The activation of protein kinase B by H_2O_2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2

Morag SHAW[1], Philip COHEN and Dario R. ALESSI

MRC Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, U.K.

Protein kinase B (PKB) isoforms became activated [and glycogen synthase kinase-3 (GSK3) became inhibited] when mouse Swiss 3T3 fibroblasts were exposed to oxidative stress (H_2O_2) or heat shock, but not when they were exposed to osmotic shock (0.5 M sorbitol or 0.7 M NaCl), chemical stress (sodium arsenite), the protein-synthesis inhibitor anisomycin, or UV radiation. In contrast, all seven stimuli activated mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-K2). The activation of MAPKAP-K2 was suppressed by the drug SB 203580, but not by inhibitors of phosphoinositide (phosphatidylinositide, PI) 3-kinase. In contrast, the activation of PKB isoforms and the inhibition of GSK3 by oxidative stress or heat shock were prevented by inhibitors of PI 3-kinase, but not by SB 203580. Thus the activation of PKB by oxidative stress or heat shock is mediated by PI 3-kinase and not by MAPKAP-K2. PKBα and PKBγ were also activated by heat shock and oxidative stress in human embryonic kidney 293 cells and PKBγ was activated by heat shock in NIH 3T3 cells; in each case activation was suppressed by inhibitors of PI 3-kinase. The activation of PKB isoforms by H_2O_2 may underlie some of the insulin-mimetic effects of this compound.

INTRODUCTION

Protein kinase Bα (PKBα, also known as c-Akt1) becomes activated via a phosphoinositide (phosphatidylinositide, PI) 3-kinase-dependent pathway when cells or tissues are stimulated with insulin or growth factors [1,2]. In this pathway, phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], the product of the PI 3-kinase reaction, interacts with the pleckstrin homology domain of PKBα, recruiting it to the plasma membrane where it becomes activated by the phosphorylation of Thr308 and Ser473 (reviewed in [3]). The phosphorylation of Thr308 is catalysed by PDK (3-phosphoinositide-dependent protein kinase) 1 [4–7] and the phosphorylation of Ser473 by an as-yet-unidentified kinase, termed PDK2 [3]. Phosphorylation of either site alone produces partial activation, but far-larger increases in activity are observed when PKBα is phosphorylated at both sites [8]. The second messenger PtdIns(3,4,5)P_3 plays at least two roles in the activation process. It not only recruits PKBα to the plasma membrane, bringing it into close proximity with PDK1 (and probably PDK2), but also induces a conformational change in PKBα that converts it into a substrate for PDK1 (and probably PDK2). Two closely related isoforms, termed PKBβ and PKBγ, are activated by insulin in a similar manner to PKBα (reviewed in [3]).

PKB phosphorylates serine and threonine residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr-Phe/Leu motifs [9], and appears to have multiple substrates *in vivo*. These include another protein kinase, termed glycogen synthase kinase-3 (GSK3) [10] and the cardiac isoform of 6-phosphofructo 2-kinase [11,12]. Phosphorylation by PKB inhibits GSK3 and this is thought to underlie the insulin-induced dephosphorylation and activation of eukaryotic protein-synthesis-initiation factor 2B [13] and glycogen synthase [10], which contribute to the stimulation of protein synthesis and glycogen synthesis by this hormone. PKB phosphorylates and activates 6-phosphofructo-2-kinase and appears to underlie the insulin-induced stimulation of cardiac-muscle glycolysis. The overexpression of PKBα mimics the stimulatory effects of insulin on glucose [14,15] and amino acid [15] uptake, and on the transcription of the obesity gene product leptin [16]. The overexpression of PKBα also mimics the ability of insulin-like growth factor-1 (IGF-1) to inhibit apoptosis induced in a variety of ways (reviewed in [3,17]).

Two recent papers report that PKBα can be activated when NIH 3T3 fibroblasts are stressed in a variety of ways [18,19]. Importantly, these studies claim that, unlike the effects of insulin or growth factors, the stress-induced activation of PKB is not prevented by inhibitors of PI 3-kinase. If correct, these observations imply that stresses activate PKB by a distinct mechanism, perhaps via the pathway that leads to the activation of mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-K2). MAPKAP-K2, which is activated *in vivo* by stress-activated protein kinase-2a [also called p38 mitogen-activated protein (MAP) kinase] (reviewed in [20]), has been shown to activate PKB 5-fold *in vitro* by phosphorylating Ser473 without phosphorylating Thr308 [8].

The present study was initiated to elucidate the mechanism by which stresses activate PKB. We confirm that oxidative stress and heat shock do indeed trigger the activation of PKB isoforms (and inhibit GSK3) in mouse Swiss 3T3 fibroblasts and human embryonic kidney 293 cells. However, in contrast with earlier reports, we find that these effects are completely prevented by inhibitors of PI 3-kinase and at the same concentrations that prevent the activation of PKB (and the inhibition of GSK3) by insulin or IGF-1. Morever, the activation of PKB (and inhibition of GSK3) by heat shock or oxidative stress is not prevented by the drug SB 203580, which suppresses the activation of MAPKAP-K2 [20]. Thus the activation of PKB induced by oxidative stress or heat shock is mediated via PI 3-kinase and not by MAPKAP-K2 or a novel mechanism.

