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Aggravation of necrotic death of glucose-deprived cells by the MEK1 inhibitors U0126 and PD184161 through depletion of ATP

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Abstract

The extracellular-regulated kinases (ERK) modulate cell proliferation and survival in response to several different stimuli and are therefore important drug targets. ERKs are activated by the dual phosphorylation kinase MEK1 and MEK1 inhibitors PD98059, U0126 and CI-1040 are now widely used to inhibit ERKs in cell and animal studies. In an analysis of ERK functions in astrocytes we found that PD98059 (100 μM) failed to inhibit ERK phosphorylation but U0126 (50 μM) inhibited ERK phosphorylation to ~80%. Surprisingly, U0126 also caused profound depletion of ATP in glucose-deprived cells, leading to death by necrosis. Since glucose-deprived cells depend mainly on mitochondrial ATP-synthase for ATP production, we tested whether U0126 or PD184161, a derivative of CI-1040, might inhibit ATP synthase activity, using $143B^{Rho0}$ cells (which lack a functional F0 subunit) to further parse this effect. We found that the F1F0ATPase activity extracted from U0126- or PD184161-treated parental 143B cells or astrocytes was indeed inhibited by ≥80% suggesting a covalent change in the enzyme. However, F1F0ATPase activity extracted from similarly treated $143B^{Rho0}$ cells was spared. Because F1F0ATPase activity in isolated mitochondria was not inhibited directly, we propose that U0126 and PD184161 inhibit ATP-synthase via an indirect action on F0. The MEK1 inhibitors also induced necrosis of other glucose-deprived cell types including primary neurons at the same concentrations required for inhibition of ERK phosphorylation. Thus, the MEK1/ERK signalling pathway may modulate ATP synthase function, and its inhibition may cause cells unable to perform glycolysis to die by necrosis.

Keywords: ATP-synthase; Astrocytes; MAPK/ERK; Mitochondria; Necrosis; Sympathetic neurons

1. Introduction

Much use is being made of kinase inhibitors in studies of kinase functions in cellular processes and disease. However, kinase inhibitors can also exert effects unrelated to their primary target. Outside of the obvious possibility of cross-inhibition of other kinases [1–3], competition with ATP for other ATP binding proteins [4–6] or formation of aggregates that absorb/adsorb to proteins and alter their activity [7] have been reported. The allosteric MEK1 inhibitors U0126 [8] and PD98059 [9,10] both of which are non-competitive with ERK or ATP [8,9] are heavily used in studies of MAPK/ERK function in cultured cells. Though U0126 was reported to form inhibitory aggregates as distinct from PD98059 [7], both have been reported to have effects on cells that are additional to MEK1 inhibition. For example, Pereira et al. [11] demonstrated that both U0126 and PD98059 altered glutamate release from hippocampal synaptosomes in addition to the inhibition of ERKs, with U0126 potentiating depolarization-induced calcium-independent glutamate release, and PD98059 partially inhibiting calcium entry through voltage-gated Ca^{2+} channels. In another study, PD98059 was reported to inhibit human platelet cyclooxygenase activity directly [12]. It is important to understand these additional effects as such activities in drug analogues that are being developed as ERK pathway inhibitors [13] might be advantageous under the right circumstances rather than deleterious.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular regulated kinases (or mitogen-activated protein kinases); MEK1, MAPK and ERK kinase; oligA, oligomycin A; PD184352, 2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropylmethoxy-3,4-difluoro-benzamide; PD184161, 2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropylmethoxy-3,4-difluoro-5-bromo-benzamide; PD98059, 2′-amino-,3′-methoxy-flavone; U0126, (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene); SCG, superior cervical ganglion

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While we were investigating the roles of ERK in the protection against glucose-deprivation induced death in astrocytes [14], we noted that U0126 caused rapid necrosis of the glucose-deprived but not glucose-maintained cells. This inhibition occurred with the same dose-response that characterizes the inhibition of ERK phosphorylation by U0126. Because glucose-deprived astrocytes rely on mitochondrial ATP-synthase for their survival, as the mitochondrial ATP synthase inhibitor oligomycin A caused glucose-deprived cells to die rapidly by necrosis, we suspected that the necrosis induced by U0126 could be due to inhibition of the ATP-synthase complex, a potentially important effect.

Dissection of the locus of action of a drug that inhibits ATP synthase is facilitated by using ρ0 cells [15]. In ρ0 cells, mitochondrial DNA has been ablated by low doses of ethidium bromide causing loss of a functional F0 component (the moiety responsible for proton translocation) as well as loss of a functional respiratory chain. In these cells the ATP synthase (carried by the F1 component) becomes an ATPase, consuming rather than producing ATP [15–17] so as to maintain a mitochondrial membrane potential [15–17]. These characteristics make ρ0 cells critically dependent on glucose and glycolysis as a source of ATP. As a result of loss of F0 function, oligomycin A, a drug that normally inhibits ATPase activity by binding to F0, ceases to function while azide, which inhibits the ATPase activity through F1, causes ρ0 cells to accumulate ATP. Hence, if a drug inhibits ATP synthase through the F0 component it will lose its effects on ρ0 cells. If, however, the drug inhibits F1, it will increase cellular ATP levels in ρ0 cells. Analysing the effects of a drug that inhibits F1F0ATPase in F0-replete cells or ρ0 cells therefore provides a test of whether the drug's effects are mediated by F0 or F1.

In this study we show that U0126 and a novel more affine MEK1 inhibitor, PD184161 (a derivative of PD184352 or CI-1040) [1,13] both promote death by necrosis of several types of glucose-deprived cells. Based on a comparison of their effects in parental 143B cells and 143Bρ0 cells [15], we propose that the MEK1 inhibitors deplete ATP by inhibition of ATP-synthase activity through an inhibitory effect on F0.

