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Clotrimazole decreases glycolysis and the viability of lung carcinoma and colon adenocarcinoma cells

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Abstract

Glycolysis is known to be the primary energy source in most cancer cells. We investigated here the effect of clotrimazole (1-(α-2-chlorotrityl)imidazole), the antifungal azole derivative, which was recently recognized as calmodulin antagonist, on the levels of glucose 1,6-bisphosphate and fructose 1,6-bisphosphate, the two stimulatory signal molecules of glycolysis, and on ATP content and cell viability in LL/2 Lewis lung carcinoma cells and CT-26 colon adenocarcinoma cells. We found that clotrimazole induced a significant, dose- and time-dependent reduction in the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, ATP, and cell viability. These findings suggest that clotrimazole causes a reduction in glycolysis and ATP levels, which eventually leads to cell destruction after 3 h of treatment. Since cell proliferation was also reported to be inhibited by calmodulin antagonists, this substance is most promising agent in treatment of cancer by inhibiting both cell proliferation and the glycolytic supply of ATP required for cancer cell growth.

Keywords: Cancer; Calmodulin antagonist; Clotrimazole; Glycolysis; ATP

1. Introduction

Cancer cells are known to exhibit a high rate of glycolysis, even under aerobic conditions, which is their primary energy source (Beckner et al., 1990; Eigenbrodt et al., 1985; Fiechter and Gmunder, 1989; Greiner et al., 1994). Recent molecular studies have revealed that several of the multiple genetic alterations that cause tumor development directly affect glycolysis (Dang and Semenza, 1999). One of the important mechanisms which controls glycolysis is through allosteric regulators, such as glucose 1,6-bisphosphate and fructose 1,6-bisphosphate (for reviews, see (Beitner, 1979, 1984, 1985, 1990, 1993, 1998; Hue and Bartons, 1985; Lowry, 1966).

Previous studies from our laboratory have revealed that the growth-promoting hormones, insulin and growth factors, stimulate glycolysis by increasing the binding of glycolytic enzymes to cytoskeleton (a rapid and transient effect) and by raising the level of glucose 1,6-bisphosphate (a slower and consistent effect). This compound acts as an intracellular signal which, through its allosteric effects on the key enzymes of carbohydrate metabolism, regulates the different pathways of glucose metabolism under different conditions (for reviews, see Beitner, 1979, 1984, 1985, 1990, 1993, 1998). Glc-1,6-P_2 is a potent activator of cytosolic phosphofructokinase (PFK) (Beitner et al., 1978; Beitner, 1985, 1990), the rate-limiting enzyme of glycolysis (Beitner, 1979, 1990, 1993; Chen-Zion et al., 1992; Livnat et al., 1995), and, thereby, regulates ATP production by cytosolic glycolysis. The involvement of glucose 1,6-bisphosphate in the mechanism of insulin action was also found in other laboratories (Bauer et al., 1986; Carreras et al., 1988). Our experiments have also revealed that all these stimulatory effects of insulin and growth factors on glucose 1,6-bisphosphate levels and glycolysis could be prevented by treatment with calmodulin antagonists (Bassukevitz et al., 1992; Beitner, 1993; Chen-Zion et al., 1992; Glass-Marmor et al., 1999; Livnat et al., 1995). These results strongly suggest that Ca^{2+}/calmodulin is involved in the stimulatory action of insulin and growth factors on glycolysis, which supplies energy for cell growth. Calmodulin is a multifunctional Ca^{2+} binding protein that has been implicated in the regulation of numerous cellular events, including that of normal and abnormal cell growth and proliferation (Hait and Lazo, 1986; Rasmussen and Means, 1987; Reddy, 1994; Veigl et al., 1984). It was

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reported that the calmodulin level was higher in cancer cells than in normal cells and there was a positive correlation between the growth rate and calmodulin level of cancer cells (Liu et al., 1996). Calmodulin antagonists were reported to inhibit cellular proliferation of various cells (Al-Ani et al., 1988; Hait et al., 1994; Ito and Hidaka, 1983; Mac Neil et al., 1984, 1993; Susuki et al., 1986).

Based on all these findings we reasoned that calmodulin antagonists may reduce glycolysis in cancer cells, thereby decreasing the ATP supply required for the cancer cells proliferation.