Abbreviations used: PKB, protein kinase B; MAP, mitogen-activated protein; MAPKAP-K2, mitogen-activated protein kinase-activated protein kinase-2; GSK3, glycogen synthase kinase-3; PI, phosphoinositide (phosphatidylinositide); PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PDK, 3-phosphoinositide-dependent protein kinase; IGF-1, insulin-like growth factor-1; PP2A$_1$, protein phosphatase 2A$_1$.

[1] To whom correspondence should be addressed (e-mail MShaw@BAD.dundee.ac.uk).

MATERIALS AND METHODS

Materials

Protein G–Sepharose was from Pharmacia (Milton Keynes, U.K.). Peptides were synthesized at Dundee by Mr. F. B. Caudwell. Sodium arsenite and H_2O_2 (Aristar grade) were purchased from Merck (Lutterworth, Leics, U.K.), anisomycin and wortmannin from Sigma-Aldrich (Poole, Dorset, U.K.), SB 203580 and LY 294002 from Calbiochem (Nottingham, U.K.). Sources of other materials are given elsewhere [4,5]. The PKBα-specific antibody was raised in sheep against the N-terminal 176 residues of human PKBα [21]. Antibodies specific for PKB α, β and γ [21], MAPKAP-K2 [22] and GSK3α [10] were raised in sheep against the peptides RYDSLGSLELDQRTH (corresponding to residues 455–469 of human PKBβ), RMNCSPTSQIDNI (residues 116–128 of rat PKBγ), KEDKERWEDVKEEMTS (residues 343–358 of human MAPKAP-K2) and QAPDATPTLTNSS (residues 471–483 of rat GSK3α), and affinity-purified on CH-Sepharose columns to which the appropriate peptide was coupled covalently. The antibodies used in this study may be obtained from UBI (Lake Placid, NY, U.S.A.).

Cell culture, stimulation and cell lysis

Mouse Swiss 3T3 fibroblasts [23] and human embryonic kidney 293 cells [8] were cultured to confluence and incubated for 16 h in Dulbecco's modified Eagle's medium from which fetal calf serum was omitted. NIH 3T3 cells were cultured in an identical manner. All cells were stimulated, for the times indicated, with IGF-1 (100 ng/ml), sodium arsenite (0.2–5 mM), H_2O_2 (0.1–5 mM), sorbitol (0.5 M), NaCl (0.7 M), anisomycin (10 μg/ml), UV radiation (200 J/m^2), or incubated at 40–45 °C. The cells were lysed in 1.0 ml of ice-cold buffer A [50 mM Tris-acetate (pH 7.5)/1 % (w/v) Triton X-100/1 mM EDTA/1 mM EGTA/50 mM sodium fluoride/10 mM sodium β-glycerophosphate/5 mM sodium pyrophosphate/1 mM sodium orthovanadate/1 μM microcystin-LR/0.1 % (v/v) 2-mercaptoethanol/'complete' proteinase-inhibitor cocktail (1 tablet per 50 ml)] and the lysates frozen immediately in liquid nitrogen and stored at −80 °C until use. Protein concentrations were determined according to Bradford [24] using BSA as a standard.

Immunoprecipitation and assay of PKB isoforms, MAPKAP-K2 and GSK3

Each PKB isoform (α, β and γ) was immunoprecipitated separately from 0.1 mg of cell lysate using isoform-specific antibodies [21] and assayed using the peptide GRPRTSSFAEG [10]. MAPKAP-K2 was immunoprecipitated from 50 μg of cell lysate and assayed using the peptide KKLNRTLSVA [22]. GSK3α was immunoprecipitated from 50 μg of cell lysate, and the immunoprecipitates were incubated with or without protein phosphatase 2A$_1$ (PP2A$_1$; 25 m-units/ml), and assayed using phospho-glycogen synthase peptide-1 [25]. Activity (1 unit) was defined as the amount which catalysed the phosphorylation of 1 nmol of substrate in 1 min.

RESULTS

PKB was activated by heat shock and oxidative stress in Swiss 3T3 cells

Mouse Swiss 3T3 fibroblasts were subjected to a variety of stresses, or exposed to IGF-1, the most potent activator of PKB in these cells. PKB isoforms and MAPKAP-K2 were then immunoprecipitated from the cell lysates and assayed.

