Dexamethasone blocks antioestrogen- and oxidant-induced death of pituitary tumour cells

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Abstract

The oestrogen receptor is fundamental to the growth and survival of the rat pituitary tumour cell line, GH3. Our previous studies have shown that antioestrogens such as RU 58668 and ZM 182780 will reduce the rate of cell division and also induce cell death. Death of these cells in response to antioestrogen treatment appears to be due to a heightened sensitivity to reactive oxygen species (ROS). As part of a study to determine the cross-talk between steroid receptor systems in these cells, we have observed that the glucocorticoid, dexamethasone (Dex), inhibits antioestrogen-induced cell death. Cell death induced by H2O2 is enhanced by ZM 182780 and this effect is also blocked by Dex. As apoptotic cell death in a number of systems involves an early loss of mitochondrial membrane potential (ΔΨm), we have performed detailed studies on the time-course of ΔΨm loss in relation to the loss in cell membrane function. These studies have indicated that a loss of ΔΨm parallels a loss of cell membrane function – this is more characteristic of necrosis than of apoptosis.

From microscopic observations of these cells in response to H2O2, it has been noted that early cell membrane blebbing, induced by H2O2, is blocked in the presence of ZM 182780. Cell membrane blebbing can precede necrosis as well as apoptosis and it is thought to involve cytoskeletal changes, for which localised glycolytic reactions provide ATP. These observations, together with those showing that removal of glucose, but not inhibition of mitochondrial function, enhances ROS-induced cell death, prompted studies on the glycolytic pathway. As a strong candidate mechanism, it would appear that, via an effect on one of the rate-limiting glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase, Dex is able to overcome the antioestrogen-enhanced loss of glycolytic function following exposure of cells to ROS. This report contributes to the growing body of evidence showing that glucocorticoids provide a survival advantage to both normal and tumour cell types.

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Introduction

A characteristic response of tumour cell types that contain the oestrogen receptor (ER) is that oestrogens induce growth (Katzenellenbogen et al. 1979). There are exceptions to this rule, however. Although a considerable amount of evidence has accumulated that prolactin-secreting human pituitary tumours contain the ER (Friend et al. 1994), there is little strong evidence to indicate that these tumours are oestrogen-induced or even oestrogen dependent for growth. In contrast, this is not the case for the rat. Several strains of rat develop oestrogen-induced prolactin-secreting tumours (Wiklund et al. 1981). An ER-containing rat pituitary cell often used for studies on the effect of estrogens is the GH3 cell line (Tashjian et al. 1968). Although these cells were not originally obtained from an oestrogen-induced tumour, both growth and prolactin secretion are suppressed by the addition of antioestrogens to cells in culture (Newton et al. 1994a). Despite these observations with antioestrogens, a peculiarity of these cells is that growth is not always responsive to oestrogens (Haug & Gautvik 1976, Sorrentino et al. 1976).

A few years ago this problem was solved by studies using the pure antioestrogen, ZM 182780, where it was shown that the ER within these cells functions as a transcription factor in the absence of oestrogens (Newton et al. 1994b). It was apparent from these studies that the receptor could be made transcriptionally active by estrogens and also in response to signalling pathways activated by the growth factors, insulin, insulin-like growth factor-I (Newton et al. 1994c) and the cytokine, interleukin–2 (Newton et al. 1994a).

More recent work with this cell line has demonstrated that extended culture in the presence of the pure steroidal antioestrogen, ZM 182780, induces cell death, an effect that is fully reversed by oestrogens (Newton 1995).
mechanism for this has been partially elucidated by studies showing that cell death is due to the accumulation of reactive oxygen species (ROS), and that sensitivity to these agents is enhanced by antioestrogen-induced loss of ER function (Newton et al. 1999). A major conclusion from these studies was that functional ablation of the ER sensitises cells to ROS-induced metabolic collapse, leading to cell death with some of the characteristics of apoptosis (Schwartzman & Cidlowski 1993).

In addition to the ER, the other members of the superfamily of nuclear receptors (Hurd & Moudgil 1998) known to exist within the GH3 cells are the progesterone receptor (Newton et al. 1994a), the glucocorticoid receptor (Perrone et al. 1980), the thyroid hormone receptor and the retinoic acid receptors (Morita et al. 1990). During our experiments on the role that the cellular bioenergetics status plays in the response of these cells to both the antioestrogen and to the glucocorticoid, Dex.