In the present research, we investigated the effects of clotrimazole (1-(α-2-chlorotrityl)imidazole), the antifungal azole derivative, that was recently recognized as calmodulin antagonist (Hegemann et al., 1993; Mac Neil et al., 1993), on the levels of the two signal molecules of glycolysis, glucose 1,6-bisphosphate and fructose 1,6-bisphosphate, in LL/2 Lewis lung carcinoma cells and CT-26 colon adenocarcinoma cells. Fructose 1,6-bisphosphate is the product of phosphofructokinase reaction, and its levels are elevated in cancer cells (Eigenbrodt et al., 1985). Fructose 1,6-bisphosphate is also, similarly to glucose 1,6-bisphosphate, an allosteric activator of phosphofructokinase and pyruvate kinase. We also studied here the effects of clotrimazole on the concentration of ATP and on the viability of LL/2 Lewis lung carcinoma cells and CT-26 colon adenocarcinoma cells. In the present study, we investigated the subcellular sites damage during clotrimazole treatment, using electron microscopic technique. We also studied here the effects of clotrimazole on the ionic cell content, using X-ray microanalysis approach.

2. Materials and methods

2.1. Materials

Clotrimazole was purchased from Sigma (St. Louis, MO, USA). Other chemicals and enzymes were either from Sigma or from Boehringer Mannheim (Mannheim, Germany). Tissue culture reagents were purchased from Biological Industries (Beit Haemek, Israel).

2.2. Cell culture

CT-26 murine colon adenocarcinoma cells were grown in tissue culture flasks in RPMI-1640 medium (Roswell Park Memorial Institute medium) supplemented with 10% fetal calf serum and antibiotics, at 37 °C in humidified atmosphere at 5% CO_2 and 95% air. Cells were passaged two times weekly.

LL/2 Lewis lung carcinoma cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and antibiotics, at 37 °C in humidified atmosphere at 5% CO_2 and 95% air. Cells were passaged three times weekly.

2.3. Treatment of culture

CT-26 murine colon adenocarcinoma cells and LL/2 Lewis lung carcinoma cells (8×10^5 cell ml^{-1}) were seeded in tissue culture plates (10 cm). After 48 h, cells were washed twice with phosphate buffer saline (PBS). Then the cells were incubated at 37 °C in PBS containing 5 mM glucose in the absence and presence of clotrimazole for different times and concentrations. Clotrimazole was dissolved in water containing acidified 3% polyethylene glycol 400. The solvent was added to the controls.

2.4. Extraction of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP

The cells were washed twice with ice-cold PBS, placed on ice and 1.5 ml HCl 0.05 N was added to the dish. Ten minutes later, the cells were scraped with a rubber policeman and collected in centrifuge tube at 0 °C. Samples (10 μl) were removed for protein determination. Cells were mixed with 120 μl of 70% cold perchloric acid containing 130 mM EDTA. After 5 min, the extract was neutralized with KOH. The precipitated potassium perchlorate was removed by centrifugation at 4 °C for 15 min at $5000\times g$, and the clear supernatant was used for determination of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP.

2.5. Measurements of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP levels

Glucose 1,6-bisphosphate was measured by the fluorometric method of Passonneau et al. (1969); glucose 1,6-bisphosphate, being a cofactor of phosphoglucomutase reaction, was coupled with glucose-6-phosphate dehydrogenase and $NADP^+$. The buffer consisted of 50 mM imidazole–HCl, pH 7, containing 1 mM magnesium acetate, 0.1 mM EDTA, and 0.01% bovine serum albumin. First phosphoglucomutase (4 U/ml) was activated in the buffer for 1 h. The reaction mixture contained: 15 μM glucose-1-phosphate, 2 mU/ml of activated phosphoglucomutase, 0.05 mM $NADP^+$ and 1 U/ml glucose-6-phosphate dehydrogenase in 1 ml buffer. The reaction was started by adding 2–50 μl from the extract. Calculation was made from a standard of the glucose 1,6-bisphosphate between 1×10^{-8} and 5×10^{-9} M.

Fructose 1,6-bisphosphate and ATP were measured by the method of Lowry et al. (1964).