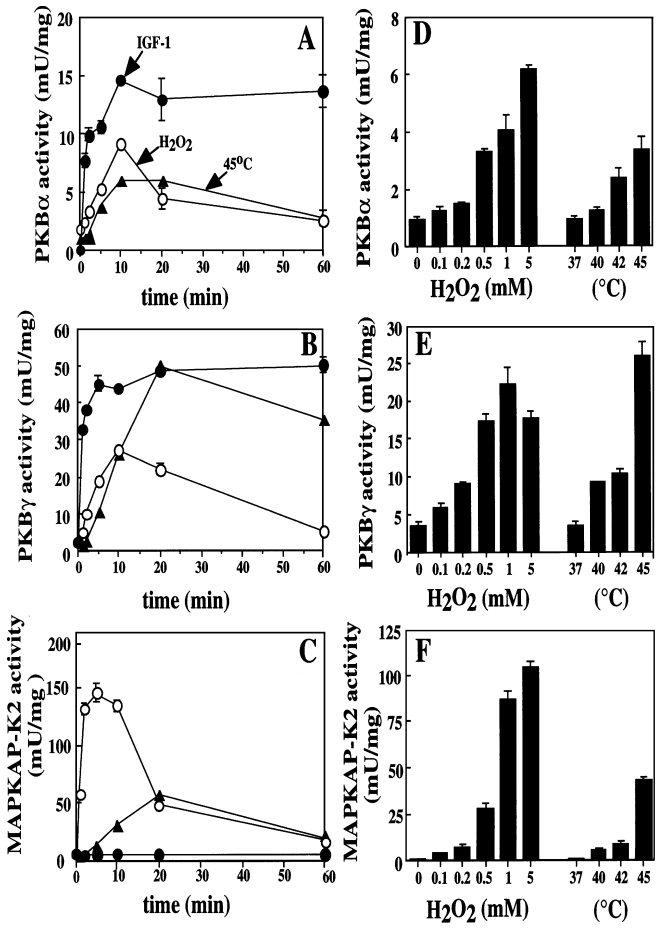

Figure 1 Time course and dose-dependence of the activation of PKB and MAPKAP-K2 by H_2O_2 and heat shock

(**A**–**C**) Swiss 3T3 cells were stimulated for the times indicated with IGF-1 (100 ng/ml, ●), H_2O_2 (1 mM, ○) or incubated at 45 °C (▲). The cells were lysed and PKBα (**A**), PKBγ (**B**) and MAPKAP-K2 (**C**) were immunoprecipitated and assayed. (**D**–**F**) Swiss 3T3 cells were either stimulated for 10 min with the indicated concentrations of H_2O_2 or incubated for 20 min at the indicated temperatures. The cells were lysed and PKBα (**D**), PKBγ (**E**) and MAPKAP-K2 (**F**) were immunoprecipitated and assayed. The data are presented as the means ± S.E.M. for three separate experiments with each determination carried out in triplicate.

H_2O_2 (an oxidative stressor) or a 45 °C heat shock induced a marked activation of PKBα (Figure 1A) and PKBγ (Figure 1B), as well as MAPKAP-K2 (Figure 1C), with no significant appearance of PKBβ activity (results not shown). The IGF-1- or H_2O_2-induced activation of PKB was maximal after 10 min, and the heat shock-induced activation was maximal after 10–20 min. The H_2O_2-induced activation of MAPKAP-K2 was maximal after 2–5 min and the heat shock-induced activation was maximal after 20 min. IGF-1 did not induce any activation of MAPKAP-K2. The effects of H_2O_2 were near maximal at 1–5 mM, whereas the effects of heat shock were maximal for all three enzymes at the highest temperature that could be tested (45 °C) (Figures 1D–1F). All further experiments were therefore carried out using 1 mM H_2O_2 or (for heat shock) a temperature of 45 °C.

A number of other stresses that activated MAPKAP-K2 in Swiss 3T3 cells [sodium arsenite, the protein synthesis inhibitor anisomycin, 0.5 M sorbitol or 0.7 M NaCl (osmotic stresses) and

