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Activation of ERK1/2, JNK and PKB by hydrogen peroxide in human SH-SY5Y neuroblastoma cells: role of ERK1/2 in H_2O_2-induced cell death

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Abstract

Reactive oxygen species including H_2O_2 activate an array of intracellular signalling cascades that are closely associated with cell death and cell survival pathways. The human neuroblastoma SH-SY5Y cell line is widely used as model cell system for studying neuronal cell death induced by oxidative stress. However, at present very little is known about the signalling pathways activated by H_2O_2 in SH-SY5Y cells. Therefore, in this study we have investigated the effect of H_2O_2 on extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK) and protein kinase B (PKB) activation in undifferentiated and differentiated SH-SY5Y cells. H_2O_2 stimulated time and concentration increases in ERK1/2, JNK and PKB phosphorylation in undifferentiated and differentiated SH-SY5Y cells. No increases in p38 MAPK phosphorylation were observed following H_2O_2 treatment. The phosphatidylinositol 3-kinase (PI-3K) inhibitors wortmannin and LY 294002 ((2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) inhibited H_2O_2-induced increases in ERK1/2 and PKB phosphorylation. Furthermore, H_2O_2-mediated increases in ERK1/2 activation were sensitive to the MAPK kinase 1 (MEK1) inhibitor PD 98059 (2′ -amino-3′ -methoxyflavone), whereas JNK responses were blocked by the JNK inhibitor SP 600125 (anthra[1-9-cd]pyrazol-6(2H)-one). Treatment of SH-SY5Y cells with H_2O_2 (1 mM; 16 h) significantly increased the release of lactate dehydrogenase (LDH) into the culture medium indicative of a decrease in cell viability. Pre-treatment with wortmannin, SP 600125 or SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; p38 MAPK inhibitor) had no effect on H_2O_2-induced LDH release from undifferentiated or differentiated SH-SY5Y cells. In contrast, PD 98059 and LY 294002 significantly decreased H_2O_2-induced cell death in both undifferentiated and differentiated SH-SY5Y cells. In conclusion, we have shown that H_2O_2 stimulates robust increases in ERK1/2, JNK and PKB in undifferentiated and differentiated SH-SY5Y cells. Furthermore, the data presented clearly suggest that inhibition of the ERK1/2 pathway protects SH-SY5Y cells from H_2O_2-induced cell death.

Keywords: Neuroblastoma cell; Hydrogen peroxide; Protein kinase B; MAP (mitogen-activated protein) kinase

1. Introduction

Oxidative stress is widely implicated in the neuronal cell death that is associated with a variety of chronic neurodegenerative disorders such as Parkinson's disease (Zhang et al., 2000), Alzheimer's disease (Behl, 1999) and Huntington's disease (Alexi et al., 2000). In addition, reactive oxygen species including hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical (OH) and peroxynitrate ($ONOO^-$) which are generated during reperfusion are known to play an important role in neuronal cell death following ischaemic stroke (Chan, 1996, 2001).

Oxidative stress-induced neuronal cell death involves necrosis and apoptosis, the form of death being dependent upon the severity of the oxidative insult (Slater et al., 1996). Necrosis is, in general, a rapidly occurring form of cell death, whereas apoptosis is a delayed form of cell death that occurs as a result of activation of a genetic programme. Previous studies have shown that H_2O_2 induces apoptosis in rat pheochromocytoma PC12 cells (a model system for catecholamine-containing neurons; Yamakawa et al., 2000) and human neuroblastoma SH-SY5Y cells (Zhang et al., 1997). However, the molecular mechanisms involved in oxidative stress-induced apoptotic neuronal cell death are complex and

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not fully understood (Chandra et al., 2000; Sastry and Rao, 2000).

Apoptotic cell death is classically associated with the activation of proteases called caspases. To date, the caspase family comprises of 14 members, which can be broadly classified as either upstream "initiator" caspases or downstream "effector" caspases. When activated caspases cleave a variety of intracellular proteins, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery and a number of protein kinases (Earnshaw et al., 1999). Two major pathways for the activation of caspases have been elucidated (for extensive reviews on apoptosis, see Reed, 2001; Strasser et al., 2000). The extrinsic pathway is triggered by members of the TNF (tumour necrosis factor)-family of cytokine receptors such as TNFR1, Fas and TRAIL receptors. Stimulation of these receptors leads to the activation of initiator caspases in particularly caspase-8. Activated caspase-8 then cleaves and activates downstream effector caspases such as caspase-3, -6 or -7. In contrast, the intrinsic pathway is triggered by release of cytochrome *c* from the mitochondria. In the cytosol, cytochrome *c* binds to Apaf1 resulting in the activation of the initiator caspase-9, which then cleaves and activates caspase-3. The mitochondrial pathway is suppressed by anti-apoptotic Bcl-2 family proteins, which prevent cytochrome *c* release. Previous studies have shown that H_2O_2 induces apoptotic neuronal cell death via extrinsic (death receptor-mediated) and intrinsic (mitochondrial-dependent) pathways (Facchinetti et al., 2002; Yamakawa et al., 2000; Chandra et al., 2000).

Reactive oxygen species including H_2O_2 activate an array of intracellular signalling cascades that are closely associated with both cell death and cell survival pathways (Kamata and Hirata, 1999). For example, H_2O_2 activates members of the mitogen-activated protein kinase (MAPK) family and protein kinase B (PKB) (Kamata and Hirata, 1999). The MAPK family members activated by H_2O_2 include extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNK1 and JNK2) and p38 MAPKs. It is generally accepted that ERK1/2 and PKB activation promotes cell survival by activating anti-apoptotic signalling pathways, whereas activation of JNK and p38 MAPK is associated with neuronal cell death (Xia et al., 1995; Brunet et al., 2001; Harper and LoGrasso, 2001). For example, H_2O_2-induced ERK1/2 activation is thought to play a critical role in cell survival in response to oxidative stress in rat PC12 cells and primary cortical neurons (Guyton et al., 1996; Crossthwaite et al., 2002). Although activation of the phosphatidylinositol 3-kinase (PI-3K)/PKB pathway is involved in neuronal survival (Brunet et al., 2001) and H_2O_2 has been shown to activate PKB in primary cortical neurons (Crossthwaite et al., 2002) it is not known whether H_2O_2-induced PKB stimulation directly protects neuronal cells from oxidative stress.