2.6. Cell viability determination

After incubation in absence and presence of calmodulin antagonists, the cells were harvested with trypsin (0.25%)–EDTA (0.05%) and centrifuged for 10 min at $270\times g$. The precipitated cells were suspended in PBS and counted in a hemozitometer (Neubauer). Plasma membrane permeability

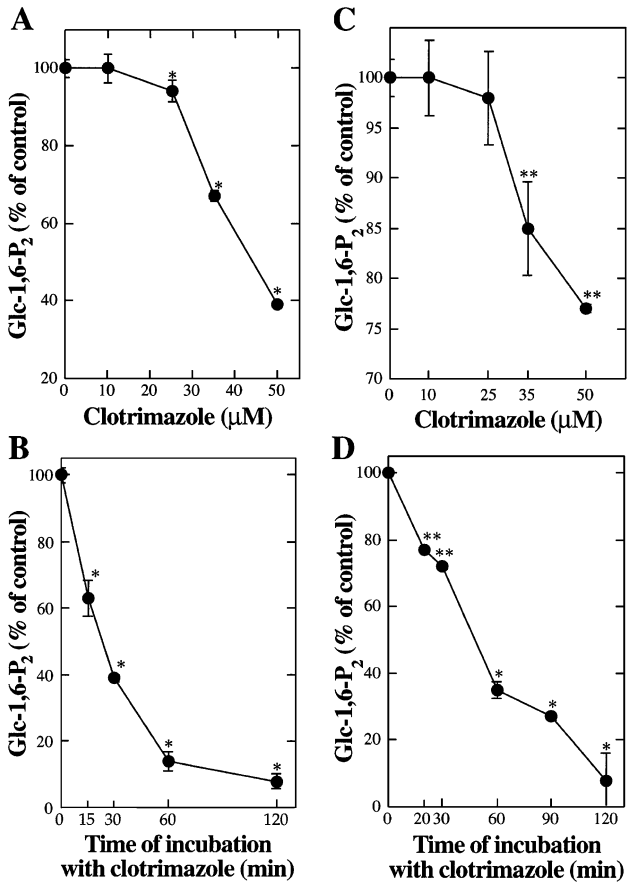

Fig. 1. Dose–response and time–response curves of the effects of the clotrimazole on glucose 1,6-bisphosphate (Glc-1,6-P_2) levels in CT-26 colon adenocarcinoma (A,B) and LL/2 Lewis lung carcinoma cells (C,D). (A) Cells were incubated for 30 min in the absence and presence of different concentrations of clotrimazole; (B) cells were incubated for different time in the absence and presence of 50 μM clotrimazole. 100% glucose 1,6-bisphosphate refers to 6.35±0.16 (nmol/mg protein); (C) cells were incubated for 20 min in the absence and presence of different concentrations of clotrimazole; (D) cells were incubated for different time in the absence and presence of 50 μM clotrimazole. 100% glucose 1,6-bisphosphate refers to 0.55±0.01 (nmol/mg protein). Each point is the mean±S.E. of 2–3 separate experiments which were performed in triplicate. *P<0.005, **P<0.05.

as a measure of cell death was determined by trypan blue dye exclusion.

2.7. Protein measurement

Protein was measured by the method of Bradford (1976) with crystalline bovine serum albumin as a standard.

2.8. Transmission electron microscopy (TEM)

Immediately after the clotrimazole treatment, clotrimazole-treated and untreated control cultures were fixed in 2.5% glutaraldehyde/paraformaldehyde in phosphate buffer solution pH 7.4 at room temperature for 1 h. The cells were removed with a rubber policeman, post-fixed in 1% osmium tetroxide (OsO_4) and uranyl acetate. The cells were dehydrated with ethanol and embedded in Epon 812. Thin sections were prepared using an LKB Ultratome III (Stockholm, Sweden), stained with uranyl acetate followed by lead citrate. The EM "staining" is the result of heavy metal (Os, Pb, U) impregnation of the specimen macromolecules. The cells were examined by a Jeol 1200EX (Tokyo, Japan) transmission electron microscope.