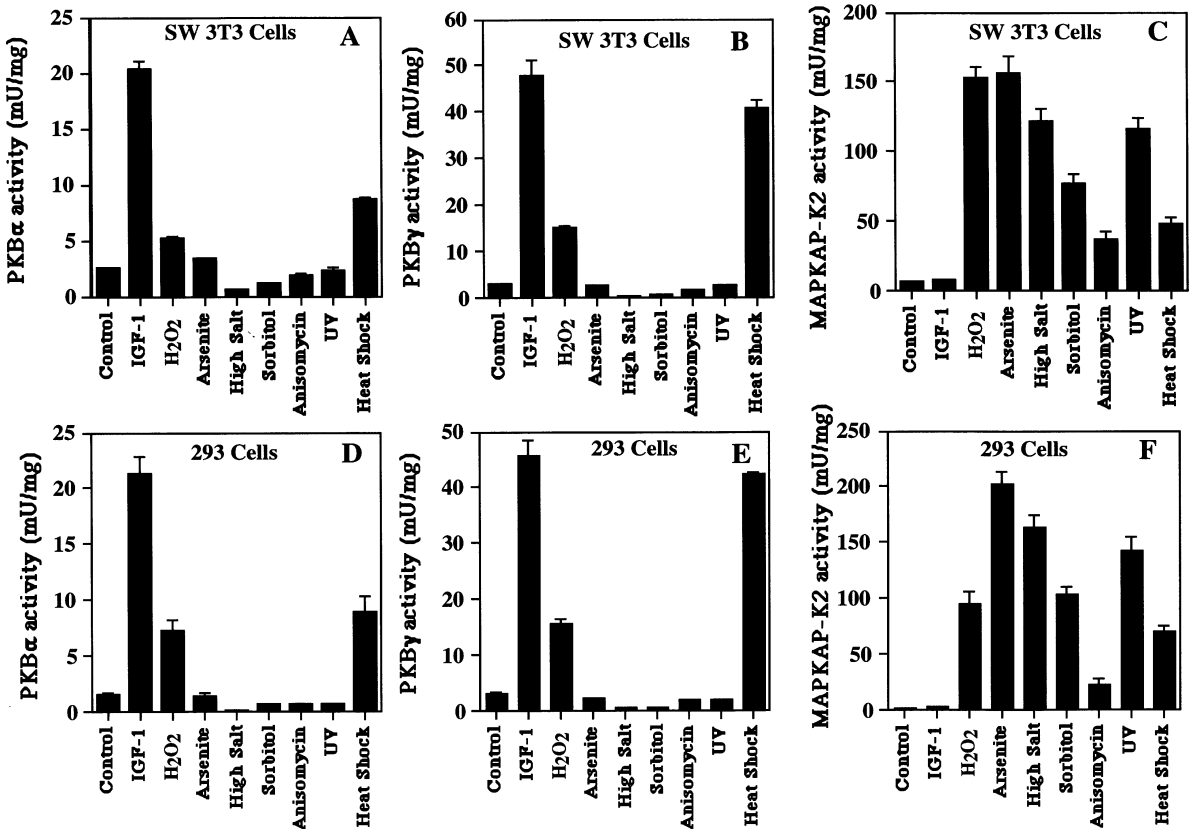

Figure 2 Activation of PKB by H_2O_2 and heat shock in Swiss 3T3 and 293 cells

Swiss 3T3 (**A**–**C**) and 293 cells (**D**–**F**) were stimulated with buffer (Control), IGF-1 (100 ng/ml, 5 min), H_2O_2 (1 mM, 10 min), sodium arsenite (0.5 mM, 30 min), NaCl (High Salt, 0.7 M, 30 min), sorbitol (0.5 M, 30 min), anisomycin (10 μg/ml, 30 min), UV radiation (200 J/m^2, then left at 37 °C for 30 min) or heat-shocked (45 °C for 20 min). The cells were lysed and PKBα (**A**, **D**), PKBγ (**B**, **E**) and MAPKAP-K2 (**C**, **F**) were immunoprecipitated and assayed. The data are presented as the means ± S.E.M. for 3 separate experiments with each determination carried out in triplicate.

UV radiation; Figure 2C] did not lead to significant activation of any PKB isoform (Figures 2A and 2B). Sodium arsenite at up to 5 mM did not induce activation of any PKB isoform, although MAPKAP-K2 was fully activated by 0.2 mM sodium arsenite (results not shown).

The activation of PKB isoforms by H_2O_2 and heat shock was prevented by inhibitors of PI 3-kinase

Incubation of Swiss 3T3 cells with inhibitors of PI 3-kinase, wortmannin and LY 294002, prior to stimulation with H_2O_2 or 45 °C heat shock completely blocked the activation of PKBα (Figures 3A and 3B) and PKBγ (Figures 3C and 3D). Inhibition of PKB activation by H_2O_2 or heat shock occurred at concentrations of wortmannin and LY 294002 that were similar to those needed to inhibit the IGF-1-induced activation of PKB (Figure 3). In contrast, the activation of MAPKAP-K2 was unaffected by wortmannin or LY 294002 (Figure 3E).

Activation of PKB isoforms by H_2O_2 or heat shock was unaffected by SB 203580

Incubation of Swiss 3T3 cells with SB 203580 prior to treatment with H_2O_2 or a 45 °C heat shock had no effect on the activation of PKBα or PKBγ by H_2O_2, heat-shock or IGF-1 (Figure 4A). However, it did block the activation of MAPKAP-K2 by these stresses (Figure 4B), as well as by arsenite, anisomycin, UV radiation and osmotic shock (results not shown).

H_2O_2- or heat shock-induced inactivation of GSK3 was prevented by inhibitors of PI 3-kinase, but not by SB 203580

Exposure of Swiss 3T3 cells to H_2O_2, heat-shock or IGF-1 resulted in 30%, 40% and 60% inhibition of GSK3α activity, respectively. This was prevented by prior incubation of the cells with wortmannin, but not by incubation with SB 203580 (Figure 5). Like the inhibition of GSK3 induced by insulin or IGF-1 [26], the inhibition of GSK3α by H_2O_2 or heat shock was completely reversed by incubating the immunoprecipitates with the serine/threonine-specific protein phosphatase PP2A (Figure 5). Exposure of Swiss 3T3 cells to stresses that did not activate PKB isoforms (anisomycin, UV irradiation or sorbitol) did not result in any significant inhibition of GSK3α (results not shown).