2. Materials and methods

2.1. Cell culture

Astrocytes were prepared from newborn 129/Ola mice using a protocol [18] approved by the Home Office and the University of Cambridge ethical review committee. After 12 days growth in medium containing modified DMEM [14] and 10% foetal bovine serum (Hyclone) cultures were shaken in an orbital shaker at 180 rpm for 16 h, washed thoroughly with modified DMEM to remove contaminating microglia, and then dissociated by incubation with 0.25% trypsin-EDTA for 15 min. After pre-plating for 15 min to remove remaining microglia, 2 ml of suspended cells (2×10^5 cells/ml) were seeded in 35 mm diameter culture dishes and grown for 12 days in modified DMEM containing 10% foetal bovine serum followed by 7 days in medium containing 5% FBS, then washed in serum-free modified DMEM and grown in serum-free DMEM for 5 days. Astrocyte purity was approximately 98%. 143B and 143Bρ0 cells were the kind gift of Dr. Michael Murphy (MRC Dunn Nutrition Unit, Cambridge, UK) and were grown as described previously [17]. HeLa cells and HepG2 cells were grown in DMEM containing 10% foetal bovine serum. Rat primary sympathetic neurons from the superior cervical ganglion (SCG) were grown as described previously [19] in RPMI medium containing 100 ng/ml NGF and either 1.2% glucose or no glucose. Oligomycin A, and NaN_3 were obtained from Sigma, U0126 and PD98059 from Calbiochem, and PD184161 was a kind gift from Dr. Alexander Bridges (Pfizer, Ann Arbor, MI, USA).

2.2. Immunoblotting

Cells were washed with ice-cold PBS, scraped off in lysis buffer pH 7.6 (50 mM Tris pH 7.6, 1 mM Na_2-EDTA, 1 mM Na_3VO_4, 10 mM NaF, 1% Triton X-100, 0.1 mM phenyl-methyl-sulfonyl chloride, 250 mM sucrose, 1 mM EGTA, 1 μg/ml leupeptin and 2 μg/ml benzamidine) and transferred to a microfuge tube. After pipetting up and down about 30 times, cells were incubated on ice for 20 min with occasional vortexing and centrifuged at $10{,}000 \times g$ for 5 min. The supernatant was kept at −80 °C until analysis. Bicinchoninic acid (BCA) (Sigma) was used to determine protein concentration using 5 μl of cell lysate. Equivalent amounts of protein were resolved by SDS–PAGE, blotted onto nitrocellulose (0.2 μm), and analysed by enhanced chemiluminescence (reagents from Amersham Biosciences or prepared in the lab) using Kodak X-OMAT AR film. Antibodies against phospho-ERK(T202/Y204), phospho-MEK1/2(S217/S221) (#9121S), and total-MEK1/2 (#9122) were from Cell Signalling Technologies, and anti-ERK (MK12) was from Transduction Labs (BD Biosciences). Secondary antibodies were from Jackson ImmunoResearch Labs. Films were scanned using a flat bed scanner (HP Scanjet 5470C) and intensities of the bands representing phospho- and total kinase forms were determined from two to three different exposures (within the linear detection range) using ImageQuant analysis software (Molecular Dynamics, Amersham Biosciences).

2.3. ATP measurement

ATP was measured using an ATP bioluminescence HS assay kit (Roche Diagnostics). Cells were washed with ice-cold PBS and excess liquid was removed before adding 200 μl lysis buffer provided in the kit. Cells were scraped off with a rubber policeman and transferred to a new microfuge tube. The cell extracts were then boiled at

100 °C for 2 min followed by centrifugation at 10,000 × *g* for 1 min to pellet unlysed cell debris. Before measurements, the luciferase-luciferin reagent was prepared following the manufacturer's instructions. Then, 100 μl of supernatant was mixed with 250 μl the luciferase-luciferin reagent and luminescence was measured using a Jade luminometer (Labtech International). Readings were recorded every 10 s for 90 s. The amount of ATP was determined from a standard curve constructed with known concentrations of ATP (measurements ranging from 1 to 1000 nM were linear, $r^2 = 1$). In some cases samples were diluted so that the readings fell within the range of the standard curve. The amount of ATP was normalised to either total cell number or total protein.

2.4. Glycogen assay

Glycogen was assayed according to Sorg and Magistretti [20]. Briefly, astrocytes were washed twice with ice-cold PBS, lysed in 30 mM HCl and sonicated for 10 min. Cell lysates (100 μl) were mixed with 300 μl of 0.1 M acetate buffer (pH 4.6) with or without 0.1 mg/ml amyloglucosidase (Roche), incubated at room temperature for 30 min, then 2 ml of the reaction buffer containing 0.1 M Tris–HCl (pH 8.1), 3.3 mM $MgCl_2$, 330 μM ATP, 38 μM NAP (Roche), and 6 μg/ml hexokinase/glucose-6 dehydrogenase 2:1 (Roche) were added and incubated at room temperature for 30 min. Fluorescence was measured (excitation, 340 nm; emission, 450 nm) against a standard curve of glucose (10 nM to 10 mM).

2.5. F1F0ATPase activity

The methods for preparing and measuring F0F1ATPase were taken from James et al. [21]. Briefly, PBS-washed cells were suspended in ice-cold buffer at pH 7.4 (250 mM sucrose, 5 mM Tris–HCl, 1 mM EGTA) and disrupted using 15 strokes in a dounce glass homogenizer. After sedimentation at 1000 × *g* for 4 min, the supernatant was sedimented at 10,000 × *g* for 10 min. Mitochondria were suspended in buffer at pH 7.4 (120 mM KCl, 10 mM Hepes/KOH, 1 mM EGTA) and disrupted by three freeze-thaws in an ethanol/dry ice bath. F1F0ATPase activity was assayed by following the decrease in absorbance of NADH at 340 nm in buffer at pH 8 containing 100 mM Tris–HCl pH 8, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 20 μM NADH, 1 mM phosphoenopyruvate, 2.5 mM MgATP, 10 U/ml pyruvate kinase, 5 U/ml lactate dehydrogenase, and 1 mM ouabain (the latter five compounds were from Sigma). For each sample, activity was measured in parallel in the absence or presence of 1.7 μg/ml oligomycin A to determine nonspecific activity and net activity was calculated as the difference between the two measurements. As mitochondria from 143Bρ0 cells are insensitive to oligomycin A, 1 mM NaN_3 was added instead to obtain a measure of nonspecific activity.