2.9. Determination of ionic cell content by X-ray microanalysis

CT-26 murine colon adenocarcinoma cells and LL/2 Lewis lung carcinoma cells were grown on Thermanox

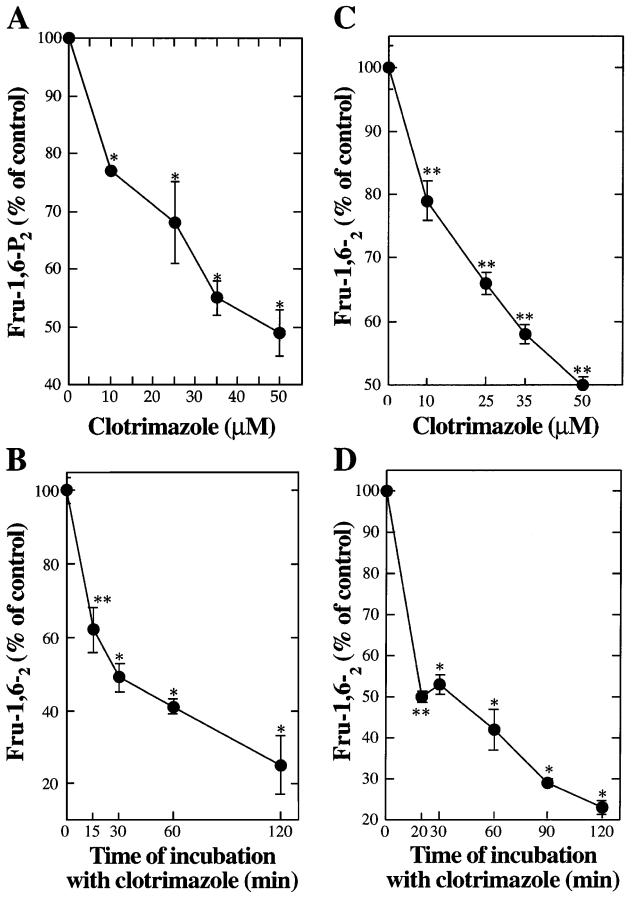

Fig. 2. Dose–response and time–response curves of the effects of the clotrimazole on fructose 1,6-bisphosphate (Fru-1,6-P_2) levels in CT-26 colon adenocarcinoma (A,B) and LL/2 Lewis lung carcinoma cells (C,D). (A) Cells were incubated for 30 min in absence and presence of different concentrations of clotrimazole; (B) cells were incubated for different time in the absence and presence of 50 μM clotrimazole. 100% fructose 1,6-bisphosphate refers to 8.24±0.12 (nmol/mg protein); (C) cells were incubated for 20 min in the absence and presence of different concentrations of clotrimazole; (D) cells were incubated for different time in the absence and presence of 50 μM clotrimazole. 100% fructose 1,6-bisphosphate refers to 5.31±0.19 (nmol/mg protein). Each point is the mean±S.E. of 2–3 separate experiments which were performed in triplicate. *P<0.005, **P<0.05.