Effect of H_2O_2 and heat shock on the activation of PKB in human embryonic kidney 293 cells and NIH 3T3 cells

In order to examine whether the effects of cellular stresses on the activation of PKB were cell-specific, we also studied their effects in human embryonic kidney 293 cells and NIH 3T3 cells. In 293 cells, PKBα (Figure 2D) and PKBγ (Figure 2E) became activated in response to H_2O_2 or heat shock in a similar manner to Swiss 3T3 cells and, like Swiss 3T3 cells, activation of PKBγ was

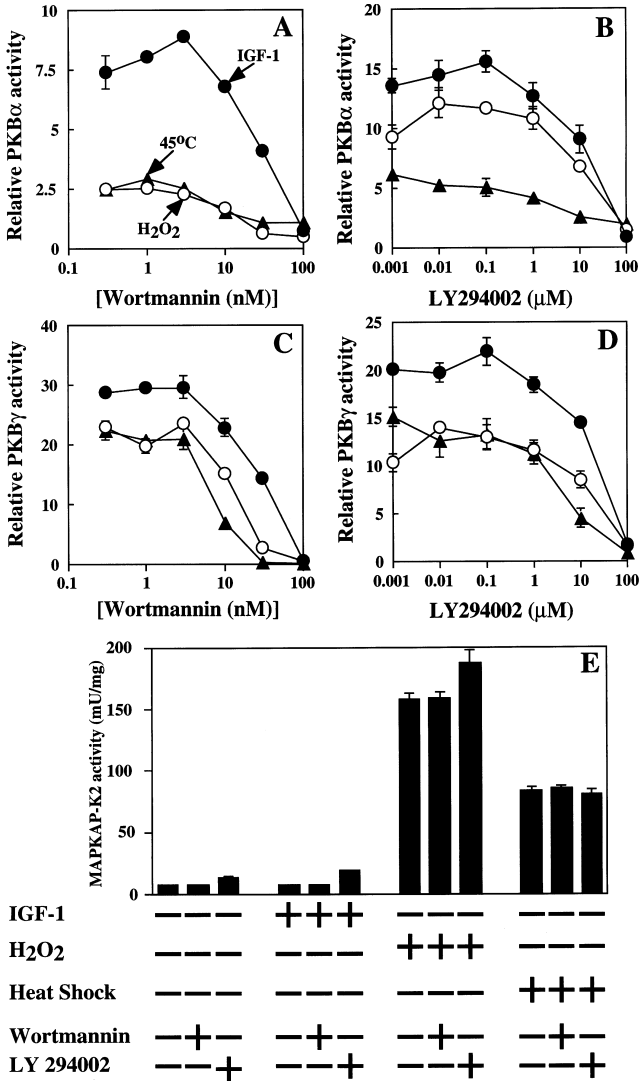

Figure 3 Activation of PKB by H_2O_2 and heat shock was prevented by inhibitors of PI 3-kinase

Swiss 3T3 cells were incubated with the indicated concentrations of wortmannin (**A**, **C**) and LY 294002 (**B**, **D**) prior to stimulation with IGF-1 (100 ng/ml, 5 min, ●), H_2O_2 (1 mM, 10 min, ○) or incubation at 45 °C for 20 min (▲). The cells were lysed and PKBα (**A**, **C**), PKBγ (**B**, **D**) and MAPKAP-K2 (**E**) were immunoprecipitated and assayed. The data are presented as the means ± S.E.M. for 3 separate experiments with each determination carried out in triplicate.

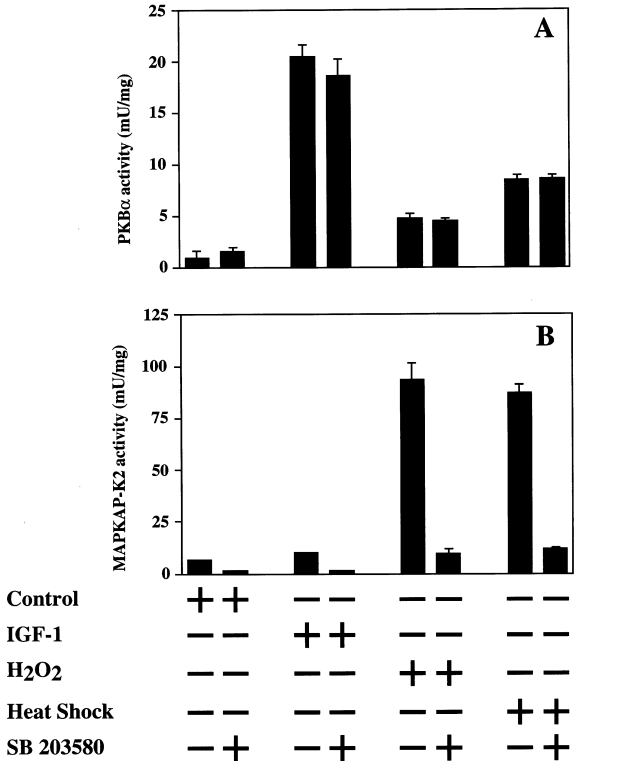

Figure 4 Activation of PKB isoforms by H_2O_2 or heat shock was unaffected by SB 203580

