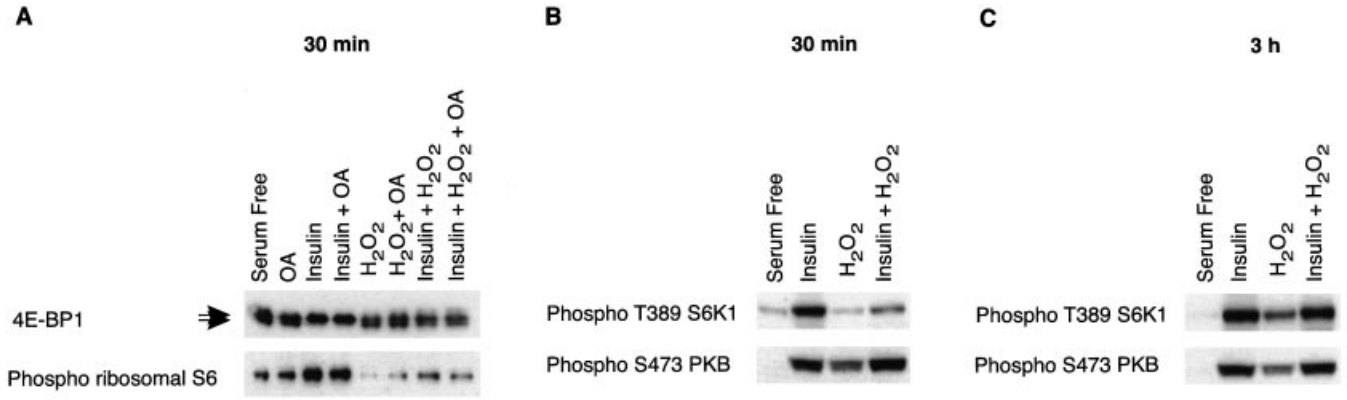

Figure 5 H_2O_2 action is not lost in the presence of okadaic acid, but antagonizes the phosphorylation of the hydrophobic motif of S6K1

H4IIE cells were serum-starved overnight prior to a pre-incubation with 1 μM okadaic acid (OA) as indicated for 30 min, and incubation with 10 nM insulin, 3 mM H_2O_2 and okadaic acid (1 μM), as indicated, for 30 min (**A** and **B**) or 3 h (**C**). Cells were lysed and subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with antibodies as labelled (phospho, phospho-specific antibody). Similar results were obtained from two experiments carried out in duplicate.

of amino acids [38,39]. We have demonstrated previously [3,13] that rapamycin or amino acid withdrawal antagonizes insulin regulation of IGFBP-1 (but not G6Pase or PEPCK) gene expression. Therefore we examined whether H_2O_2 had a rapamycin-like effect on the regulation of mTOR-dependent signalling. Treatment of H4IIE cells with 3 mM H_2O_2 for 30 min reduced basal as well as insulin-induced S6K1 activity (Figure 4A). The effect of H_2O_2 was partial (approx. 60%) and full inhibition was not obtained with higher concentrations of H_2O_2 (results not shown). However, after a 3 h incubation, the effect of H_2O_2 on S6K activity and regulation was greatly diminished (Figure 4B). Similarly, insulin-mediated phosphorylation of endogenous S6 protein (an S6K substrate) was reduced following H_2O_2 treatment of H4IIE cells for 30 min (Figure 4C), but was not affected after a 3 h incubation (Figure 4D). Thus H_2O_2 transiently antagonizes the insulin regulation of S6K.