### Materials and Methods

#### Reagents

All reagents for cell culture were obtained from Life Technologies (Paisley, Strathclyde, UK). The cell death ELISA was obtained from Roche Diagnostics Ltd (Lewes, E Sussex, UK). Fluorescent probes for flow cytometry were obtained from Molecular Probes (Cambridge Bioscience, Cambridge, Cambs, UK). Other flow cytometry reagents were obtained from Beckton Dickinson UK Ltd, Oxford, UK. All other reagents were obtained from Sigma–Aldrich Co. Ltd (Poole, Dorset, UK). The pure antioestrogen was obtained as a gift from Zeneca Pharmaceuticals Ltd (Macclesfield, Cheshire, UK).

#### Cell culture

Pituitary GH3 cells as previously described (Newton et al. 1994b) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with phenol red, penicillin (50 U/ml) streptomycin (50 µg/ml), amphotericin (2 µg/ml) and 10% foetal calf serum (Myoclone; Gibco, Paisley, Strathclyde, UK). For the majority of studies reported here, experiments were conducted on cells seeded onto 48-well culture plates at densities over the range 4000 to 10 000 cells per cm2. Cells were kept at 37 °C in an atmosphere of 5% CO2 in air. For flow cytometry and enzyme measurements, cells were harvested from 75 cm2 culture flasks by the addition of trypsin/EDTA and resuspended in culture medium. For flow cytometry, cells were treated in suspension at 37 °C in a gas atmosphere of 5% CO2 in air.

#### Transfection

Plasmids for transfection were the MMTV-Luc and ΔMTV-Luc as previously described (Newton et al. 1994c). After seeding and cell attachment, media were changed to phenol red-free DMEM containing 10% charcoal-stripped serum and 24 h later transfections were made with 1 µg plasmid DNA and the reagent FUGENE, according to the manufacturer’s instructions (Roche Diagnostics Ltd). After a further 24 h, luciferase activity was measured in the cell lysates using the Promega luciferase assay system (Madison, WI, USA). All values obtained as relative light units, measured with a luminometer, were expressed as fold control (no treatment).

#### Determination of cell numbers and cell death

We have previously shown that viable cell numbers can be quantitated by the addition of the diazo dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazium bromide (MTT) to cells for 4 h to give final concentration of 0·1 mg/ml (Newton 1995). The blue crystals formed were solubilised by the addition of sodium dodecylsulphate in HCl to give final concentrations of 10% and 10 mM respectively. Absorbance values were determined at 550 nm. For the experiment shown in Fig. 3, a direct estimate of cell numbers was made using a Coulter Counter (Beckman Coulter Ltd, High Wycombe, UK). Prior to assay, cell monolayers were washed twice with phosphate-buffered saline (PBS) to remove poorly attaching, dead cells.

As described previously (Newton et al. 1999), a quantitative measure of cell death was made by determining DNA fragmentation using the ELISAPLUS assay (Roche Diagnostics Ltd).

#### Fluorescence microscopy

Normal light (Image Modulated Contrast) and fluorescence microscopy were performed with a Leica DMIL.
fitted with a SPOT JUNIOR digital camera (Diagnostic Instruments, Oxnard, CA, USA). Photo-quality pictures were produced with a Hewlett Packard 970 Cxi. To determine the integrity of cell membranes, a mixture of propidium iodide (PI) and Hoechst 33342 was added to the cell culture medium to give final concentrations of 10 µg/ml and 1 µg/ml respectively. After 5 min at 37 °C, cells were visualised following exposure to normal and UV light (380 nm). Cells with disrupted membranes preferentially gave red nuclear fluorescence due to the uptake of PI. Cells with intact membranes gave blue nuclear fluorescence, due to the uptake of the cell-permeable Hoechst 33342 fluorochrome.