Human neuroblastoma SH-SY5Y cells are widely used as model cell system for studying neuronal cell death induced by oxidative stress. For example, treatment of SH-SY5Y cells with H_2O_2 induces apoptotic cell death as shown by DNA fragmentation and the presence of apoptotic bodies (Zhang et al., 1997). Although, H_2O_2 activates PKB in a number of cell lines (Ushio-Fukai et al., 1999; Nishida et al., 2000) and mouse primary cortical neurons (Crossthwaite et al., 2002) it is not known whether H_2O_2 induces PKB activation in human neuroblastoma SH-SY5Y cells. Therefore, one of the aims of this study was to investigate whether H_2O_2 activates PKB in undifferentiated and differentiated SH-SY5Y cells. SY-SY5Y cells can be differentiated using retinoic acid into neuronal-like cells, which acquire morphological, neurochemical and electrophysiological properties characteristic of neurons (Bielder et al., 1978; Cole et al., 1985; Farooqui, 1994; Itano and Nomura, 1995; Uberti et al., 1997). We have also compared the effect of H_2O_2 on ERK1/2, JNK and p38 MAPK activation in undifferentiated and differentiated SH-SY5Y cells. Finally, the role of ERK1/2, JNK1/2, p38 MAPK and PKB in H_2O_2-induced cell death was assessed using a range of pharmacological inhibitors of the various kinase pathways. The results presented suggest that ERK1/2 may be involved in H_2O_2-induced cell death in human neuroblastoma SH-SY5Y cells.

2. Materials and methods

2.1. Cell culture

The human neuroblastoma cell line SY-SY5Y was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). SH-SY5Y cells were cultured in 75 cm^2 flasks in Dulbecco's modified Eagles medium (DMEM) supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere until confluency and subcultured (1:10 split ratio) using trypsin (0.05% w/v)/EDTA (0.02% w/v). Cells for determination of ERK1/2, JNK, p38 MAPK and PKB activation were grown in 6-well cluster dishes, whereas cell viability assays were performed using 96-well plates. Cells for differentiation were initially seeded at 10^3 cells/well (96-well plates) or 5×10^4 cells/well (6-well plates) and cultured for 24 h in DMEM containing 10% (v/v) foetal calf serum. The medium was then changed to DMEM supplemented with 2 mM L-glutamine, 2% (v/v) foetal calf serum and 10 μM retinoic acid and the cells allowed to differentiate for 1 week.

2.2. Western blot analysis of ERK1/2, JNK, p38 MAPK and PKB activation

Undifferentiated and differentiated SH-SY5Y cells were grown in 6-well plate cluster dishes and when 80–90% confluent placed in DMEM medium containing 0.1%

bovine serum albumin for 16 h. Serum-starved cells were then washed once with Hanks/HEPES buffer, pH 7.4, and incubated at 37 °C for 30 min in 500 μl/well of the same medium. Where appropriate kinase inhibitors were added during this incubation period. H_2O_2 was subsequently added in 500 μl of medium and the incubation continued for 5 min (unless otherwise stated) at 37 °C. Stimulation's were terminated by aspiration of the medium and the addition of 300 μl of ice-cold lysis buffer [150 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, 1% (v/v) IGEPAL CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 0.1 mM phenylmethylsulphonylfluoride, 10 μg/ml aprotinin and 5 μg/ml leupeptin]. Cells were then incubated on ice for 5 min, after which the cell lysates were removed and placed into Eppendorf microcentrifuge tubes and vortexed. Insoluble material was removed by centrifugation and 250 μl of the cell lysate removed and stored at −20 °C until required. Protein determinations were made using the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Protein samples (20 μg) were separated by Sodium Dodecyl Sulphate/Polyacrylamide Gel Electrophoresis (SDS/PAGE; 10% acrylamide gel) using a Bio-Rad Mini-Protean III system (1 h at 200 V). Proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot system (1 h at 100 V in 25 mM Tris, 192 mM glycine and 20% MeOH). Following transfer, the membranes were washed with Tris buffered saline (TBS) and blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in TBS. Blots were then incubated overnight at 4 °C with primary antibodies in 5% (w/v) skimmed milk powder dissolved in TBS-Tween 20 (0.1% by vol). Primary antibodies were removed and the blot extensively washed with TBS/Tween 20. Blots were then incubated for 2 h at room temperature with the secondary antibodies (swine anti-mouse or anti-rabbit antibody coupled to horseradish peroxidase; 1:1000 dilution) in 5% (w/v) skimmed milk powder dissolved in TBS/Tween 20. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence detection system (Amersham) and quantified using the programme QuantiScan (BioSoft). The uniform transfer of proteins to the nitrocellulose membrane was routinely monitored by transiently staining the membranes with Ponceau S stain (Sigma) prior to application of the primary antibody.

2.3. Cell viability assays

The effects of H_2O_2 on SH-SY5Y cell viability were determined by measuring the activity of lactate dehydrogenase (LDH) released into the culture medium (Cytotox 96 LDH assay, Promega, UK). The assay was performed in accordance with the manufacturer's protocol using undifferentiated and differentiated SH-SY5Y cells seeded at 10^3 cells/well in 96-well plates. Cells were subsequently exposed to 0.75 and 1.0 mM H_2O_2 for 16 h in the absence or presence of various pharmacological kinase inhibitors.

2.4. Data analysis

Statistical significance was determined by Student's unpaired *t*-test ($P<0.05$ was considered statistically significant). All data are presented as mean ± S.E.M. The *n* in the text refers to the number of separate experiments.

2.5. Materials

Aprotinin, bovine serum albumin, Dulbecco's modified Eagles medium, foetal calf serum, hydrogen peroxide, and leupeptin, were obtained from Sigma (Poole, Dorset, UK). LY 294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one), PD 98059 (2′-amino-3′-methoxyflavone), SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole) and wortmannin were from Calbiochem (Nottingham, UK). SP 600 125 (anthra[1-9-cd]pyrazol-6(2*H*)-one) was obtained from Tocris Cookson Phospho-specific ERK1/2 (Thr^{202}/Tyr^{204}) and p38 MAPK (Thr^{180}/Tyr^{182}) antibodies were purchased from Sigma. Phospho-specific JNK (Thr^{183}/Tyr^{185}) antibody was from Promega. Phospho-specific PKB (Ser^{473}) was from New England Biolabs. Total (unphosphorylated) ERK1/2, PKB and JNK1/2 antibodies were obtained from New England Biolabs. All other chemicals were of analytical grade.