Next, we examined the hyperphosphorylation of 4E-BP1, a postulated physiological substrate for mTOR. 4E-BP1 is reported to migrate on SDS/PAGE as distinct species (α, β and γ) [40]. The α band (fastest migration) is the least phosphorylated form, whereas the γ band (slowest migration) is the most phosphorylated. Insulin induces formation of the γ species in an mTOR- and amino acid-dependent fashion (for review see [37]). We observed at least two of these species (the β and γ species) using an antibody to the native 4E-BP1 or an antibody that recognized 4E-BP1 phosphorylated at Thr-70 (Figures 4C and 4D). When H4IIE cells were deprived of serum, 4E-BP1 migrated as the β and γ species, with these components being present in similar amounts (Figures 4C and 4D). However, the presence of 10 nM insulin induced a shift towards the most highly phosphorylated γ species, whereas treatment with H_2O_2 alone increased its mobility towards the less highly phosphorylated β form. The ability of insulin to induce the shift toward the γ species was antagonized by treatment of the cells with H_2O_2 for 30 min (Figure 4C), but not by treatment for 3 h (Figure 4D). Thus H_2O_2 antagonizes insulin regulation of two mTOR-dependent signalling pathways. A reduction in 4E-BP1 phosphorylation has also been observed in H_2O_2-treated cardiac myocytes [41]; however, insulin regulation of PKB was also reduced in these cells, suggesting an antagonism of more than just mTOR signalling. Similarly, insulin, but not platelet-derived growth factor ('PDGF'), activation of both S6K and PKB is reduced upon H_2O_2 treatment of adipocytes [42]. However, our data demonstrate that H_2O_2 does not antagonize the activation of PKB in H4IIE cells, but does impair insulin signalling through the mTOR pathway. This may be achieved by the direct inhibition of mTOR, or through the transient activation of a phosphatase that selectively targets 4E-BP1 and S6K. H_2O_2 regulation of a protein phosphatase 1 (PP1)/protein phosphatase 2A (PP2A)-type phosphatase has been reported previously [43]. Therefore we examined whether okadaic acid blocked the action of H_2O_2 (Figure 5A). The presence of okadaic acid, at concentrations known to inhibit both PP1 and PP2A, did not reduce the H_2O_2 antagonism of insulin action. This was the case whether phosphorylation of S6 or hyperphosphorylation of 4E-BP1 was measured (Figure 5A). Therefore H_2O_2 does not require activation of PP1 or PP2A to antagonize insulin regulation of mTOR signalling, at least in H4IIE cells.

S6K and PKB are both members of the protein kinases A-, G- and C-related ('AGC') kinase family (for review see [44]) and are regulated by insulin-induced phosphorylation of two specific residues, one within the T-loop and one within a hydrophobic motif present within each protein kinase domain [44]. Interestingly, using phospho-specific antibodies, we found that H_2O_2 impaired insulin-induced phosphorylation of S6K at Thr-389 (the hydrophobic motif residue), but not the phosphorylation of the equivalent regulatory residue on PKB, namely Ser-473 (Figure 5B). This effect was observed after treatment of H4IIE cells with H_2O_2 for 30 min, but lost after a 3 h incubation (Figures 5B and 5C). Since the phosphorylation of Thr-389 of S6K (but not Ser-473 of PKB) is sensitive to rapamycin, it is likely that this effect is due to a direct inhibition of mTOR by H_2O_2. Thus we hypothesize that H_2O_2 antagonizes mTOR activity/signalling and that this ultimately blocks insulin regulation of IGFBP-1 gene expression.

Conclusions

Although many studies have indicated that generation of H_2O_2 would have beneficial effects on glucose disposal and hepatic glucose production in insulin-resistant states [21–29], our finding that it also antagonizes mTOR signalling has major implications for the use of H_2O_2-generating agents as insulin mimetics. As

rapamycin is currently being used as an immunosuppressant in kidney transplantation, it will soon be possible to determine whether there are risks associated with inhibition of mTOR. Indeed, it will be interesting to determine whether these patients develop any defects in glucose homoeostasis. Conversely, oxidative stress, leading to reduced mTOR signalling, may play a role in the development of insulin resistance. Recent epidemiological evidence proposes a role for inflammation in the development of Type II diabetes [45]. In the present study, we show that H_2O_2 antagonizes insulin regulation of IGFBP-1 gene expression in H4IIE cells, and that this is mediated through a reduction of mTOR-regulated pathways and not PI 3-kinase or PKB activity. This is not only consistent with an important role for mTOR activity in the regulation of IGFBP-1 gene transcription, but also provides another example of distinct regulation of PEPCK and IGFBP-1 gene expression. Indeed, the data suggest that oxidative stress would block mTOR signalling in the liver, while stimulating events downstream of PKB signalling. This may have beneficial effects on some aspects of insulin resistance, but would also provide a reduction in the ability of insulin to regulate protein synthesis and expression of specific genes (e.g. IGFBP-1, Na^+/P_i-cotransporter-1 [46], and hexokinase-II [47]), thereby producing a form of insulin resistance.

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