Flow cytometry for changes in mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was determined by a previously published method (Decaudin et al. 1997). Control and steroid pretreated (24 h) cells were harvested from 75 cm² dishes, sonicated and then centrifuged at 1500 g. The supernatant was then diluted with a further 1 ml distilled water and an aliquot of the crude cytosol containing supernatant was then centrifuged at 1500 g and an aliquot of the crude cytosol containing supernatant was taken for spectrophotometric analysis of glutathione. All values given in the figures are expressed as means ± s.d. For clarity, error bars have been omitted from some figures.

Results

Functional activation of steroid receptors and effect of ligands on ZM 182780-induced cell death

To determine the functional activity of the steroid receptors present in GH3 cells, transfection was performed with the MMTV–ERE–Luc and ΔMTV–ERE–Luc reporter plasmids (Newton et al. 1994b) followed by a 24-h treatment period with the progestin, promogestone (R5020, 10 nM), the androgen, dihydrotestosterone (DHT, 10 nM), the glucocorticoid, Dex (100 nM) and the oestrogen, oestradiol (E2, 1 nM). To ensure the removal of endogenous steroids, cells were maintained prior to treatment in phenol red-free medium containing charcoal-stripped serum. Figure 1 shows that all steroids induced reporter gene expression and that the respective receptor antagonists, ZK 98299 (progesterone receptor (PR), 10 µM), RU 486 (glucocorticoid receptor (GR), 10 µM), hydroxyflutamide (androgen receptor (AR), 10 µM) and ZM 182780 (ER, 100 nM) blocked transcription of the reporter genes. It should be noted that for transfections performed with the ΔMTV–ERE–Luc plasmid, the anti-oestrogen reduced luciferase expression below that of the oestrogen-free control. This is consistent with earlier studies showing that the ER within these cells is transcriptionally active in the absence of oestrogen (Newton et al. 1994).

To determine whether the ligand-activated receptors altered the response of cells to the anti-oestrogen, cultures
were maintained in the presence of 100 nM ZM 182780 and a dose range of Dex, R5020, DHT, oestrone (E1) and E2. Figure 2 demonstrates the response of GH3 cells measured as metabolic activity by the addition of the redox sensitive diazo dye, MTT. In comparison to E1 and E2, where metabolic activity was restored to that of cells untreated with antioestrogen, only Dex was observed to partially reverse the antioestrogen-induced decrease of metabolic activity. For estimates of the proportion of viable cells, determined by the addition of the fluorochromes, Hoechst 33342 and PI, Fig. 2b shows that Dex completely blocked the antioestrogen-induced loss of cell viability. It should be mentioned that unlike E2, E1 was able to maintain cell viability at concentrations 100-fold lower than for E2. As E1 has a lower affinity for the ER than E2, this suggests that either the effect of E2 is mediated via an ER isoform to which the antioestrogen fails to bind, or that the effect of E1 is mediated via an ER-independent pathway.

To provide confirmation of the effect of Dex, a further experiment was performed over a time-course of 9 days, where cell numbers were measured using a Coulter Counter. Figure 3 shows that in the presence of ZM 182780 alone, cell numbers fell by day 5, but growth continued at a low rate in the combined presence of 100 nM ZM 182780 and 100 nM Dex.

**Antioestrogen- and exogenous oxidant-induced cell death is blocked by Dex**

We have previously shown that death induced by ZM 182780 results in extensive DNA fragmentation (Newton et al. 1999). Using the same ELISA method, we determined the effect of Dex on the time-course of antioestrogen-induced DNA fragmentation. Figure 4a shows that over a time-course of 7 days, DNA fragmentation began to rise from day 4 and this was completely blocked by 100 nM Dex. Given that we have previously shown that oxidants are involved in antioestrogen-induced death of GH3 cells, we next tested the response of cells to the pro-oxidant, H2O2. Previously untreated control cells and cells exposed to 100 nM ZM 182780 for 48 h were treated with a dose range of H2O2 for 2 h. At concentrations of H2O2 around 100–200 µM, DNA fragmentation was markedly enhanced for antioestrogen-exposed cells (Fig. 4b). As indicated previously (Newton...
et al. 1999), over a time-period of 1–2 h, GH3 cells paradoxically fail to show DNA fragmentation at the higher pro-oxidant doses. Figure 4b also shows that pretreatment of cells with 100 nM Dex in the absence or presence of ZM 182780 markedly blocked DNA fragmentation induced by the low doses of H2O2.