3. Results

3.1. H_2O_2-induced activation of ERK1/2 in SH-SY5Y cells

Increases in ERK1/2 activation were monitored by Western blotting using a phospho-specific ERK1/2 (Thr^{202}/Tyr^{204}) antibody as described previously (Robinson and Dickenson, 2001). Stimulation of differentiated human SH-SY5Y neuroblastoma cells with 1 mM H_2O_2 produced a marked increase in the phosphorylation (Thr^{202}/Tyr^{204}) status of ERK1 (44 kDa) and ERK2 (42 kDa) (Fig. 1A). Maximal increases in ERK1/2 phosphorylation occurred after 10 min, after which phosphorylation levels slowly declined towards basal levels. Furthermore, the increases in ERK1/2 phosphorylation induced by H_2O_2 were concentration-dependent (Fig. 1B). H_2O_2 also triggered similar time- and concentration-dependent increases in ERK1/2 phosphorylation in undifferentiated SH-SY5Y cells (data not shown). Pre-treatment with the MEK1 inhibitor, PD 98059 (50 μM; 30 min; Dudley et al., 1995) completely inhibited H_2O_2-induced increases in ERK1/2 phosphorylation in differentiated SH-SY5Y cells (Fig. 2). Previous studies have reported that phosphatidy-

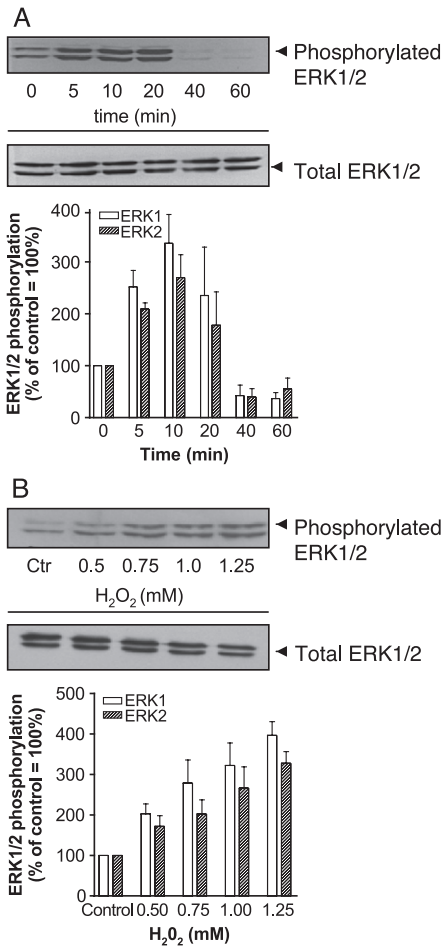

Fig. 1. H_2O_2-induced ERK1/2 activation in differentiated human SH-SY5Y neuroblastoma cells. Cell lysates (20 μg protein) were analysed for ERK1 (44 kDa) and ERK2 (42 kDa) activation by Western blotting using a phospho-specific ERK1/2 antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognises unphosphorylated (total) ERK1/2 to confirm equal loading on each lane. Representative Western blots for each experiment are shown in the upper panels. (A) Time-course profile for H_2O_2-induced ERK1/2 phosphorylation in differentiated SH-SY5Y cells treated with vehicle (time zero) or H_2O_2 (1 mM) for the indicated periods of time. (B) Concentration–response curve for H_2O_2 in differentiated SH-SY5Y cells treated with vehicle (control) or the indicated concentrations of H_2O_2 for 10 min. Combined results represent the mean ± S.E.M. from four independent experiments. Data are presented as the percentage of the basal level of ERK1 and ERK2 phosphorylation (control = 100%) in the absence of H_2O_2.

linositol 3-kinase (PI-3K) is involved in H_2O_2-induced ERK1/2 activation in primary cortical neurons and rat neonatal cardiomyocytes (Nishida et al., 2000; Crossthwaite et al., 2002). We therefore examined the role of PI-3K in the activation of ERK1/2 by H_2O_2 in differentiated SH-SY5Y cells using the PI-3K inhibitors wortmannin and LY 294002. Pre-treatment of cells (30 min) with wortmannin (100 nM) and LY 294002 (30 μM) significantly inhibited 1 mM H_2O_2-induced increases in ERK1/2 phosphorylation (Fig. 2). These observations suggest that PI-3K is involved in H_2O_2-mediated ERK1/2 activation in human SH-SY5Y neuroblastoma cells.

3.2. H_2O_2-induced activation of PKB in SH-SY5Y cells

H_2O_2 activates PKB in mouse primary cortical neurons (Crossthwaite et al., 2002), however it is not known whether H_2O_2 induces PKB activation in human neuroblastoma SH-SY5Y cells. PKB activation in SH-SY5Y cells was detected by Western blotting using a phospho-specific PKB (Ser^{473}) antibody as described previously (Germack and Dickenson, 2000; Dickenson, 2002). Stimulation of differentiated human SH-SY5Y neuroblastoma cells with 1 mM H_2O_2 produced a marked increase in PKB phosphorylation (Fig. 3A). Maximal increases in PKB phosphorylation occurred after 10 min comparable to that of ERK1/2 phosphorylation after which phosphorylation levels slowly declined towards basal levels (Fig. 3A). Furthermore, H_2O_2-mediated increases in PKB phosphorylation were concentration-dependent (Fig. 3B). H_2O_2 also triggered comparable time- and concentration-dependent increases in PKB phosphorylation in undifferentiated SH-SY5Y cells (data not shown). Recent studies have shown a requirement for PI-3K in H_2O_2-induced PKB activation in primary cortical neurons and rat neonatal

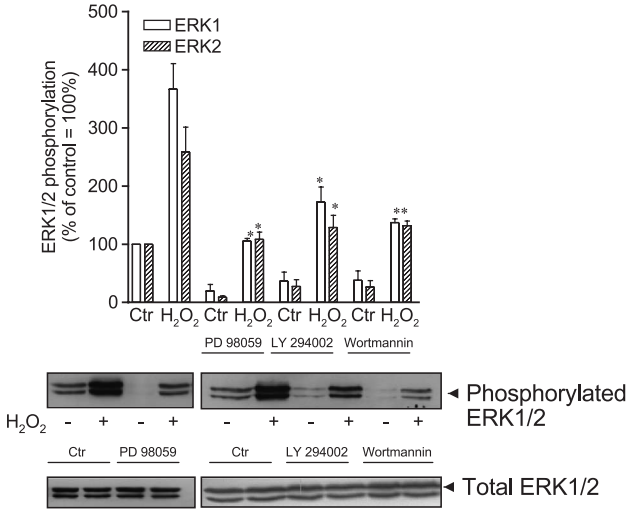

Fig. 2. Role of MEK1 and PI-3K in H_2O_2-induced ERK1/2 activation in differentiated human SH-SY5Y neuroblastoma cells. Cell lysates (20 μg protein) were analysed for ERK1 (44 kDa) and ERK2 (42 kDa) activation by Western blotting using a phospho-specific ERK1/2 antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognises unphosphorylated (total) ERK1/2 to confirm equal loading on each lane. Differentiated SH-SY5Y cells were pre-treated for 30 min with the MEK1 inhibitor PD 98059 (50 μM) and the PI-3K inhibitors LY 294002 (30 μM) and wortmannin (100 nM) prior to stimulating with H_2O_2 (1 mM) for 10 min. Combined results represent the mean ± S.E.M. from four independent experiments. Data are presented as the percentage of the basal level of ERK1 and ERK2 phosphorylation (control = 100%) in the absence of H_2O_2 and kinase inhibitors. *Significantly different ($P < 0.05$) from the control response to 1 mM H_2O_2.