Swiss 3T3 cells were incubated for 30 min in the presence or absence of 10 μM SB 203580 prior to stimulation with IGF-1 (100 ng/ml, 5 min), H_2O_2 (1 mM, 10 min) or incubation at 45 °C for 20 min. The cells were lysed and PKBα (**A**) or MAPKAP-K2 (**B**) were immunoprecipitated and assayed. The data are presented as the means ± S.E.M. for 3 separate experiments with each determination carried out in triplicate.

blocked by wortmannin or LY 294002 (results not shown). As in Swiss 3T3 cells, PKB was not activated significantly in response to arsenite, 0.7 M NaCl, 0.5 M sorbitol, anisomycin or UV radiation in 293 cells (Figures 2D and 2E), although these stimuli activated MAPKAP-K2 (Figure 2F).

As in Swiss 3T3 and 293 cells, PKBγ was activated in response to heat shock but, unlike in the other cells studied, it was also activated to a small extent in response to arsenite, anisomycin or UV radiation (Figure 6), but not by osmotic shock (results not shown). The activation of PKB by all the stimuli was blocked by inhibitors of PI 3-kinase (Figure 6), but not by SB 203580 (results not shown). In contrast to Swiss 3T3 cells or 293 cells, H_2O_2 (1 mM) produced almost no activation of PKBγ (Figure 6). Surprisingly, there was little activation of PKBα in response to any stress in NIH 3T3 cells, although PKBα was strongly activated in response to IGF-1 (results not shown).

DISCUSSION

It has been reported previously that the activation of the endogenous PKBα in NIH 3T3 and Chinese hamster ovary cells can be triggered in response to heat shock and that transfected PKB isoforms in COS-7 cells can be activated by several stressful stimuli, including heat shock and H_2O_2. However, unlike insulin- or growth factor-induced activation of PKB, the stress-induced activation of PKB was reported not to be prevented by inhibitors of PI 3-kinase [18]. These findings implied that adverse stimuli might trigger the activation of PKB via a novel pathway, and prompted us to examine these effects in more detail.

Oxidative stress and heat shock are known to activate stress-activated MAP-kinase cascades, including that which culminates in the activation of MAPKAP-K2 [20]. Since MAPKAP-K2 has been shown to activate PKBα 5-fold *in vitro* by phosphorylating Ser473 [8], one hypothesis was that MAPKAP-K2 might mediate the activation of PKBα by oxidative stress or heat shock. However, this mechanism has been excluded in the present study by a variety of experiments. First, other stresses that induce MAPKAP-K2 activation as effectively as H_2O_2 or heat shock did

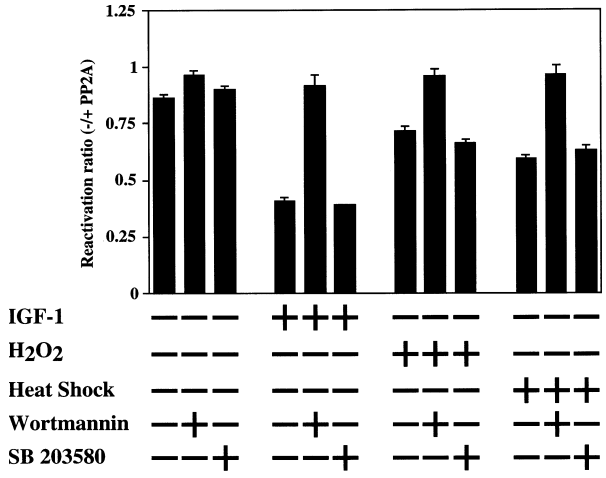

Figure 5 H_2O_2- or heat shock-induced inactivation of GSK3 is prevented by inhibitors of PI 3-kinase but not by SB 203580

Swiss 3T3 cells were incubated in the presence or absence of 10 μM SB 203580 (30 min) or 100 nM wortmannin (10 min) prior to stimulation with IGF-1 (100 ng/ml, 5 min), H_2O_2 (1 mM, 10 min) or incubated at 45 °C (20 min). The cells were lysed and GSK3α was immunoprecipitated, incubated without or with 25 m-units/ml of $PP2A_1$ and then assayed. GSK3α activity is expressed as a re-activation ratio; i.e. GSK3 activity measured without $PP2A_1$ treatment divided by GSK3 activity after $PP2A_1$ treatment. The data are presented as the means $\pm$ S.E.M. for 3 separate experiments with each determination carried out in triplicate. The specific activity of GSK3α after immunoprecipitation from unstimulated cells followed by treatment with $PP2A_1$ was 70 $\pm$ 10 m-units/mg.