Characteristics of oxidant-induced death

As loss of $\Delta \Psi_m$ is one of the earliest events in apoptosis, the effect of H2O2 in combination with ZM 182780 alone, with Dex and with the antiglucocorticoid, RU 486 was determined as a function of time after treatment. Cells pretreated for 48 h with ZM 182780 (100 nM) alone or in combination with Dex (100 nM) and RU 486 (10 µM) were exposed to 150 µM H2O2 for various times over a 4- to 5-h period, prior to the addition of a mixture of the $\Delta \Psi_m$-sensitive fluorochrome, DiOC6, and the marker for membrane integrity, PI, as described in Materials and Methods. For all experiments conducted ($n=3$), it was observed that after 90-min exposure to H2O2 the $\Delta \Psi_m$ decreased for cells pre-exposed to ZM 182780 and this was paralleled by the uptake of PI. Figure 5 shows the results from one experiment typical of all three. It is clear that in the presence of ZM 182780, a second lower intensity peak was observed for DiOC6 and this correlated with a large uptake of PI as indicated by the adjacent profiles. Both the lower intensity fluorescence peak, corresponding to cells with a low $\Delta \Psi_m$, and the high intensity red fluorescence peak, indicating PI uptake, were markedly attenuated by Dex; a response reversed by RU 486. It should be noted that, in this experiment, no marked effect on $\Delta \Psi_m$ and PI uptake could be observed when H2O2 was added alone to cells (control). Although only the 90-min exposure time is shown in Fig. 5, for all experiments conducted, Dex was still able to significantly reduce H2O2-induced PI uptake after exposure to H2O2 for 4 h (ZM 182780+H2O2 vs ZM 182780+Dex+H2O2; 67 ± 12% vs 26 ± 8%, $P<0.01$; paired t-test at 90 min, 1, 2, 3 and 4 h for data from three experiments).

For all three experiments, the percentage of the cell population with a low $\Delta \Psi_m$ was correlated with the proportion of the cell population showing PI uptake, for the 90 min, 2, 3 and 4 h sampling times (Fig. 6).

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analysis reveals a correlation coefficient of 0.914 and a slope of 1.07. This analysis shows that at no time-point following H$_2$O$_2$ exposure, could the loss of the $\Delta \Psi_m$ be disassociated from the uptake of PI.

A further possibility that might explain the above findings is that membrane integrity is lost following a catastrophic loss of mitochondrial function. To address this, GH$_3$ cells were stained with the mitochondrial
Mechanism for Dex blockade of antioestrogen- and oxidant-induced death

Cellular ATP concentrations in response to H₂O₂

Cell death involving a loss of membrane integrity is often associated with insufficient cellular ATP to maintain plasma membrane Na⁺/K⁺-ATPase activity. Experiments were conducted to determine the ATP status of cells in relation to the time at which PI uptake occurred following challenge with H₂O₂. Figure 8a shows that after 30-min exposure of cells to 1 mM H₂O₂, ATP concentrations had fallen to 10% of control values. At this time-point, Fig. 8b shows that PI uptake had not occurred. After a further 30 min, Fig. 8a shows that at the low pro-oxidant concentration of 150 µM, ATP concentrations continued to fall and that this was followed by the uptake of PI, indicating a loss of cell membrane integrity. That ATP levels had fallen so dramatically at the high H₂O₂ doses and yet cell death (loss of membrane integrity) failed to occur for several hours, suggested that it was not the absolute cellular ATP concentration that was the factor determining cell survival.

Effect of inhibiting glycolysis and mitochondrial function on response to H₂O₂

A further finding was that at the low oxidant doses cells displayed marked membrane blebbing. Figure 9 shows that in comparison with cells treated with a low dose of H₂O₂ (Fig. 9b), for high pro-oxidant doses (Fig. 9c), or where cells were pretreated with ZM 182780 (Fig. 9d), membrane blebbing failed to occur. Membrane blebbing is a process of cytoskeletal arrangement and it can precede apoptosis and necrosis (Schwartzman & Cidlowski 1993). Studies have indicated that glycolytic enzymes are associated with actin and also tubulin cytoskeletal filaments and that glycolysis provides the ATP necessary for the cytoskeletal changes involved in membrane blebbing (Glass-Marmor & Beitner 1997).