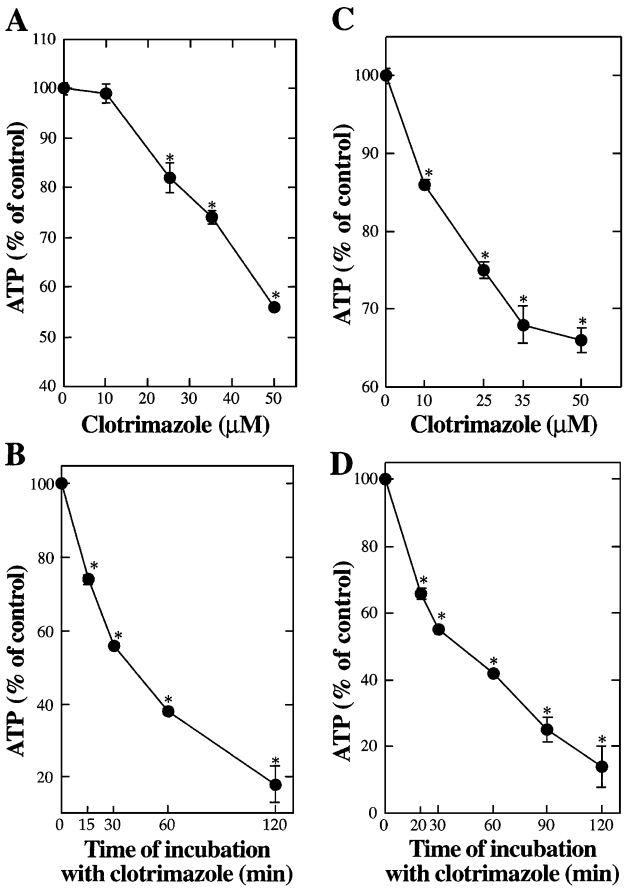

Fig. 3. Dose–response and time–response curves of the effects of the clotrimazole on ATP levels in CT-26 colon adenocarcinoma (A,B) and LL/2 Lewis lung carcinoma cells (C,D). (A) Cells were incubated for 30 min in the absence and presence of different concentrations of clotrimazole; (B) cells were incubated for different time in the absence and presence of 50 μM clotrimazole. 100% ATP refers to 30.46±0.21 (nmol/mg protein); (C) cells were incubated for 20 min in the absence and presence of different concentrations of clotrimazole; (D) cells were incubated for different time in the absence and presence of 50 μM clotrimazole. 100% ATP refers to 34.54±0.19 (nmol/mg protein). Each point is the mean±S.E. of 2–3 separate experiments which were performed in triplicate. *P<0.005.

plastic coverslips (Sigma). After incubation with and without clotrimazole, coverslips were rinsed in 0.15 M ammonium acetate solution at pH 7. Thereafter, the Thermanox-attached cells were rapidly frozen with liquid nitrogen, freeze-dried at −80 °C and then coated with a layer of carbon (Schoenfeld et al., 1994). X-ray microanalysis was performed on 10 cells from each sample, using an eXL Link system attached to a scanning electron microscope Jeol 840. In this method, the specimen is bombarded by an electron beam, and the excited elements emit X-ray fluorescence with specific energy.

3. Results

The results presented in Fig. 1 show that clotrimazole induced a concentration and time-dependent decrease in the levels of glucose 1,6-bisphosphate levels in CT-26 murine colon adenocarcinoma cells (Fig. 1A,B) and LL/2 Lewis lung carcinoma cells (Fig. 1C,D). Fig. 2A–D shows that clotrimazole also exerted a dose and time-dependent reduction in the levels of fructose 1,6-bisphosphate in both cell types. The results presented in Fig. 3 show the effects of the clotrimazole on ATP levels in colon adenocarcinoma cells (Fig. 3A,B) and lung carcinoma cells (Fig. 3C,D). It can be seen that clotrimazole markedly reduced ATP levels. The results presented in Fig. 4 show that clotrimazole induced a time-dependent reduction in the viability of CT-26 colon adenocarcinoma cells (Fig. 4A) and LL/2 Lewis lung carcinoma cells (Fig. 4B), which resulted in marked cell detachment from culture plates. Total cells death of CT-26 colon adenocarcinoma (Fig. 4A) and LL/2 Lewis lung

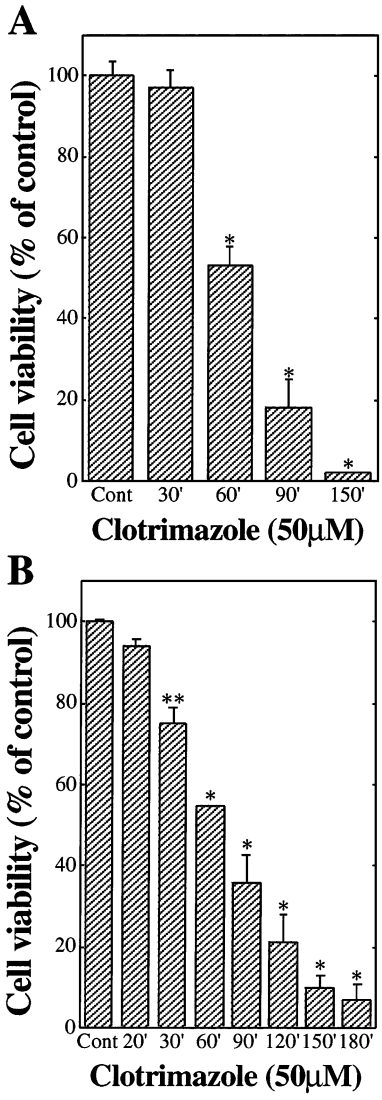

Fig. 4. Effects of clotrimazole on cell viability of CT-26 colon adenocarcinoma cells (A) and LL/2 Lewis lung carcinoma cells (B). Cells were incubated for different time in absence and presence of 50 μM clotrimazole. 100% cell viability refers to 5×10^6 cells ml^{-1}. Each point is the mean±S.E. of 2–3 separate experiments which were performed in triplicate. *P<0.005, **P<0.05.