2.6. Statistics

Significance of differences between means was tested using two-tailed unpaired Student's *t*-test.

3. Results

3.1. Cell death of glucose-deprived astrocytes is accelerated by the MEK1 inhibitor U0126

We have shown previously that glucose-deprivation of astrocytes promotes sustained phosphorylation of ERK1/2 and that these cells survive for several days [14]. To investigate its possible role in cell survival, ERK activity was inhibited using the MEK1/2 inhibitor U0126 [8] as PD98049 [9,10] used at concentrations up to 100 μM had no inhibitory effect (data not shown). The efficacy of U0126 as an inhibitor was also relatively low compared to published IC_{50} values [1,8] (Fig. 1A) requiring about 25 μM to achieve half-maximal inhibition. At 50 μM U0126, which inhibited ERK phosphorylation by about 70–80%, the death of glucose-deprived astrocytes occurred within 6 h (Fig. 1B). No cell death was observed when astrocytes were incubated with 50 μM U0126 in the presence of glucose (Fig. 1B) showing that the compound was not generally toxic.

3.2. U0126 causes necrosis by depletion of ATP

Because astrocyte death was apparently necrotic (as virtually all dead cells were stained with propidium iodide, data not shown), we investigated whether ATP might be depleted in U0126-treated cells. Indeed, ATP levels were dramatically reduced by treatment with 50 μM U0126 (Fig. 1B). Both ATP depletion measured 6 h after onset of glucose-deprivation, and P-ERK inhibition measured after 5 h in the presence of glucose to prevent cell death from occurring, followed similar dose–response curves (Fig. 1C). No decrease in ATP or cell death was observed with 100 μM U0124, a structural analogue of U0126 that does not inhibit MEK1 activity [8] (data not shown).

As necrosis in the presence of U0126 occurred only in glucose-deprived astrocytes, we reasoned that ATP depletion could be due to inhibition of glycogen mobilization, limited provision of glycolytic pyruvate to the mitochondria, and/or inhibition of ATP synthase (F1F0ATPase) itself. However, there was no significant difference in the extent of glycogen breakdown when astrocytes were deprived of glucose in the absence or presence of 50 μM U0126 (Table 1). There was also no increase in ATP levels or decrease in cell death when medium was enriched with 1 mM pyruvate (Table 1). Given the lack of effect of U0126 on these two processes, these data suggested that U0126 was inhibiting ATP production by the mitochondria, whose supply of ATP is critical for survival of astrocytes maintained in the absence of glucose [22,23].

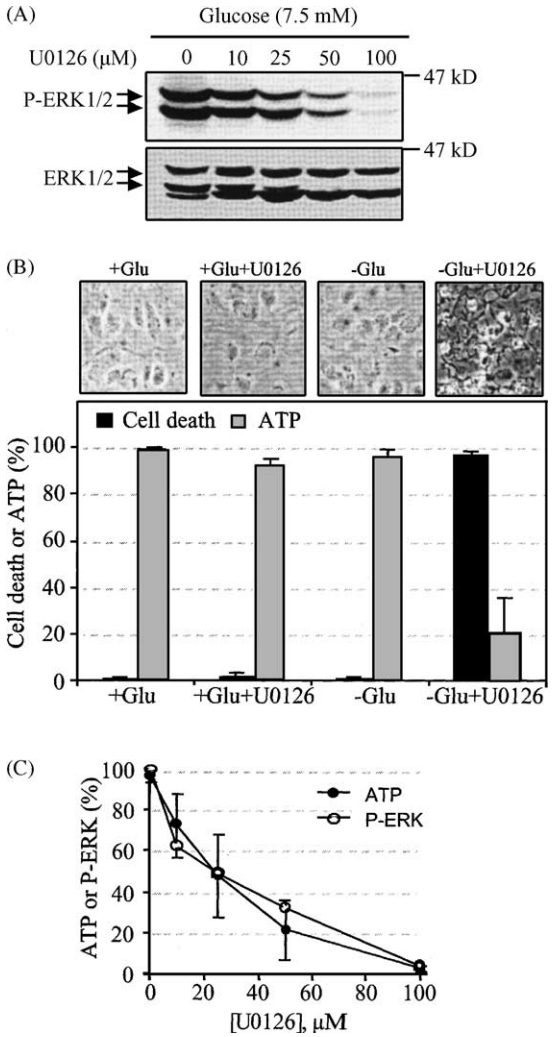

Fig. 1. The MEK1/2 inhibitor U0126 inhibits ERK phosphorylation and causes necrosis of glucose-deprived astrocytes by depletion of ATP. (A) Astrocytes grown in the presence of 7.5 mM glucose were incubated with different concentrations of U0126 for 5 h whereupon extracts were collected and analysed by immunoblotting for ERK phosphorylation using an anti-phosphoT202/Y204-ERK antibody. Blot was stripped and reprobed with an anti-ERK antibody to control for loading (active ERK is also detected as shifted bands above p42/p44). (B) Astrocytes were incubated in the absence or presence of glucose alone or in the absence or presence of 50 μM U0126. ATP was measured 4 h after onset of treatment using a firefly luciferase assay while cell viability was determined 6 h after onset of treatment using propidium iodide staining to indicate necrotic cells, and Hoechst 33342 staining to indicate total number of cells. The amount of ATP derived from glucose-maintained astrocytes was set to 100% (mean ± S.D., three independent experiments. $P < 0.05$, −Glu + U0126 against all other conditions; all other changes are not significant). Cells were photographed after 6 h under phase contrast. (C) Correlation between reduction in ATP levels and loss of ERK phosphorylation. Relative intensities of P-ERK (p44/p42) bands detected on the blot are plotted alongside relative ATP levels as a function of U0126 concentration.

3.3. The inhibitors U0126 and PD184161 suppress production of ATP by F0F1ATPase

To investigate whether F0F1ATPase activity is impaired by U0126, and whether this inhibition occurs through the F1 or F0 components of ATP synthase, we examined the responses of 143B cells and F0-deficient 143Bρ0 cells [15]. To further examine whether ERK inhibition contributed to the loss of ATP we observed, we also compared the effects of U0126 with those of the less affine drug PD98059 [9] and the potentially more affine drug PD184161, reasoning that if ATP reduction is due to ERK inhibition the efficacy of each drug for inhibiting both processes should be similar. The minimum effective inhibitory dose of each compound was determined (data not shown) which was then used in further studies.