To determine the relative role of glycolytic and mitochondrial ATP generation in the response to H₂O₂, cells were incubated in medium lacking glucose or with the protonophore, carbonyl cyanide m-chlorophenylhydrazone (mClCCP) (100 µM; Sigma) and the succinate dehydrogenase inhibitor, 3-nitropropionic acid (3NP; 2 mM). As indicated in Fig. 7b the protonophore collapses the ΔΨₘ whilst 3NP will reduce the delivery of electrons to the electron transport chain and lower the ΔΨₘ (Keller et al. 1998). Figure 10 shows that the removal of glucose markedly enhanced cell death induced by H₂O₂. Surprisingly, both mClCCP and 3NP gave some degree of protection against the oxidant-induced cell death.

Role of GADPH in the response of cells to antioestrogen and Dex

As the experiments above strongly pointed to a fundamental role of glycolysis in the response of these cells to oxidants, experiments were performed to determine the extent to which ZM 182780 and Dex exposure altered GAPDH activity, a rate-limiting enzyme of glycolysis (Sahlin 1995). For cells treated with ZM 182780 and Dex for 48 h, enzyme activity was measured in cell lysates. Table 1 shows that ZM 182780 and Dex treatment alone had no effect on enzyme activity. This experiment was then repeated where cells exposed to ZM 182780 and Dex for 48 h were exposed to 150 µM H₂O₂.
Figure 7  Fluorescence microscopy for ΔΨₘ with JC-1 (a, b, c, e, g), and for membrane integrity with Hoechst 33342 and PI (d, f, h). In comparison with cells treated with JC-1 alone (a) where red ‘J’ aggregates are present (high ΔΨₘ) (b) shows only green fluorescence (low ΔΨₘ) in the presence of the protonophore, mClCCP (100 µM). In a separate experiment, (c–h) show the response to treatment with H₂O₂ for 90 min. Control cells with JC-1 and Hoechst 33342 are shown in (c) and (d) respectively, whilst (e) and (f) show the effect of low concentrations of H₂O₂ (150 µM) with JC-1 and Hoechst 33342 respectively. (The same field for JC-1 and Hoechst 33342 could not be used as JC-1 and Hoechst 33342 gave mutual quenching.) Finally (g) and (h) show the response to the high concentration of H₂O₂ (1 mM) with JC-1 and Hoechst 33342 respectively.
30 min prior to harvesting for enzyme measurement. As observed in the first experiment, antioestrogen or Dex treatment alone failed to provoke a response but, this time, a fall in enzyme activity occurred for cells exposed to H$_2$O$_2$ for 30 min. This decrease of enzyme activity was substantially enhanced in the presence of ZM 182780 and this enhancement was reversed by the inclusion of Dex in the culture medium.

Figure 8 ATP concentrations (a) and cell membrane integrity (b) measured at various times after the addition of H$_2$O$_2$ (150 µM). Values are expressed as per cent control.

Figure 9 Image Modulated Contrast light microscopy images of GH$_3$ cells showing membrane blebbing on exposure to 150 µM H$_2$O$_2$ for 45 min (b), in comparison with lack of blebbing for cells treated with 1 mM H$_2$O$_2$ (c) or for cells treated with 150 µM H$_2$O$_2$, following pre-exposure to ZM 182780 (d). Control, untreated cells are shown in (a).
Discussion

The expansion of a cell mass is dependent on stimuli that activate cell cycle events and those that allow the survival of cell progeny. With regard to the growth and development of rat pituitary tumours, oestrogens are the endocrine agents that would appear to fulfill both these requirements. Therefore, the rat pituitary tumour cells used here are not only dependent on the ER for growth but, in the presence of the pure antioestrogens, ZM 182780 and RU 58668 (Van de Velde et al. 1994), they are killed. During studies to determine the role of other steroid-activated pathways in this response, we have observed that death induced by antioestrogens was blocked by the glucocorticoid, Dex.

These observations contribute to the growing controversy concerning the role of glucocorticoids in cell survival.