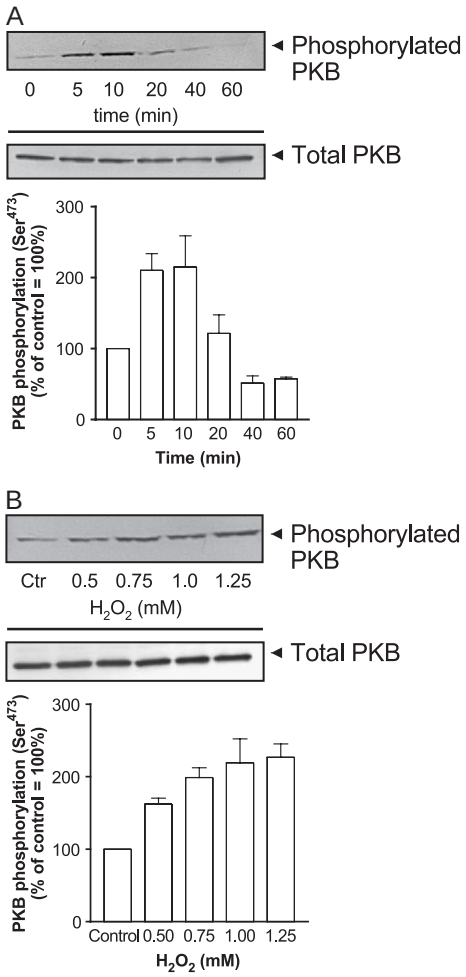

Fig. 3. H_2O_2-induced PKB activation in differentiated human SH-SY5Y neuroblastoma cells. Cell lysates (20 μg protein) were analysed for PKB activation by Western blotting using a phospho-specific PKB (Ser^{473}) antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognises unphosphorylated (total) PKB to confirm equal loading on each lane. Representative Western blots for each experiment are shown in the upper panels. (A) Time-course profile for H_2O_2-induced PKB phosphorylation in differentiated SH-SY5Y cells treated with vehicle (time zero) or H_2O_2 (1 mM) for the indicated periods of time. (B) Concentration–response curve for H_2O_2 in differentiated SH-SY5Y cells treated with vehicle (control) or the indicated concentrations of H_2O_2 for 10 min. Combined results represent the mean ± S.E.M. from four independent experiments. Data are presented as the percentage of the basal level of PKB phosphorylation (control = 100%) in the absence of H_2O_2.

cardiomyocytes (Nishida et al., 2000; Crossthwaite et al., 2002). The role of PI-3K in H_2O_2-induced PKB activation in differentiated human SH-SY5Y neuroblastoma cells was therefore explored using the PI-3K inhibitors wortmannin and LY 294002. H_2O_2-mediated (1 mM) increases in PKB phosphorylation were completely blocked following pre-treatment (30 min) of differentiated SH-SY5Y cells with 100 nM wortmannin and 30 μM LY 294002 (Fig. 4). These observations demonstrate that a PI-3K-dependent pathway mediates H_2O_2-induced increases in PKB phosphorylation in human SH-SY5Y neuroblastoma cells.

3.3. H_2O_2-induced activation of JNK1/2 in SH-SY5Y cells

Previous studies have shown that H_2O_2 (at 1 mM) activates JNK1/2 in primary cortical neurons (Crossthwaite et al., 2002). JNK1/2 activation in SH-SY5Y cells was detected by Western blotting using a phospho-specific JNK1/2 (Thr^{183}/Tyr^{185}) antibody. Stimulation of differentiated human SH-SY5Y neuroblastoma cells with 1 mM H_2O_2 produced a marked increase in JNK1 (46 kDa) and JNK2 (54 kDa) phosphorylation (Fig. 5A). These increases were maximal after 10 min after which phosphorylation levels slowly declined towards basal levels (Fig. 5A). Furthermore, H_2O_2-mediated increases in JNK1/2 phosphorylation were concentration-dependent (Fig. 5B). H_2O_2 also triggered comparable time- and concentration-dependent increases in JNK1/2 phosphorylation in undifferentiated SH-SY5Y cells (data not shown). H_2O_2-mediated increases in JNK1/2 phosphorylation were sensitive to JNK1/2 inhibitor SP 600 125 (10 μM; Fig. 6; Bennett et al., 2001). Finally, H_2O_2 (1 mM) did not stimulate measurable increases in p38 MAPK phosphorylation in undifferentiated and differentiated SH-

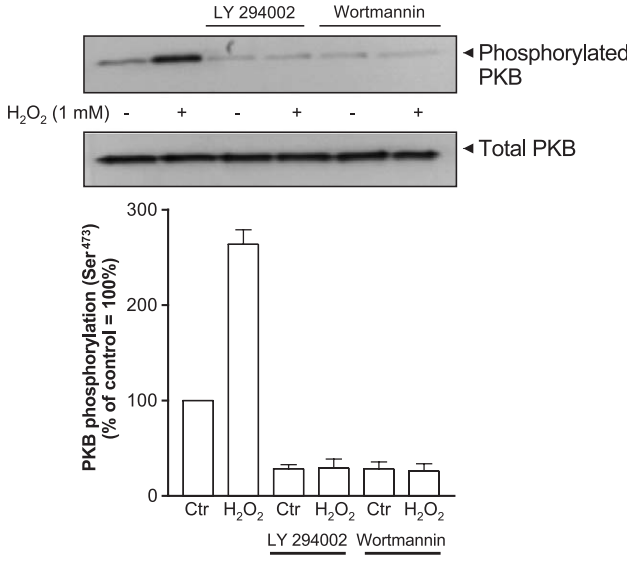

Fig. 4. Role of PI-3K in H_2O_2-induced PKB activation in differentiated human SH-SY5Y neuroblastoma cells. Cell lysates (20 μg protein) were analysed for PKB activation by Western blotting using a phospho-specific PKB (Ser^{473}) antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognises unphosphorylated (total) PKB to confirm equal loading on each lane. A representative Western blot is shown in the upper panel. Differentiated SH-SY5Y cells were pre-treated for 30 min with the PI-3K inhibitors LY 294002 (30 μM) and wortmannin (100 nM) prior to stimulating with H_2O_2 (1 mM) for 10 min. Combined results represent the mean ± S.E.M. from four independent experiments. Data are presented as the percentage of the basal level of PKB phosphorylation (control = 100%) in the absence of H_2O_2 and kinase inhibitors.