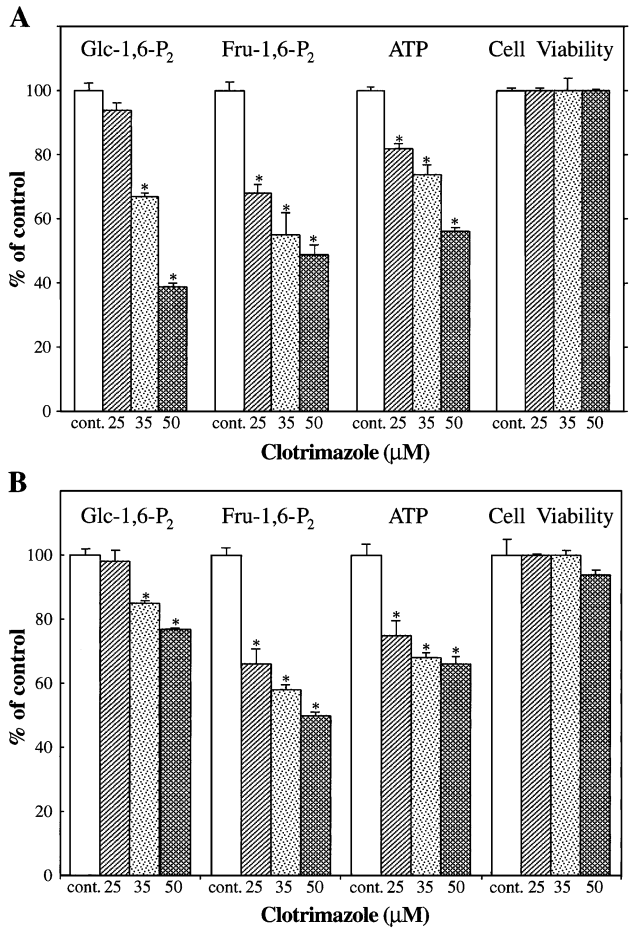

Fig. 5. Effect of clotrimazole on cell viability and its relation to the levels of ATP, glucose 1,6-bisphosphate (Glc-1,6-P_2) and fructose 1,6-bisphosphate (Fru-1,6-P_2). (A) CT-26 colon adenocarcinoma cells were incubated for 30 min in the absence and presence of different concentrations of clotrimazole. 100% ATP, glucose 1,6-bisphosphate and fructose 1,6-bisphosphate levels refer to 30.46±0.21, 6.35±0.16, and 8.24±0.12 nmol/mg protein, respectively. (B) LL/2 Lewis lung carcinoma cells were incubated for 20 min in absence and presence of different concentrations of clotrimazole. 100% ATP, glucose 1,6-bisphosphate and fructose 1,6-bisphosphate levels refer to 34.54±0.19, 0.55±0.01, and 5.31±0.19 nmol/mg protein, respectively. 100% cell viability refers to 5×10^6 cell ml^{-1}. Values are the mean±S.E.M. of 2–3 separate experiments, which were performed in triplicate. *P<0.005.

carcinoma cells (Fig. 4B) occurred after about 3 h of incubation with 50 μM clotrimazole. The results in Fig. 5 clearly show that the decrease in the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP, induced by clotrimazole, preceded the decrease in cell viability of CT-26 colon adenocarcinoma (Fig. 5A) and LL/2 Lewis lung carcinoma cells (Fig. 5B) and was not due to the cytotoxic effects of the drug, since their concentration was already significantly reduced by clotrimazole before any cytotoxic effect was observed, in 100% viable cells. Afterwards, there was a close correlation between the decrease in ATP levels and the reduction in cell viability (Figs. 3 and 4).

The activity of the Na^+/K^+-ATPase pump is one of the major cellular functions dependent on ATP cellular level. Thus, a rapid reduction in cellular content of ATP after clotrimazole treatment was expected to reduce Na^+/K^+-ATPase activity. Indeed, the cytosolic ion concentrations were altered as we can see from X-ray spectra from control and clotrimazole-treated cells (Fig. 6). The spectrum from control CT-26 colon adenocarcinoma cells (Fig. 6A,B) shows small peaks for Na and Cl. In contrast, the spectrum from clotrimazole-treated cells shows high Na and Cl peaks after 30 min of incubation, which should be read as the alterations, which preceded the decrease in cell viability. More prolonged clotrimazole-treatment (Fig. 6B) revealed an increase in the cellular level of Na^+, Cl^- and Ca^{2+}, while the K^+ level decreased, compared with controls. As regards LL/2 Lewis lung carcinoma cells (Fig. 6C), incubation with