A classical model of apoptosis is the glucocorticoid-induced death of thymocytes (Schwartzman & Cidlowski 1994). Further support for a pro-death role of glucocorticoids comes from the studies on Alzheimer’s disease. Alzheimer’s disease is associated with aging and hippocampal cell death and there is an age-related increase in the activity of the hypothalamus–pituitary axis (Behl et al. 1997). These observations have prompted studies showing that Dex enhances oxidant-induced hippocampal cell death (Behl et al. 1997, Reagan & McEwen 1997). In addition to this, studies have demonstrated that glucocorticoids enhance neuronal ischaemic necrosis (Sapolsky & Pulsinelli 1995). Although necrosis can be due to physical cell injury, in the majority of cases it is due to severely compromised cell energetics (Eguchi et al. 1997, 1999). During ischaemic injury, mitochondrial function is restricted by low O\textsubscript{2} concentrations, rendering cells dependent on glycolytic function (Maxwell & Lip 1997). Glucocorticoid-enhanced necrosis under these circumstances may be due to the observed down-regulation of glucose uptake by these hormones (Rist & Naftalin 1991).

Contrary to these reports, it has been demonstrated that Dex prevents cell death in a neonatal rat model of hypoxic–ischaemic encephalopathy (Ekert et al. 1997). Here it was reported that cell death was by apoptosis. Similarly, there are other reports to indicate that liver cell, neutrophil, pancreatic acinar cell and breast cell apoptosis is blocked by glucocorticoids (Feng et al. 1995, Harris et al. 1995, Liles et al. 1995, Iida et al. 1998). From these conflicting studies it would appear that the effect of glucocorticoid exposure is very much cell-type dependent.

Our earlier reports on these pituitary tumour cells maintained that cell death in response to ZM 182780 and to exogenous oxidants had more of the characteristic of apoptosis than necrosis (Newton 1995, Newton et al. 1999). Having performed more extensive studies on these cells, we now believe that cell death induced by antioestrogens and oxidants is more characteristic of necrosis. The confusion with regard to assigning the correct mode of cell death has arisen for several reasons. The first of these is that antioestrogens cause considerable cell shrinkage prior to rapid death – this is a characteristic of apoptosis in a number of systems (Schwartzman & Cidlowski 1993). We have now performed experiments showing that a reduction in cell volume in response to ZM 182780 is not always followed by cell death (data not shown). Secondly, using an ELISA assay that fails to detect necrosis in endothelial cells (Newton et al. 1999), marked DNA fragmentation was detected in response to antioestrogen and oxidants. The third factor that has given rise to this confusion is the unusual dose-response to H\textsubscript{2}O\textsubscript{2}. In
the majority of cell systems, low oxidant doses induce apoptosis and high doses induce necrosis. For GH3 cells, we have observed that low doses of H2O2 induce rapid death whilst death at higher doses is delayed for several hours.

A re-evaluation of the characteristics of oxidant-induced cell death, as presented here, has provided the findings that the loss of ΔΨm, a characteristic response of early apoptosis in a number of systems, cannot be dissociated from a loss of cell membrane integrity (see Figs 5 and 6). In practical terms, at every time-point of ΔΨm measurement with the cationic fluorochrome, DiOC6, PI uptake correlates with the fall in ΔΨm. This strongly suggests that a catastrophic loss of membrane function has occurred resulting in the loss of mitochondrial function. Data presented in Fig. 7 showing that at high H2O2 doses, cell membrane integrity is intact when the ΔΨm has collapsed indicate that cell membrane function is not lost as a secondary response to a collapse of mitochondrial function and ATP generation. Indeed data presented in Fig. 8 indicate that the absolute level of ATP, measured in the whole population of cells under treatment, does not determine the viability of the cells. Therefore, high doses of H2O2 induce a large fall (95%) in ATP concentrations and yet cell membrane integrity is maintained for several hours. In contrast, at the lower doses of H2O2, loss of cell membrane function is apparent when ATP concentrations have only fallen to around 30–40% of control values. Our observations of a rapid uptake of PI indicate a catastrophic loss of membrane function for a proportion of the cell population. One of the possibilities we have considered is that such an effect was due to oxidation of membrane lipids. Although not shown, we have attempted to address this by determining glutathione (GSH) concentration with the fluorescent probe, Cell Tracker Green (Molecular Probes). In our earlier publication we have shown that GSH is a major antioxidant in these cells (Newton et al. 1999). In the presence of GSH and the enzyme glutathione-S-transferase, Cell Tracker Green is converted to a green fluorescent compound. Using this method, we have been unable to detect a loss of GSH in response to H2O2 until the time at which cell membrane rupture has occurred. For this reason, we believe that the loss of membrane integrity has, paradoxically, more to do with the energetic status of the cells than the antioxidant status. In particular, we propose that high oxidant doses induce a ‘shut-down’ of cellular activities, sparing the remaining ATP for the maintenance of cell membrane function. At the lower doses of H2O2, ATP-consuming cellular processes will not be as markedly inhibited and therefore the demand on the diminishing pool of ATP will be higher. As the cell energetics continue to fall in response to oxidative damage, a crisis point will be reached at which demand outstrips supply, leading to a rapid and catastrophic loss of plasma membrane Na+/K+-ATPase and membrane integrity. Our inability to measure this rapid fall in ATP concentrations on an individual cell basis before membrane integrity is lost is due to the current lack of suitable methodology.