Table 1
MEK1 inhibitors do not prevent glycogen mobilization or limit pyruvate supply to mitochondria

	Assay		
	No glucose	No glucose + U0126	
Glycogen (%)	29.7 ± 4.3*	35.1 ± 2.2*	
	100 μM pyr	100 μM pyr	1 mM pyr
ATP (%)	90.4 ± 2.0	21.0 ± 14§	29.4 ± 10§
Cell death (%)	0.7 ± 1.3	81.3 ± 14¶	68.6 ± 12¶

Astrocytes maintained in the presence or absence of 7.5 mM glucose were treated with 50 μM U0126 for 4 h before lysates were prepared and assayed for glycogen content. Glycogen values are relative to the value obtained from glucose-maintained cells (100%). In some cultures, the glucose-free medium containing 50 μM U0126 was supplemented with 1 mM pyruvate (pyr) for 6 h after which ATP and cell death were measured. Values are mean ± S.D., three independent experiments. None of comparisons achieved statistical significance ($^{*,\S,\P}P \geq 0.3$).

As use of PD184161 has not been reported upon previously, the structures of the three compounds are shown in Fig. 2A. Fig. 2B shows that all three compounds inhibited ERK phosphorylation in 143B and in 143Bρ0 cells, irrespective of the presence or absence of glucose. PD184161 was most potent, showing complete inhibition of ERK phosphorylation at 5–10 μM while inhibition of ERK phosphorylation by 75 μM PD98059 in 143B cells was incomplete (∼90%). In the presence of glucose, neither oligomycin A nor NaN_3, bona fide ATP-synthase inhibitors, reduced ERK phosphorylation over the time studied, showing that prevention of ERK phosphorylation by the MEK1 inhibitors is not obligatorily linked to ATP-synthase activity. Intriguingly, as in astrocytes, ERK phosphorylation was substantially activated by glucose-deprivation in both 143B cells and in 143Bρ0 cells (Fig. 2B).

To obtain a better understanding of the mechanism of PD184161, we also examined its effects on MEK1 phosphorylation as U0126 and PD184352 (from which PD184161 was derived) were reported to inhibit MEK1 activation (as well as MEK1 activity) while PD98059 only inhibited MEK1 activity [1]. As shown in Fig. 2C, in 143B cells U0126 inhibited MEK1 phosphorylation by about 60% at concentrations that inhibited P-ERK almost completely but neither PD184161 or PD98059 had such an effect. Similar results were obtained with 143Bρ0 cells and HeLa cells (data not shown). Thus PD184161 is different from U0126 on two counts, it is 5- to 10-fold more potent