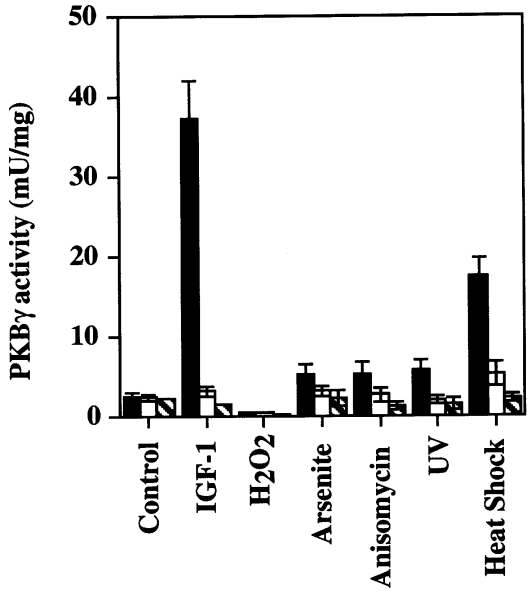

Figure 6 Activation of PKB by cellular stress in NIH 3T3 cells is suppressed by inhibitors of PI 3-kinase

Cells were stimulated with buffer (control), IGF-1 (100 ng/ml, 5 min), H_2O_2 (1 mM, 10 min), sodium arsenite (0.5 mM, 30 min), anisomycin (10 μg/ml, 30 min), UV radiation (200 J/m^2, then left at 37 °C for 30 min) or heat-shocked (45 °C for 20 min). The cells were lysed and PKBγ immunoprecipitated and assayed. The experiments were carried in the absence of PI 3-kinase inhibitors (black bars), after pre-incubation of the cells for 10 min with 100 nM wortmannin (open bars) or 100 μM LY 294002 (hatched bars). The data are presented as the means $\pm$ S.E.M. for 3 separate experiments with each determination carried out in triplicate (apart from the effects of LY294002 on arsenite, anisomycin and UV-induced activation of PKBγ, where only 2 separate experiments were performed).

not activate PKBα or PKBγ in Swiss 3T3 or 293 cells (Figure 1). Secondly, preventing the activation of MAPKAP-K2, by inhibiting its upstream activator p38 MAP kinase with the drug SB 203580, had no effect on the activation of PKBα by oxidative stress or heat shock in Swiss 3T3 cells (Figure 4). Thirdly, oxidative stress or heat shock activate PKBγ just as effectively as PKBα in Swiss 3T3 and 293 cells (Figures 1 and 2), but PKBγ lacks Ser473 [3], the residue phosphorylated by MAPKAP-K2. We therefore conclude that MAPKAP-K2 does not mediate the heat shock- or H_2O_2-induced activation of PKB in Swiss 3T3 cells.

We found that the activation of PKB isoforms by oxidative stress or heat shock in Swiss-3T3 (Figure 3) and 293 (results not shown) cells is completely suppressed by two structurally unrelated inhibitors of PI 3-kinase (wortmannin and LY 294002) that do not prevent the activation of MAPKAP-K2 by these stimuli in Swiss 3T3 cells (Figure 3). These observations led us to re-investigate the activation of PKB in NIH 3T3 cells, because it had been reported that the activation of PKBα by heat shock in these cells was not prevented by wortmannin. However, in the NIH 3T3 cells that we studied, PKBα was not activated significantly by exposure to any stressful stimulus, although it was strongly activated by IGF-1 (results not shown). The reason why Konishi et al. [18] observed a heat shock-induced, wortmannin-insensitive, activation of PKBα in NIH 3T3 cells is unclear. However, these investigators used mixed histones in their assay, which are much poorer substrates for PKB than the peptide GRPRTSSFAEG used in the present study [9]. Moreover, histones are excellent substrates for many other protein kinases. It is therefore possible that the histone kinase measured in the immunoprecipitates from heat-shocked NIH 3T3 cells was not PKBα, but another protein kinase present as a trace contaminant. In the present work, we found that PKBγ was activated by heat shock in NIH 3T3 cells and that this activation was largely suppressed by inhibitors of PI 3-kinase (Figure 6). Other stress stimuli that did not activate PKBγ in Swiss 3T3 and 293 cells (sodium arsenite, aniosmycin and UV radiation, see Figure 1) produced a slight activation of PKBγ in NIH 3T3 cells that could also be blocked by inhibitors of PI 3-kinase (Figure 6).

The finding that wortmannin and LY 294002 prevent the activation of PKB isoforms by oxidative stress and heat shock in several cells, and at concentrations similar to those that prevent activation by IGF-1 (Figure 3), strongly suggests that all three stimuli activate PKB by triggering the activation of class-1 PI 3-kinases. Indeed, the ability of heat shock to activate PI 3-kinase in NIH 3T3 fibroblasts has recently been reported [27]. These conclusions are supported by the observation that oxidative stress or heat shock induces a partial inhibition of GSK3 (an immediate downstream target of PKB [10]) in Swiss 3T3 cells, which is also prevented by inhibitors of PI 3-kinase (Figure 5). The smaller inhibition of GSK3 induced by oxidative stress or heat shock compared with IGF-1 is consistent with the smaller activation of PKB induced by these agonists (Figures 1 and 2).