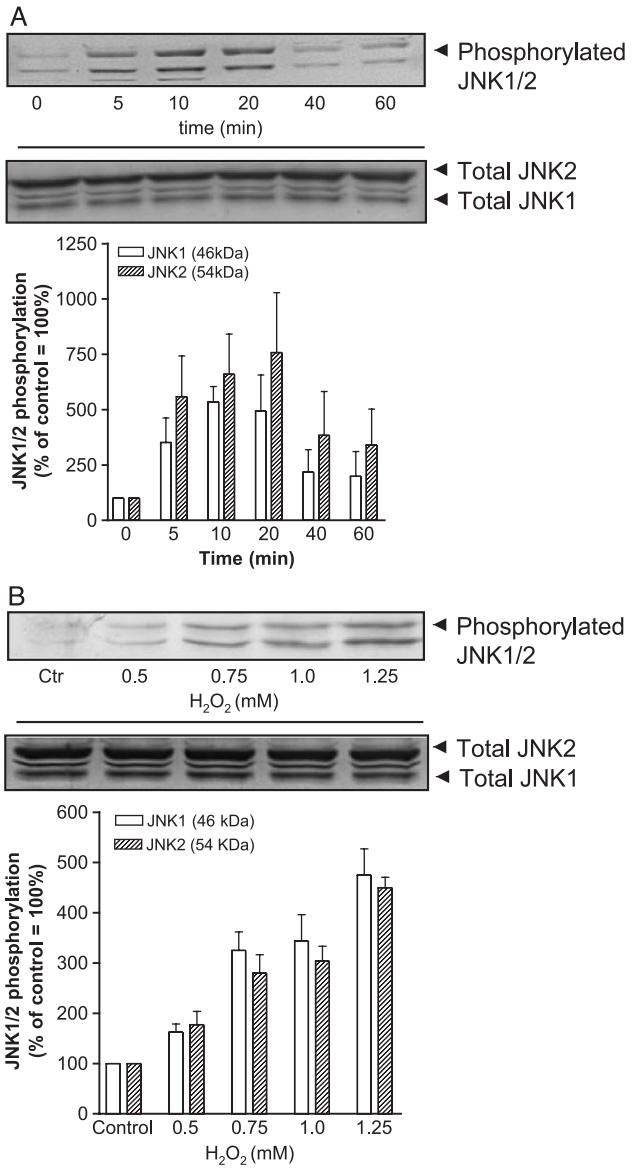

Fig. 5. H_2O_2-induced JNK activation in differentiated human SH-SY5Y neuroblastoma cells. Cell lysates (20 μg protein) were analysed for JNK1 (46 kDa) and JNK2 (54 kDa) activation by Western blotting using a phospho-specific JNK1/2 antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognises unphosphorylated (total) JNK to confirm equal loading on each lane. Representative Western blots for each experiment are shown in the upper panels. (A) Time-course profile for H_2O_2-induced JNK1/2 phosphorylation in differentiated SH-SY5Y cells treated with vehicle (time zero) or H_2O_2 (1 mM) for the indicated periods of time. (B) Concentration–response curve for H_2O_2 in differentiated SH-SY5Y cells treated with vehicle (control) or the indicated concentrations of H_2O_2 for 10 min. Combined results represent the mean ± S.E.M. from four independent experiments. Data are presented as the percentage of the basal level of JNK1/2 phosphorylation (control = 100%) in the absence of H_2O_2.

SY5Y cells during time course experiments conducted up to 60 min (data not shown). These experiments were performed using a phospho-specific p38 MAPK (Thr^{180}/Tyr^{182}) antibody and compared to marked increases in p38 MAPK phosphorylation obtained following sorbitol (0.5M) treatment.

3.4. Measurement of cell viability after treatment of SH-SY5Y cells with H_2O_2

Having shown that H_2O_2 activates ERK1/2, JNK1/2 and PKB in undifferentiated and differentiated SH-SY5Y cells we subsequently determined role of these kinase pathways in H_2O_2-induced cell death using a range of pharmacological inhibitors. Cell viability was evaluated by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium by dead and dying cells. This assay has been used previously to monitor H_2O_2-induced cell death in SH-SY5Y cells (Gao et al., 2001; Uberti et al., 2002). As shown in Fig. 7 exposure of undifferentiated SH-SY5Y cells to 1 mM H_2O_2 for 16 h induced a significant increase in cell death (76 ± 7%; $n = 5$). To investigate the role of ERK1/2, JNK1/2, p38 MAPK and PKB in H_2O_2-induced cell death undifferentiated SH-SY5Y cells were pre-treated for 30 min with the following kinase inhibitors; PD 98059 (50 μM; MEK1/2 inhibitor), LY 294002 (30 μM; PI-3K inhibitor), wortmannin (100 nM; PI-3K inhibitor), SP 600125 (10 μM; JNK1/2

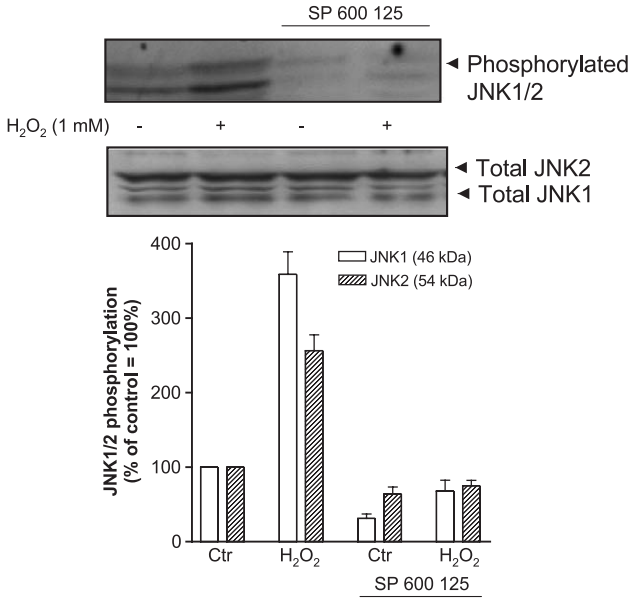

Fig. 6. Inhibition of H_2O_2-induced JNK activation in differentiated human SH-SY5Y neuroblastoma cells by SP 600 125. Cell lysates (20 μg protein) were analysed for JNK1 (46 kDa) and JNK2 (54 kDa) activation by Western blotting using a phospho-specific JNK1/2 antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognises unphosphorylated (total) JNK to confirm equal loading on each lane. A representative Western blot is shown in the upper panel. Differentiated SH-SY5Y cells were pre-treated for 30 min with the JNK1/2 inhibitor SP 600 125 (10 μM) prior to stimulating with H_2O_2 (1 mM) for 10 min. Combined results represent the mean ± S.E.M. from four independent experiments. Data are presented as the percentage of the basal level of JNK1/2 phosphorylation (control = 100%) in the absence of H_2O_2 and SP 600 125.