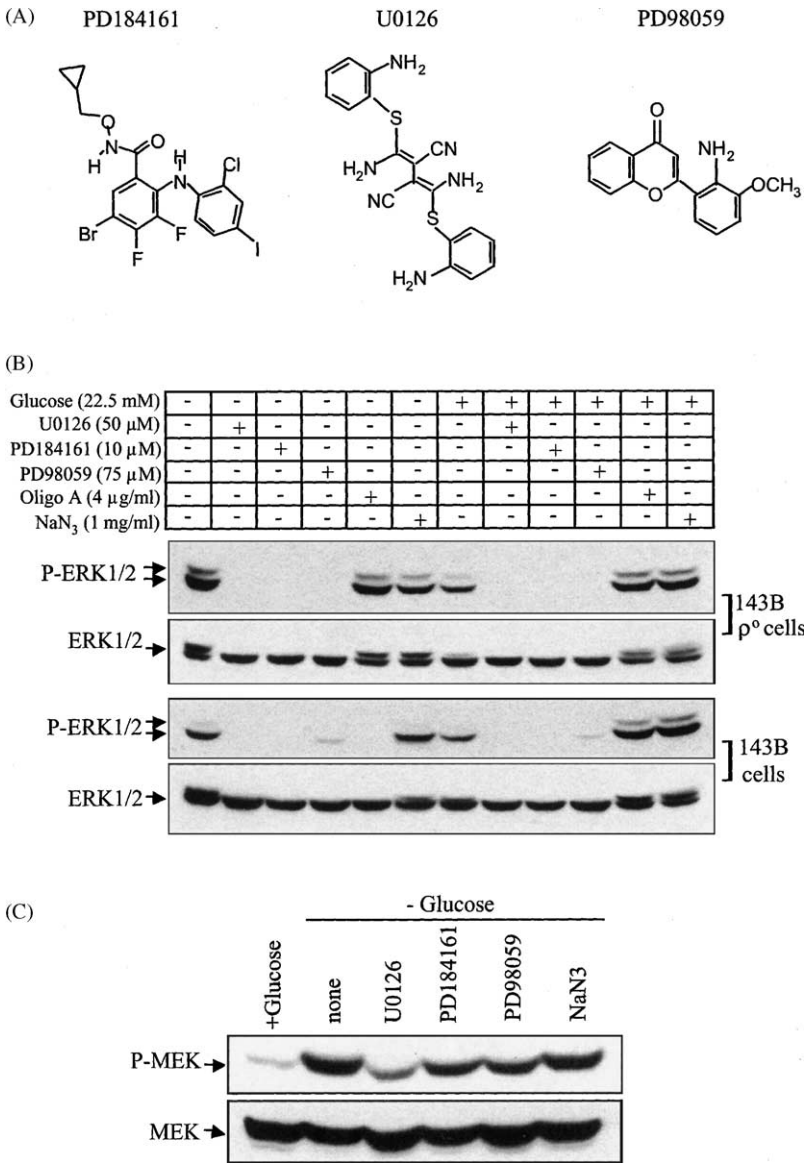

Fig. 2. The MEK1 inhibitors U0126, PD184161, and PD98059 suppress ERK phosphorylation in 143B and 143Bρ0 cells in a glucose-independent manner. (A) Structure of the MEK1/2 inhibitors PD184161, U0126, and PD98059. (B) Cells were serum-deprived for 2 h and incubated in the absence or presence of glucose (22.5 mM) and one the following drugs: U0126 (50 μM), PD184161 (10 μM), PD98059 (75 μM), oligomycin A (oligA, 4 μg/ml), or NaN_3 (1 mg/ml). The drugs were added either at the time of onset of glucose-deprivation and left to incubate for 6 h (143B cells) or were preincubated with cells in the presence of glucose for 1–2 h before glucose-deprivation (in the presence of the drugs) was initiated for 15 min (143Bρ0 cells). Extracts were analysed by immunoblotting with an antibody against phospho(T202/Y204)-ERK, and reprobed after stripping, with an anti-ERK antibody to control for loading (fold increase in ERK phosphorylation induced by glucose deprivation was 3 ± 0.4 (143Bρ0) and 2.5 ± 0.5 (143B cells), mean ± range, two independent experiments). (C) Extracts from 143Bρ0 cells treated as in B were analysed by immunoblotting with an antibody against phospho-MEK1/2 and reprobed, after stripping, with an anti-total MEK1/2 antibody (fold change in MEK1 phosphorylation compared to a value of 1 assigned to the band from cells treated with no glucose alone; U0126, 0.43; PD184161 or PD98059, 0.87; NaN_3, 1).

as an inhibitor of ERK phosphorylation and it is not an inhibitor of MEK1 activation. Due to its higher affinity, PD184161 might be predicted to be the least nonspecific of all three compounds.

When we examined the effect of the inhibitors on ATP levels in 143B and 143Bρ0 cells, neither of the inhibitors caused a significant reduction in ATP levels in the presence of glucose in either type of cell (Fig. 3A and B). However, both 50 μM U0126 and 10 μM PD184161 caused a profound decrease of up to 90% in ATP compared to controls in glucose-deprived 143B cells (Fig. 3A), on par with the inhibition caused by oligomycin A or NaN_3. PD98059 did not have any ATP-lowering effect. In 143Bρ0 cells, glucose-deprivation on its own caused rapid ATP depletion by about 80% after as little as 15 min (Fig. 3B) so there was not much scope for further lowering of ATP levels by the drugs, especially as such profound ATP depletion may prevent ERK phosphorylation in its own right [14]. However, while oligomycin A had no inhibitory effect in 143Bρ0 cells, as expected [15–17], 1 mM NaN_3 partially