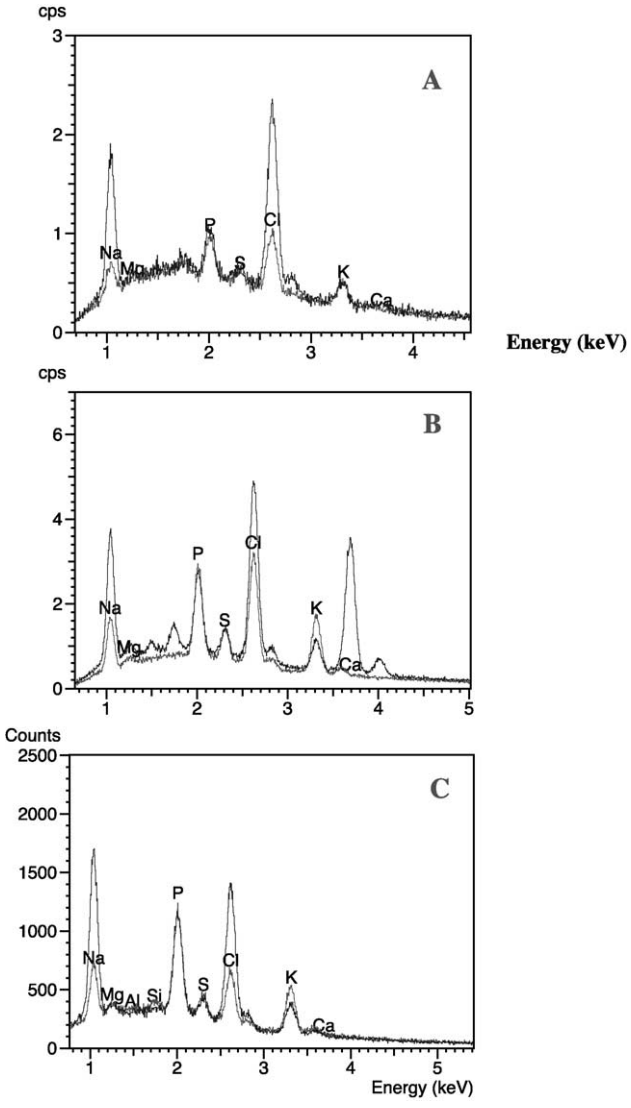

Fig. 6. Effect of clotrimazole on the ion content of clotrimazole-treated CT-26 colon adenocarcinoma and LL/2 Lewis lung carcinoma cells. X-ray microanalysis spectra of treated cells, compared with controls. (A) CT-26 cells treated with 50 μM clotrimazole for 30 min; (B) CT-26 cells treated with 50 μM clotrimazole for 1 h; (C) LL/2 cells treated with 50 μM clotrimazole for 20 min.