With regard to the mechanism by which Dex blocks antioestrogen- and oxidant-induced cell death, we have had to first identify a mechanism by which oxidants induce cell death. Given our observations in Fig. 9 concerning membrane blebbing, in the light of studies indicating that glycolysis drives the cytoskeletal changes that result in blebbing, we have tested the relative role of glycolysis and mitochondria in the response to H2O2. Data presented in Fig. 10 have shown that removal of glucose markedly enhances oxidant-induced cell death whilst, paradoxically, lowering mitochondrial function is protective. Previous studies by Sahlin (1995) have demonstrated that a key enzyme of glycolysis, GAPDH, is highly regulated by oestrogens and by Dex. In addition, studies have shown that GAPDH is a major target for oxidative damage due to the presence of a critical thiol group (Baker et al. 1989). Our data presented in Table 1 show that whilst enzyme activity is not markedly altered in response to ZM 182780 and Dex alone, in the presence of H2O2, antioestrogen pretreatment leads to an enhanced reduction of GAPDH activity following H2O2 exposure, and this effect is blocked by Dex. These data strongly indicate that one of the mechanisms by which Dex is able to block antioestrogen-enhanced, oxidant-induced cell death is that the steroid stabilises glycolytic function. In contrast to these results on glycolytic function, the results presented in Fig. 10 have provided the intriguing possibility that mitochondrial function, in part, drives the death process. Here it was shown that the two agents that lower mitochondrial activity reduce death in the presence of the low doses of H2O2.

These data presented in the current report support the previous studies on GH3 cells that antioestrogens enhance sensitivity to the toxic effect of oxidants. We now show that this effect is blocked by the glucocorticoid, Dex, possibly due to the role that the Dex-activated GR has to stimulate glycolysis – a pathway that we show here to be fundamental in the response of these cells to oxidants. These finding therefore contribute to the growing body of data to suggest that, for certain cell types, glucocorticoids decrease susceptibility to toxic insults. Cellular energetics have been shown to be fundamental with regard to how a cell responds to a particular toxic agents. Apoptosis is an energetic process and stimuli that normally induce apoptosis have been shown to induce necrosis when cellular ATP levels are reduced (Eguchi et al. 1997, 1999). By blocking necrosis, one might have expected that Dex would allow apoptosis to occur. The failure to observed apoptosis is not due an inability of GH3 cells to undergo apoptosis, as we have recently observed this mode of death in response to the calmodulin inhibitor, calmidazolium chloride (Newton et al. 2000). Our most recent unpublished observations on these cells may provide the explanation for this. In response to the topoisomerase
inhibitor, camphothecin, GH3 cells undergo nuclear fragmentation characteristic of apoptosis and this is blocked by Dex. Therefore, our studies concur with those of others showing that glucocorticoids block both necrotic and apoptotic cell death. Although we have provided some evidence to support a mechanism by which necrosis is blocked, studies are required to determine at which step in the apoptotic pathway Dex is able to block apoptosis.

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