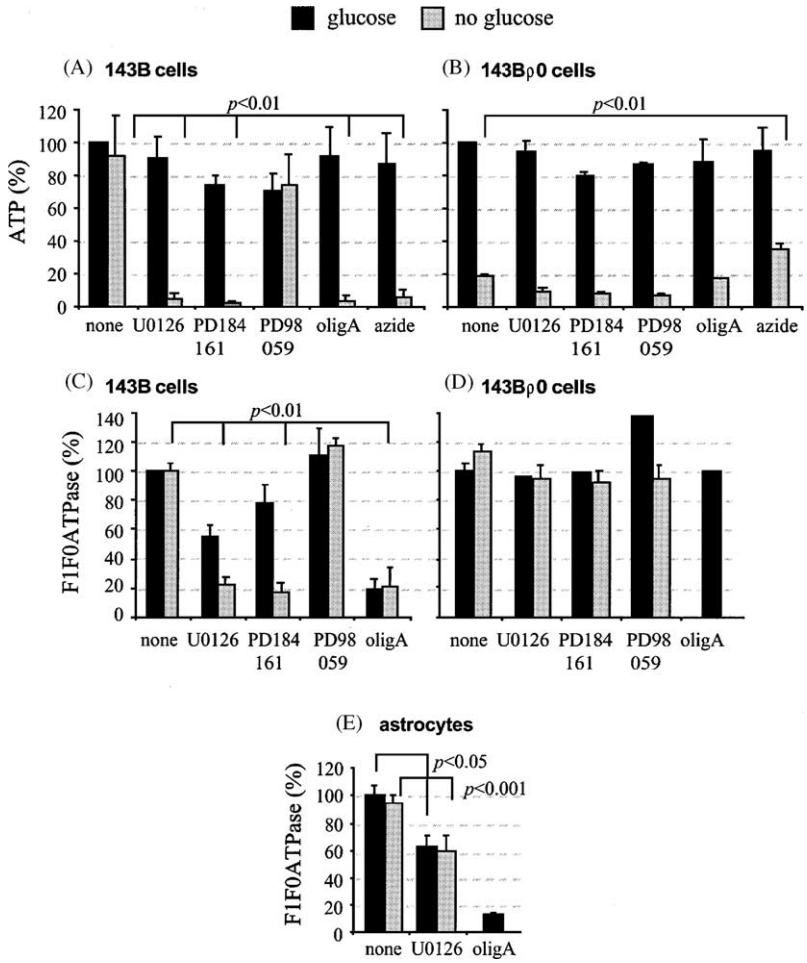

Fig. 3. ATP production and F1F0ATPase activity derived from U0126- or PD184161-treated 143B cells or astrocytes, but not from 143Bρ0 cells, is partially suppressed. (A) 143B cells were treated as described in Fig. 2A. After 6 h, extracts were prepared and ATP was measured using the firefly luciferase assay. The value measured in extracts of untreated cultures maintained in the presence of glucose was set at 100% (mean ± S.E.M., three to five independent experiments; with glucose, P values are not significantly different to those induced by azide; no glucose, $P < 0.01$ for all values except with PD98059). (B) 143Bρ0 cells were treated as described in Fig. 2A. Cultures deprived of glucose were incubated for 15 min in the appropriate medium before extraction while cultures maintained with glucose were left to incubate for another 3 h before extraction. ATP was measured using firefly luciferase assay (mean ± S.E.M. from 3 to 6 independent experiments; with glucose, P values are not significantly different to those induced by azide; no glucose, $P < 0.01$ for the higher value obtained using azide compared to control value; all other changes were not significant). (C) Mitochondrial fractions from 143B cells treated as in A were analysed for azide-inhibitable ATPase activity. Data show mean ± range (two independent experiments) for data obtained in the presence of glucose and mean ± S.E.M. (three independent experiments) for data obtained in the absence of glucose ($P < 0.01$ for values obtained using U0126, PD184161 or oligomycin A compared to control). (D) Mitochondrial fractions from 143Bρ0 cells treated as in B, or treated for 6 h in the presence of glucose and the drugs indicated, were analysed for azide-inhibitable ATPase activity (for experiments performed in the absence of glucose, data are mean ± S.E.M., four independent experiments; none of the values showed significant differences; one data set in duplicate was obtained in the presence of glucose). (E) Astrocytes were incubated in the presence or absence of glucose for 4 h without or with 50 μM U0126 after which mitochondria were prepared and assayed for oligomycin-dependent F1F0ATPase activity with 100% value being assigned to the measurement obtained in the presence of glucose alone (mean ± S.E.M., three independent experiments; $P < 0.05$ for reduction induced by U0126 in the presence of glucose, $P < 0.001$ for the reduction induced by U0126 in the absence of glucose).

reversed the reduction in ATP induced by glucose-deprivation ($P < 0.01$) but none of the inhibitors mimicked this activity. These results suggested that the drugs do not share the same mechanism of action on F1 as NaN_3, leaving open the possibility that their effects are mediated through F0.

When the activity of the F1F0ATPase was measured in mitochondria isolated from glucose-deprived 143B cells that had been treated with the drugs, both U0126 and PD184161 caused a profound inhibition of activity to 20% of control values, similar to the effect observed with oligomycin A ($P < 0.01$, Fig. 3C) but PD98059 had no effect, consistent with the measurements of ATP. Even in the presence of glucose there was about 50% inhibition in F1F0ATPase activity induced by U0126 and PD184161. As this ATPase assay was conducted using isolated mitochondria derived from the treated cells, whereas full activity of the enzyme was restored when mitochondria were derived from 143B cells that had been treated with the reversible inhibitor NaN_3 for the same period of time (data not shown), it follows that inhibition of F1F0ATPase activity was long-lived. The activity of the F0F1ATPase was also inhibited by U0126 in murine astrocytes in a

glucose-independent manner (Fig. 3E). suggesting that the effect of the drug is not species- or cell-type specific.

In the 143Bρ0 cells, however, none of the inhibitors reduced F1F0ATPase activity significantly (Fig. 3D) though the specific activity of the F1F0ATPase in untreated cells was similar to that measured in the 143B parental cell line (data not shown). To offset the possibility that the inhibitory effect on F1F0ATPase takes time to develop, the inhibitors were added to 143Bρ0 cells for 6 h in the presence of glucose before glucose deprivation was initiated. However, despite the long treatment, no reduction in F1F0ATPase activity was observed in the 143Bρ0 cells treated with U0126 or PD184161 (Fig 3D), activity being similar to that found in extracts from oligomycin A-treated cells.

To examine whether U0126 might directly inhibit F1F0ATPase activity, we tested whether it could inhibit the F1F0ATPase in preparations of purified mitochondria. However, 50 μM U0126 failed to inhibit F1F0ATPase activity in purified mitochondria derived either from rat liver, or rat brain, or pure astrocytes even after an incubation of 4 h prior to the assay (data not shown). Together, these data suggest that U0126—and by extension PD184161—inhibit F1F0ATPase activity indirectly through a stable modification of F0, as there was no inhibition of the enzyme in 143Bρ0 cells that lack a functional F0 subunit and the compounds failed to elevate ATP, unlike NaN_3. The lack of inhibition of the ATPase with PD98059 suggested, however, that the effects of the other two inhibitors was not dependent on ERK inhibition.