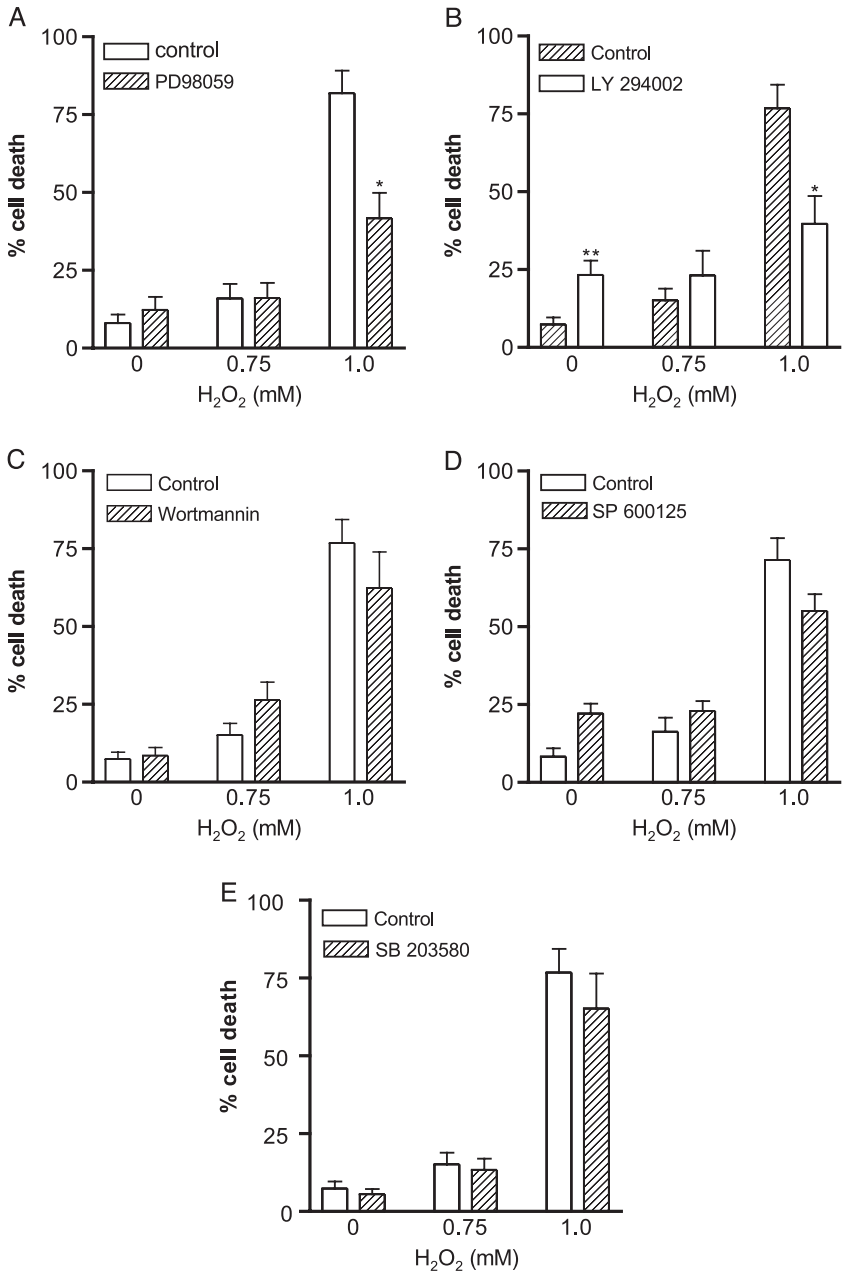

Fig. 7. Effects of MEK1/2, PI-3K, JNK1/2 and p38 MAPK inhibition on H_2O_2-induced cell death in undifferentiated human SH-SY5Y neuroblastoma cells. Undifferentiated SH-SY5Y neuroblastoma cells were exposed to 0.75 and 1.0 mM H_2O_2 for 16 h in the absence (control) or presence of (A) PD 98059 (50 μM; MEK1/2 inhibitor), (B) LY 294002 (30 μM; PI-3K inhibitor), (C) wortmannin (100 nM; PI-3K inhibitor), (D) SP 600125 (10 μM; JNK1/2 inhibitor) and (E) SB 203580 (30 μM; p38 MAPK inhibitor). Inhibitors were applied 30 min prior to the addition of H_2O_2. Each column is the mean ± S.E.M. from five independent experiments each performed in triplicate. Data are expressed as the percentage of maximum LDH release (defined as 100% cell death) induced by incubating cells for 45 min with 0.8% Triton® X-100 as detailed in the manufacturer's protocol. *Significantly different ($P<0.05$) from the control cells treated with 1 mM H_2O_2. **Significantly different ($P<0.05$) from control cells not treated with H_2O_2.

inhibitor) and SB 203580 (30 μM; p38 MAPK inhibitor). As shown in Fig. 7, wortmannin, SP 600125 and SB 203580 had no significant effect on loss of cell viability induced by 1 mM H_2O_2. However, PD 98059 and LY 294002 treatment significantly reduced cell death triggered by 1 mM H_2O_2. In addition treatment with LY 294002 alone (in the absence of H_2O_2) triggered a significant decrease in undifferentiated SH-SY5Y cell viability. The effects of H_2O_2 on SH-SY5Y cell viability were also investigated using differentiated cells. As shown in Fig. 8 exposure of differentiated SH-SY5Y cells to 1 mM H_2O_2 for 16 h also induced a significant increase in cell death (55 ± 6%; $n=9$). The effects of the various kinase inhibitors on H_2O_2-induced cell death using differentiated SH-SY5Y cells are shown in Fig. 8. Wortmannin, SP 600125 and SB 203580 had no significant effect on 1 mM H_2O_2-induced cell death, where-