IGF-1 is a survival factor that protects many cells against programmed cell death (apoptosis) induced by a variety of agonists. The effect of IGF-1 appears to be mediated via PI 3-kinase and PKB, because its anti-apoptotic effects can be replaced by expression of constitutively active forms of either enzyme (reviewed in [3,17]). The activation of PKB isoforms by oxidative stress or heat shock could therefore play a role in preventing cells from activating apoptotic programmes immediately after exposure to these adverse stimuli, giving them a chance to recover from these stresses if they are not unduly prolonged.

H_2O_2 has been known for many years to mimic many of the metabolic actions of insulin [28–32] and was at one time even

proposed to be the insulin second messenger itself [29]. The finding that H_2O_2 activates PKB isoforms, and the mounting evidence that PKB plays a key role in insulin signal transduction (reviewed in [3]), may explain at least some of the insulin-mimetic effects of H_2O_2.

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REFERENCES

1 Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. and Tsichlis, P. N. (1995) Cell **81**, 727–736
2 Burgering, B. M. T. and Coffer, P. J. (1995) Nature (London) **376**, 599–602
3 Alessi, D. R. and Cohen, P. (1988) Curr. Opin. Genet. Dev. **7**, 55–62
4 Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B. and Cohen, P. (1997) Curr. Biol. **7**, 261–269
5 Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P. R. J., Reese, C. B., MacDougall, C. N., Harbison, D. et al. (1997) Curr. Biol. **7**, 776–789
6 Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R. J., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. and Hawkins, P. T. (1997) Science **277**, 567–570
7 Stephens, L., Anderson, K., Stokoe, D., Erdjument, H., Painter, G., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., McCormick, F., Tempst, P. et al. (1998) Science **279**, 710–714
8 Alessi, D. R., Andjelkovic, M., Caudwell, F. B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. A. (1996) EMBO J. **15**, 6541–6551
9 Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A. and Cohen, P. (1996) FEBS Lett. **399**, 333–338
10 Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovic, M. and Hemmings, B. A. (1995) Nature (London) **378**, 785–789
11 Lefebvre, V., Mechin, M. C., Louckx, M. P., Rider, M. H. and Hue, L. (1996) J. Biol. Chem. **271**, 22289–22292
12 Deprez, J., Vertommen, D., Alessi, D. R., Hue, L. and Rider, M. H. (1997) J. Biol. Chem. **272**, 17269–17275
13 Welsh, G. I., Miller, C. M., Loughlin, A. J., Price, N. T. and Proud, C. G. (1998) FEBS Lett. **421**, 125–130
14 Kohn, A. D., Summers, S. A., Birnbaum, M. J. and Roth, R. A. (1996) J. Biol. Chem. **271**, 31372–31378
15 Hajduch, E., Alessi, D. R., Hemmings, B. A. and Hundal, H. (1998) Diabetes **47**, 1006–1013
16 Barthel, A., Kohn, A. D., Luo, Y. N. and Roth, R. A. (1997) Endocrinology **183**, 3559–3562
17 Marte, B. M. and Downward, J. (1997) Trends Biochem. Sci. **22**, 355–358
18 Konishi, H., Matsuzaki, H., Tanaka, M., Ono, Y., Tokunaga, C., Kuroda, S. and Kikkawa, U. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 7639–7643
19 Konishi, H., Matsuzaki, H., Tanaka, M., Takemura, Y., Kuroda, S., Ono, Y. and Kikkawa, U. (1997) FEBS Lett. **410**, 493–498
20 Cohen, P. (1997) Trends Cell Biol. **7**, 353–361
21 Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P. and Alessi, D. R. (1998) Biochem. J. **331**, 299–308
22 Clifton, A. D., Young, P. R. and Cohen, P. (1996) FEBS Lett. **392**, 209–214
23 Alessi, D. R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S. M. and Cohen, P. (1995) Curr. Biol. **5**, 283–295
24 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
25 Sutherland, C., Leighton, I. A. and Cohen, P. (1993) Biochem. J. **296**, 15–19
26 Shaw, M., Cohen, P. and Alessi, D. R. (1997) FEBS Lett. **416**, 307–311
27 Lin, R. Z., Hu, Z. W., Chin, J. H. and Hoffman, B. B. (1997) J. Biol. Chem. **272**, 31196–31202
28 Czech, M. P., Lawrence, J. C. and Lynn, A. C. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 4173–4177
29 Czech, M. P., Lawrence, J. C. and Lynn, A. C. (1974) J. Biol. Chem. **249**, 1001–1006
30 May, J. M. and Haen, C. (1979) J. Biol. Chem. **254**, 9017–9021
31 Little, S. A. and Haen, C. (1980) J. Biol. Chem. **255**, 10888–10895
32 May, J. M. and Haen, C. (1979) J. Biol. Chem. **254**, 2214–2220

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