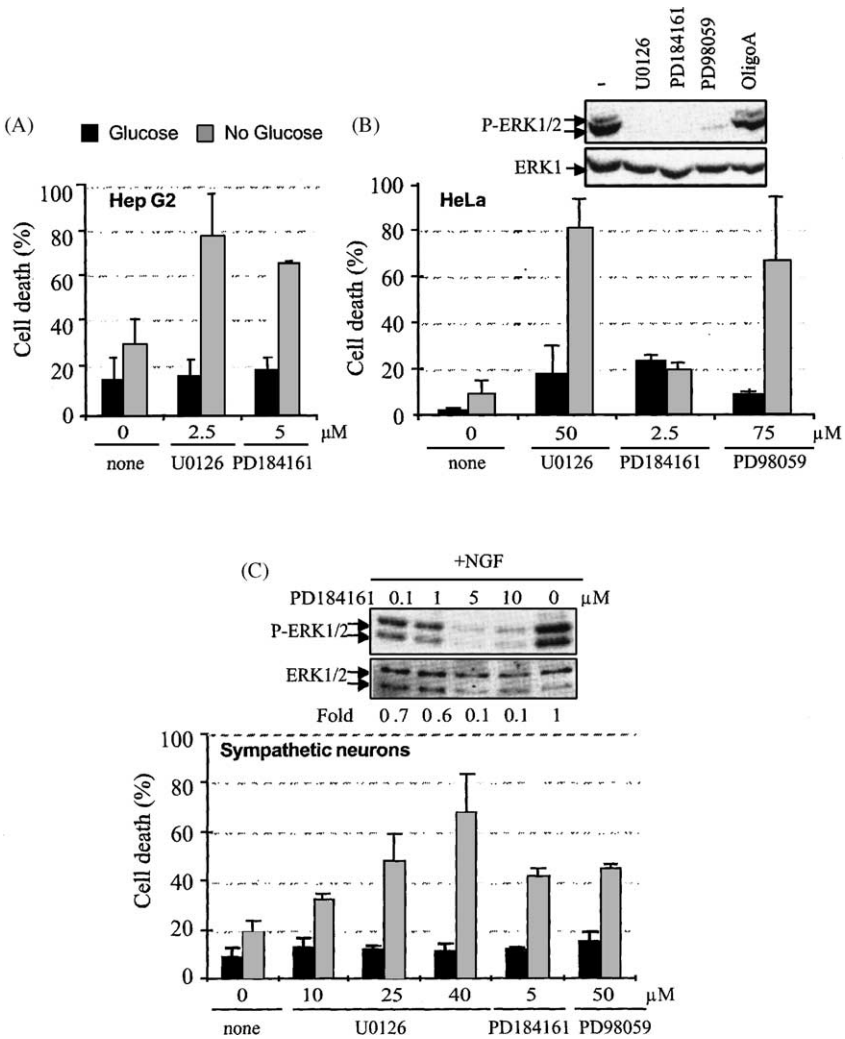

Fig. 4. MEK1 inhibitors accelerate cell death of glucose-deprived HepG2 cells, HeLa cells, and sympathetic neurons. Cell death was determined after staining with PI and Hoechst 33342. (A) HepG2 cells were incubated for 21 h in the presence or absence of 7.5 mM glucose alone, or in the same medium containing U0126 (25 μM) or PD184161 (5 μM) (mean ± S.E.M., three independent experiments, $P < 0.01$ for no glucose without and with drugs). (B) HeLa cells were incubated for 18 h in the presence or absence of 7.5 mM glucose alone, or in the same medium containing U0126 (50 μM) or PD184161 (2.5 μM) or PD98059 (75 μM). (mean ± range, two independent experiments). Note that PD184161 used at 5 μM was toxic to the cells. (C) Sympathetic neurons were incubated for 20 h in the presence or absence of glucose and/or the indicated concentrations of the drugs. U0126, mean ± S.E.M. (three independent experiments) $P < 0.01$ for value with no glucose compared to values at all concentrations of U0126; PD184161 or PD98059 (mean ± range, two independent experiments).

3.4. ERK-pathway inhibitors accelerate cell death of glucose-deprived HepG2 cells, HeLa cells, and primary sympathetic neurons

The efficacy of MEK1 inhibitors is cell-type and treatment-dependent. PD98095 especially has such low efficacy that it is not always possible to demonstrate that it is inhibitory [8]. To further enquire whether the exacerbation of cell death by MEK1 inhibitors is specific to glucose-deprived 143B cells and astrocytes, and whether PD98059 is always a benign compound in the context of glucose-deprivation, we investigated their effects in HepG2 cells, HeLa cells, and primary sympathetic neurons using in each case the minimum dose required for complete inhibition of ERK phosphorylation. Fig. 4A shows that U0126 (25 μM) and PD184161 (5 μM) induced 78 and 65% of cell death in glucose-deprived HepG2 cells after 21 h of glucose-deprivation without elevating cell death in cells maintained in the presence of glucose. Glucose deprivation by itself only promoted an increase of about 15% in HepG2 cell death. In glucose-deprived HeLa cells (Fig. 4B), 50 μM U0126 induced about 75% cell death after 18 h, close to the levels induced by oligomycin A ($97.8 \pm 1\%$). Interestingly, 75 μM PD98059 also induced 50% necrotic death but 2.5 μM PD184161 had no necrotic effect though it too inhibited ERK phosphorylation (higher concentrations were toxic even in the presence of glucose, data not shown).

In primary sympathetic (SCG) neurons deprived of glucose, all three inhibitors enhanced necrotic death, the percentage of propidium iodide positive cells rising from 20% in glucose-deprived neurons, to about 45% in the presence of 25 μM U0126, or 50 μM PD98059, or 5 μM PD184161 ($P < 0.01$). All three concentrations were also sufficient to eliminate ERK phosphorylation (Fig. 4C, [24] and data not shown). A dose-dependent effect of U0126 was noted, with 10 μM U0126 already being sufficient to induce significant necrosis (about 35%). In the presence of glucose, necrotic death was between 10 and 15% for all treatments. Hence, the death-aggravating effects of MEK1 inhibitors is observed in several different cell types when the cells are deprived of glucose, although the potency and efficacy of the different inhibitors varies between cell types. In all cases, however, the necrotic effect occurred concomitantly with inhibition of ERK phosphorylation.

4. Discussion

In most cells with a functional F1F0ATPase, production of ATP by respiration can compensate for loss of glucose by utilizing other substrates (such as amino and fatty acids) so if cells die at all they do so rather slowly (in the case of astrocytes, after 5 days [14]). However, failure to maintain ATP levels above a critical threshold produces rapid necrosis. We show here that U0126 and PD184161, two drugs that are ERK pathway inhibitors but which have very different structures, have the capacity to sensitise different types of cells to the consequences of loss of glucose by disabling ATP production by the mitochondrial ATP synthase. In U0126-treated astrocytes, the degree of inhibition of ERK phosphorylation (which required 50 μM for complete inhibition, well above its reported IC_{50} values [8]) was closely correlated with the decrease in ATP levels suggesting that the two were causally related, especially as PD98059 failed to reduce both ERK activity and ATP levels even at 100 μM concentrations. However, in 143B cells, ERK inhibition was near-complete in the presence of 50 μM PD98059 or 10 μM U0126, though neither concentration was sufficient to reduce ATP levels significantly. Moreover, in neurons, greater necrosis was observed as the concentration of U0126 increased above the concentration sufficient for >90% ERK inhibition (which is 10 μM). Though this result suggests that the drugs induce necrosis in glucose-deprived cells in an ERK-independent manner, in all cases where necrosis was produced, ERK activity was also reduced, so the possibility that inhibition of ATP production contains an ERK-dependent component cannot be excluded.

In all cells, U0126 had the most consistent effect, in keeping with the possibility that U0126 has unique properties (Fig. 2C), such as increasing the rate at which MEK1 is dephosphorylated [1]. We note, however, that all the drugs exerted their effects at concentrations that are above their reported IC_{50} values for inhibition of MEK1 activation in cells or ERK phosphorylation using pure enzymes under defined conditions [1,2]. There are precedents for experimental use of drugs well above their biochemical IC_{50} values. For example, the minimal concentration of the BCR/ABL inhibitor STI571 (Gleevac) required to inhibit growth of human leukaemic cells in mice was at minimum 6 μM, 20- to 60-fold higher than the reported biochemical IC_{50} value of 0.1–0.3 μM [25]. In the case of PD184352, the IC_{50} for its anti-proliferative effect in cells was 1 μM, but even at 10 μM there was incomplete G1 arrest [13]. Hence, micromolar concentrations well above the biochemical IC_{50} values are used and may be required to achieve a positive therapeutic outcome though loss of specificity may be a by-product.