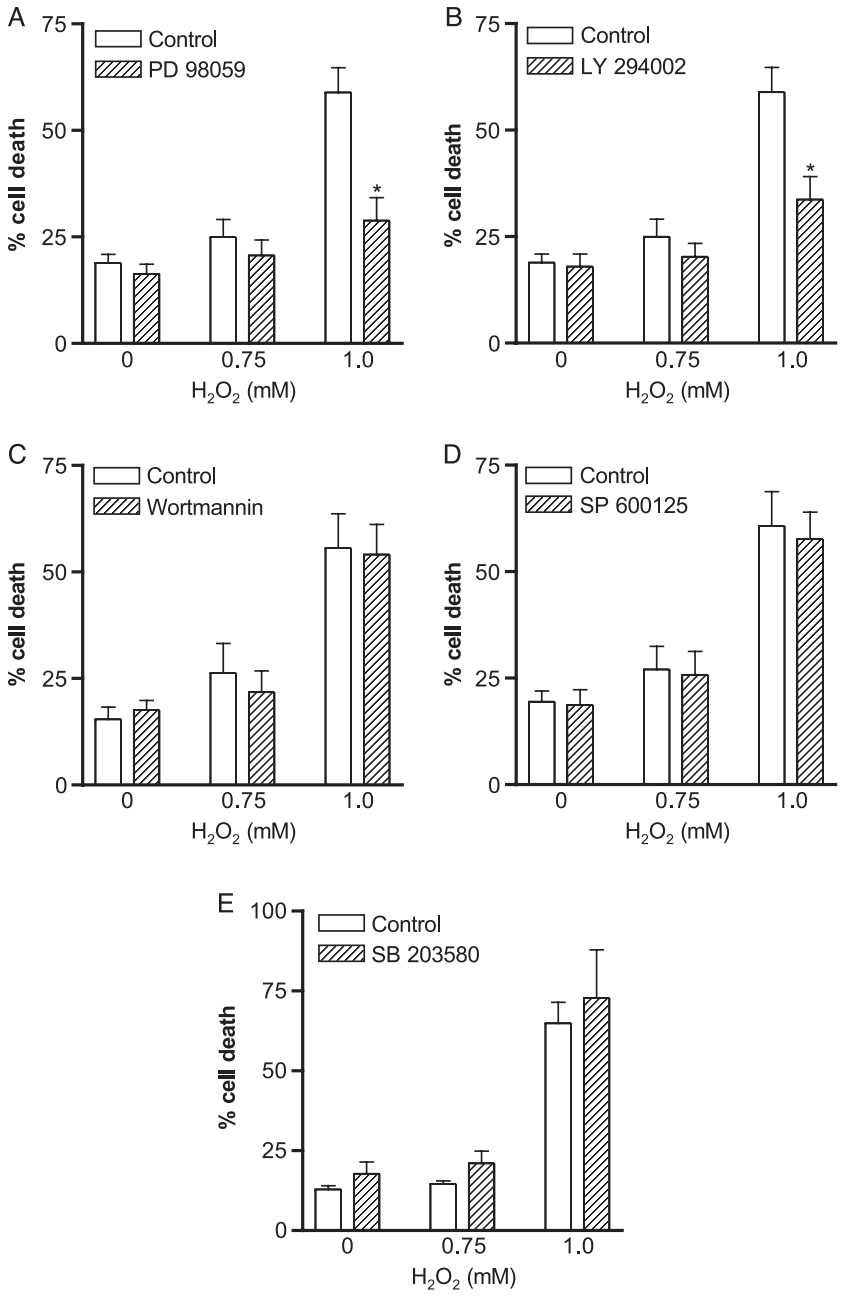

Fig. 8. Effects of MEK1/2, PI-3K, JNK1/2 and p38 MAPK inhibition on H_2O_2-induced cell death in differentiated human SH-SY5Y neuroblastoma cells. Differentiated SH-SY5Y neuroblastoma cells were exposed to 0.75 and 1.0 mM H_2O_2 for 16 h in the absence (control) or presence of (A) PD 98059 (50 μM; MEK1/2 inhibitor), (B) LY 294002 (30 μM; PI-3K inhibitor), (C) wortmannin (100 nM; PI-3K inhibitor), (D) SP 600125 (10 μM; JNK1/2 inhibitor) and (E) SB 203580 (30 μM; p38 MAPK inhibitor). Inhibitors were applied 30 min prior to the addition of H_2O_2. Each column is the mean ± S.E.M. from five independent experiments each performed in triplicate. Data are expressed as the percentage of maximum LDH release (defined as 100% cell death) induced by incubating cells for 45 min with 0.8% Triton® X-100 as detailed in the manufacturer's protocol. *Significantly different ($P < 0.05$) from the control cells treated with 1 mM H_2O_2.

as (and in agreement with undifferentiated cells) PD 98059 and LY 294002 treatment significantly reduced cell death triggered by 1 mM H_2O_2. However, in contrast, to undifferentiated cells treatment with LY 294002 alone (in the absence of H_2O_2) did not significantly decrease SH-SY5Y cell viability. Overall, these observations suggest that ERK1/2 is involved in the mechanisms resulting in neuronal cell death triggered by oxidative stress.

4. Discussion

Although human SH-SY5Y neuroblastoma cells are widely used as model cell system for studying neuronal cell death induced by oxidative stress (Zhang et al., 1997; Gao et al., 2001; Uberti et al., 2002) very little is known at present about the signalling pathways triggered by H_2O_2 in these cells. We therefore investigated whether H_2O_2 acti-

vates members of the MAPK family (ERK1/2, JNK1/2 and p38 MAPK) and PKB in undifferentiated and differentiated human SH-SY5Y neuroblastoma cells. Activation of ERK1/2, JNK1/2, p38 MAPK and PKB was monitored using commercially available phospho-specific antibodies, which recognise phosphorylated motifs within activated ERK1/2 (pTEpY), JNK1/2 (pTPpY), p38 MAPK (pTGpY) and PKB (Ser^{473}). We demonstrate that H_2O_2 stimulates time and concentration-dependent increases in ERK1/2, JNK1/2 and PKB phosphorylation in differentiated human SH-SY5Y neuroblastoma cells. In contrast, H_2O_2 did not stimulate measurable increases in p38 MAPK phosphorylation. Similar time and concentration-dependent increases in ERK1/2, JNK1/2 and PKB phosphorylation were also observed following treatment of undifferentiated SH-SY5Y cells with H_2O_2. Furthermore, H_2O_2 did not stimulate detectable increases in p38 MAPK in undifferentiated SH-SY5Y cells. Overall, these results are comparable to H_2O_2-induced increases in ERK1/2, JNK1/2 and PKB phosphorylation recently reported in primary cortical neurons (Crossthwaite et al., 2002).

ERK1/2 activation triggered by G-protein coupled receptors and growth factor receptors involves PI-3K-dependent pathways (Belcheva and Coscia, 2002). Studies using rat neonatal cardiomyocytes (Nishida et al., 2000) and primary cortical neurons (Crossthwaite et al., 2002) have shown that PI-3K is also required for H_2O_2-induced ERK1/2 activation in these cell types. In agreement with these previous studies the PI-3K inhibitors, wortmannin and LY 294002 significantly reduced H_2O_2-induced increases in ERK1/2 activation in human SH-SY5Y neuroblastoma cells suggesting a role for PI-3K activation. Furthermore, pre-treatment with wortmannin and LY 294002 also blocked H_2O_2-induced increases in PKB activation in SH-SY5Y neuroblastoma cells. Overall, these observations indicate that H_2O_2 triggers the activation of ERK1/2 and PKB survival pathways in human SH-SY5Y neuroblastoma cells via PI-3K-dependent pathways. In addition, to stimulating signalling pathways generally associated with cell survival (ERK1/2 and PKB) reactive oxygen species also trigger kinases linked with cell death such as JNK1/2 and p38 MAPK (Kamata and Hirata, 1999). In this study, we have shown that H_2O_2 triggered significant increases in JNK1/2 but had no effect on p38 MAPK. Since previous studies have reported that H_2O_2 activates p38 MAPK in neuronal cells (Tamagno et al., 2003; Yoshizumi et al., 2002) the absence of H_2O_2-induced p38 MAPK stimulation in SH-SY5Y cells (both undifferentiated and differentiated) was unexpected. However, it is likely that signalling pathways triggered by H_2O_2 will vary considerably not only between cell types and but also between cell lines of neuronal origin.