What might mediate the ERK-independent mechanisms, and are they the same for all three drugs? In addition to inhibition of MEK1 activation, U0126 was reported to inhibit SAPK2a/p38, PRAK and PKB/Akt at a four-fold lower affinity in assays using purified kinases [1]. Though we did not investigate whether PRAK was inhibited, astrocytes did not contain significant p38 activity, and we found no evidence for inhibition of PKB/Akt by U0126 in astrocytes,143B or 143Bρ0 cell lines even at the relatively high concentrations employed in our experiments (H. Yung, data not shown). Thus, the results from kinase assays cannot necessarily be extrapolated to the situation found in cells, especially given the different time frames and conditions used in the two types of assays.

Moreover, PD184352 (from which PD184161 is derived) showed no inhibition of kinases other than MEK1 and ERK in the purified kinase assay [1], yet PD184161 was the most potent inhibitor of F1F0ATPase in the astrocytes and 143B cells. The lack of effect of PD98059 on the ERKs in the astrocytes may be due to its inability to inhibit pre-activated MEK1 and its poor affinity for MEK1, similar to the situation in Jurkat cells [8]. In these cells the IC_{50} for inhibition of immunoprecipitated MEK1 by PD98059 was over 100 μM compared to a value of 0.5 μM for U0126, again values that are 7- to 10-fold higher that those reported in kinase assays with pure MEK1 [8]. As evidence for its low potency, PD98059 also left some residual ERK activity in the 143B cell lines in our study. The precise mechanism of action of these drugs as ERK pathway inhibitors in the context of cells is thus still incompletely understood.

One possibility to account for the loss of ATP synthase activity induced by the higher concentrations of the inhibitors used in our study is that they aggregate nonspecifically [7,26]. Although PD98059 was reported not to form aggregates and did not aggravate the toxicity of glucose-deprivation of 143B cells, PD98059 did enhance necrosis in glucose-deprived sympathetic neurons and HeLa cells. Moreover, there were no detectable differences in the propensity of the three drugs to aggregate when concentrations applied in this study were adsorbed to an EM grid, negatively stained and viewed by electron microscopy (A. Tolkovsky and J. Skepper, unpublished observations). It is possible that the drugs aggregate differentially inside the cells, or they are differentially metabolised to more potent compounds, or they accumulate differentially against their concentration gradients. None of these mechanisms have been sufficiently explored.

It is not surprising that glucose deprivation sensitizes cells to other insults as many signals have been reported to be induced aside from the obvious reduction that occurs in respiratory substrates and reducing equivalents required to maintain mitochondrial homeostasis. In astrocytes, glucose-deprivation has been demonstrated to lead to glutathione depletion [27] and to greatly increase the cells' vulnerability to pro-oxidants like NO [28]. In Baf-3 cells, glucose-deprivation has been suggested to contribute to a pro-apoptotic state [29] which, in DU-145 cells, occurs through a mechanism that includes ceramide production, loss of Akt phosphorylation and downregulation of FLIP [30]. Hence, glucose deprivation by itself drives more than one reactive pro-death mechanism in cells. It may be that the MEK1 inhibitors exacerbate many of these responses through inhibition of ERK activity. However, it is clear that the death effect we have observed in this study is largely due to ATP depletion. The finding that U0126 potentiated depolarization-induced Ca^{2+} independent glutamate release [11] is also consistent with a depletion of ATP by this compound.

Our results suggest that the inhibitors deplete ATP through inhibition of the F0F1ATPase. How might the inhibitors promote F0F1ATPase inhibition? It is unlikely that the inhibitors are azide mimetics, as when cells were treated with azide before the mitochondria were prepared, the inhibitory effect of azide was lost due to the reversible nature of its binding while the inhibition conferred by U0126 and PD184161 was retained. Moreover, in 143Bρ0 cells deprived of glucose, azide increased ATP levels while the inhibitors did not. These data suggest that the binding of the inhibitors is either very tight, or that their effects are mediated by phosphorylation or another covalent modification. The activity of the MEK1 inhibitors U0126 and PD184161 seemed to mimic that of oligomycin A in that inhibition of F0F1ATPase activity by the compounds failed to manifest itself in 143Bρ0 cells. However, the MEK1 inhibitors and oligomycin A are unlikely to have similar mechanisms of action as the latter inhibited F1F0ATPase activity in143B cells irrespective of the presence or absence of glucose, while U0126 and PD184161 inhibited F1F0ATPase activity much more potently in the absence of glucose. There are two reports in the literature that describe post-translational modifications of components of the F0F1ATPase [31,32] but in neither case was it demonstrated that the subunits phosphorylated were part of the ATP synthase complex or that they affected its activity. Thus it is possible that the inhibitors alter phosphorylation of one of the components of F0F1ATPase though this remains to be tested.

Over 863 references using U0126 alone, of which 149 references pertain to cell death studies, were cited in PubMed at the time this paper was submitted. In many of these studies the concentrations used are well above the IC_{50} values based on in vitro characterization. Clearly, special vigilance is required when interpreting cell death mechanisms as the outcome ERK inhibition when using MEK1 inhibitors in light of our study. The more interesting possibility raised by our study is that the F1F0ATPase activity may be modulated by the MEK1 and/or ERK signaling pathway. In terms of therapeutic outcome, inhibition of the F1F0ATPase could be a valuable adjunct to other forms of anti-proliferative therapy as it will preferentially target cells that are already compromised in glycolysis, though deleterious effects in the nervous system would warrant careful consideration. Induction of necrosis by MEK1/ERK pathway inhibitors under glucose-compromised conditions might also enhance their efficacy as anti-proliferative drugs. It might be of benefit if the structure of ERK phosphorylation inhibitors that are being developed currently as anti-cancer drugs could be modified to carry this additional function.

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