The immunoblot depicting the time course for H_2O_2-induced JNK1/2 activation, which was obtained using a phospho-specific JNK1/2 antibody, also contained a third immunoreactive band of approximate molecular weight 42-kDa (see Fig. 5A). Using the same commercially available phospho-specific JNK1/2 antibody Crossthwaite et al. (2002) also reported this additional band. These authors concluded that the phospho-specific JNK1/2 antibody is cross-reacting with dually phosphorylated ERK2 since treatment with the selective MEK1/2 inhibitor U0126 removed the 42-kDa band detected by phosho-specific JNK1/2 (Crossthwaite et al., 2002).

Having established that H_2O_2 triggers ERK1/2, JNK1/2 and PKB activation in human SH-SY5Y neuroblastoma cells we then investigated role of these protein kinase pathways in H_2O_2-induced cell death using a range of pharmacological inhibitors. SH-SY5Y cell viability was monitored by measuring lactate dehydrogenase (LDH) activity released into the culture medium following treatment with H_2O_2. The release of LDH has been widely used as a marker of cell injury in primary neuronal cell cultures and SH-SY5Y cells (Murray et al., 1998; Gao et al., 2001; Moore et al., 2002; Uberti et al., 2002). As shown in Figs. 7 and 8, pre-treatment with the selective MEK1 inhibitor PD 98059 (50 μM; Dudley et al., 1995) significantly reduced H_2O_2-induced LDH release from undifferentiated and differentiated human SH-SY5Y neuroblastoma cells. This concentration of PD 98059 completely blocked H_2O_2-induced ERK1/2 in SH-SY5Y cells (see Fig. 2). These data clearly suggest that inhibition of the ERK1/2 pathway protects SH-SY5Y cells from H_2O_2-induced cell death. These data are in agreement with previous studies, which have also shown that ERK1/2 is involved in neuronal cell death triggered by oxidative stress (Satoh et al., 2000; Stanciu et al., 2000; Stanciu and DeFranco, 2002). Furthermore, in vivo studies have shown that PD 98059 reduces cortical infarct size resulting from focal cerebral ischaemia (Alessandrini et al., 1999). Finally, ERK activation has also been implicated in cell death in a hippocampal cell culture model of seizure activity (Murray et al., 1998). Interestingly, PD 98059 also protects SH-SY5Y cells from peroxynitrite-induced cell death (Oh-hashi et al., 1999). Overall, these observations suggest that ERK1/2 is involved in oxidative stress induced neuronal death. However, several studies have also shown that the ERK1/2 pathway protects against oxidative stress-induced neuronal cell death. For example, PC12 cells constitutively expressing dominant negative RasN17 (to inhibit the ERK1/2 pathway) were more sensitive to H_2O_2-induced toxicity than control cells (Guyton et al., 1996). Furthermore, treatment of primary cortical neurons with the MEK1/2 inhibitors U0126 and PD 98059 significantly increased neuronal cell loss induced by H_2O_2 and hypoxia, respectively (Crossthwaite et al., 2002; Jin et al., 2002). Finally, ERK1/2 activation has been implicated in protein kinase C mediated protection against oxidative stress-induced neuronal cell death (Maher, 2001). Clearly, the role of ERK1/2 in determining the fate of neuronal cells (survival or death) following oxidative stress is controversial. The reasons for the varying results may reflect the use of different model systems (established cell lines versus primary cultures) or inducers and severity of neuronal cell death (H_2O_2 versus hypoxia).

Previous studies have shown that the PI-3K/PKB pathway is involved in neuronal survival (Brunet et al., 2001). In this study we have shown that H_2O_2 stimulates PKB in undifferentiated and differentiated SH-SY5Y cells via a PI-3K-dependent pathway. These observations suggest that H_2O_2-induced PKB activation may be involved in protecting neuronal cells against oxidative stress-induced cell death. In order to investigate this possibility we used the structurally unrelated PI-3K inhibitors wortmannin and LY 294002. Previous studies have shown inhibitors of PI-3K trigger cerebellar granule cell death (Shimoke et al., 1999). In this study, treatment with wortmannin had no significant effect on cell death induced by 1 mM H_2O_2, whereas LY 294002 significantly reduced cell death triggered by 1 mM H_2O_2 in both undifferentiated and differentiated SH-SY5Y cells. Since, wortmannin had no significant effect on H_2O_2-induced cell death the results obtained with LY 294002 would seem to suggest that the effects of LY 294002 are independent of PI-3K inhibition. Interestingly, Davies et al. (2000) have shown LY 294002 (at 50 μM) inhibits protein kinase CK2 with a similar potency to PI-3K, whereas the structurally unrelated wortmannin (at 1 μM) did not. Hence it is conceivable that the effects of LY 294002 on H_2O_2-induced cell death involve inhibition of protein kinase CK2 and not PI-3K. Interestingly, there is growing evidence for a role of protein kinase CK2 in cell survival and death associated with stress signalling pathways (Ahmed et al., 2002; Litchfield, 2003). However, at present it is not known whether H_2O_2 activates protein kinase CK2 in neuronal cells or if CK2 is involved in regulating neuronal cell survival.

It is also notable that treatment of undifferentiated SH-SY5Y cells with LY 294002 alone (in the absence of H_2O_2) triggered a significant reduction in neuronal cell viability (see Fig. 7B). A similar decrease in neuronal cell viability following chronic exposure to LY 294002 has been reported previously using cerebellar granule cells and primary cortical neurons (Shimoke et al., 1999; Crossthwaite et al., 2002). However, differentiated SH-SY5Y cells were not sensitive to treatment with LY 294002 alone (Fig. 8B). The reasons for the difference in sensitivity to LY 294002 between differentiated and undifferentiated SH-SY5Y cells are not clear but these observations highlight the importance of using not only undifferentiated (as in the case of most studies) but also differentiated SH-SY5Y cells.

In summary, the present study has shown that H_2O_2 stimulates robust increases in ERK1/2, JNK1/2 and PKB phosphorylation in undifferentiated and differentiated human neuroblastoma SH-SY5Y cells. In addition, we have shown that inhibition of the ERK1/2 pathway protects SH-SY5Y cells from H_2O_2-induced cell death. However, the role of ERK1/2 in determining the fate of neuronal cells (survival or death) following oxidative stress is controversial and clearly requires further investigation.

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