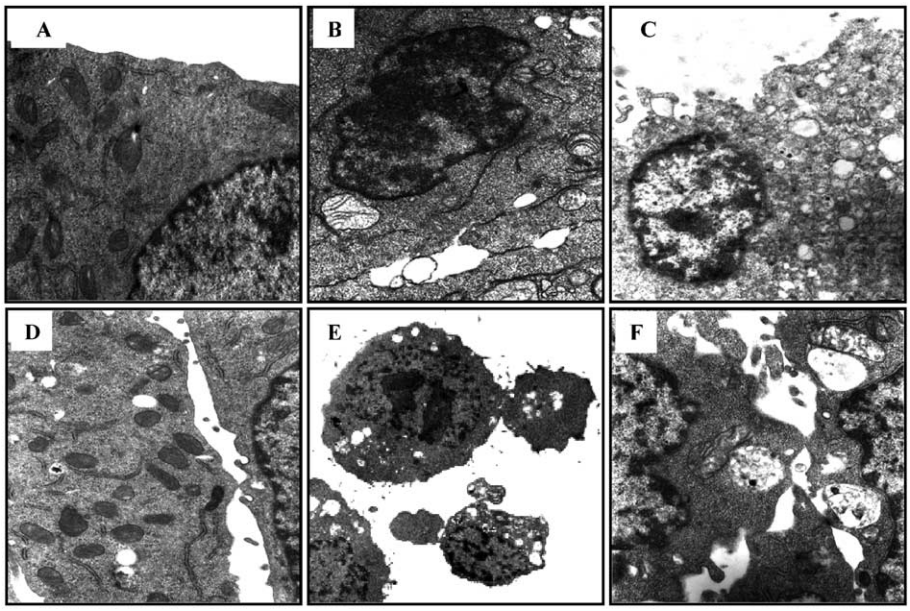

Fig. 7. Effect of clotrimazole on the ultrastructure of CT-26 colon adenocarcinoma and LL/2 Lewis lung carcinoma cells. Transmission electron microscopy images of (A) untreated CT-26 cells (magnification ×8000); (B) CT-26 cells treated with 50 μM clotrimazole for 30 min (magnification ×15,000); (C) CT-26 cells treated with 50 μM clotrimazole for 1 h (magnification ×15,000); (D) untreated LL/2 cells (magnification ×8000); (E) LL/2 cells treated with 50 μM clotrimazole for 30 min (magnification ×5000); (F) LL/2 cells treated with 50 μM clotrimazole for 45 min (magnification ×15,000).

50 μM clotrimazole for 20 min lead to quite similar changes in ion cell content, expressed as increase in Na^+ and Cl^- and decrease in K^+.

The alterations in element content during cell death were correlated with the sequence of morphological changes. Treatment of cells with clotrimazole affected the ultrastructure of the cells as revealed by transmission electron microscopy (Fig. 7). Control cells displayed normal morphology with normal appearing chromatin and normal mitochondria (Fig. 7A,D). Transmission electron microscopy showed that clotrimazole induced necrotic changes in CT-26 colon adenocarcinoma and LL/2 Lewis lung carcinoma cells. Apoptosis was originally distinguished from necrosis on the basis of its ultrastructure (Kerr et al., 1995) and electron microscopy still provides the most reliable method for recognizing the two processes. The grossly swollen mitochondria, irregular mild clumping of nuclear chromatin without severe damage to nuclei, dissolution of ribosomes and focal rupture of membranes were depicted in both cell types treated with clotrimazole (Fig. 7B,C,E,F) in comparison to control cells. At a more advanced stage of this process (Fig. 7C), all cellular components disintegrate and plasma membrane has disappeared. In LL/2 Lewis lung carcinoma cells in addition, blebbing of the plasma membrane was observed (Fig. 7E).

4. Discussion

The present results (Figs. 1 and 2) reveal that the antifungal imidazole derivative, clotrimazole, which was recently reported to display calmodulin antagonistic activity (Hegemann et al., 1993; Mac Neil et al., 1993), caused a significant dose- and time-dependent decrease in the levels of glucose 1,6-bisphosphate and fructose 1,6-bisphosphate in CT-26 colon adenocarcinoma cells and LL/2 Lewis lung carcinoma cells. These results strongly suggest that clotrimazole exerted effects on the two signal molecules of glycolysis, through inhibition of Ca^{2+}/calmodulin activity, since the relative potency of the clotrimazole in reducing glucose 1,6-bisphosphate and fructose 1,6-bisphosphate levels was similar to its reported IC_{50} for calmodulin inhibition. Antifungal azole derivatives, like many other calmodulin antagonists, were also shown to inhibit cell proliferation (Mac Neil et al., 1993).

The results presented here (Fig. 4) also reveal that clotrimazole was cytotoxic, markedly reducing viability of the CT-26 colon adenocarcinoma and LL/2 Lewis lung carcinoma cells. This reduction was closely related to the decrease in ATP content (Fig. 4). The great cytotoxicity of the azole derivatives may result also from their additional actions, e.g., inhibition of cytochrome *P*-450.

The decrease in glucose 1,6-bisphosphate and fructose 1,6-bisphosphate levels, induced by clotrimazole, was not due to the cytotoxic effect of the drugs, because the content of the two regulatory molecules was already significantly reduced under nontoxic conditions, in 100% viable cells (Fig. 5).

Our previous experiments have suggested that Ca^{2+}/calmodulin is involved in the stimulatory action of insulin and growth factors on glucose 1,6-bisphosphate levels and glycolysis in normal cells, which could be inhibited by

calmodulin antagonists (Bassukevitz et al., 1992; Beitner, 1993, 1998; Chen-Zion et al., 1992; Livnat et al., 1995). Here we show that in CT-26 colon adenocarcinoma and LL/2 Lewis lung carcinoma cells, clotrimazole markedly decreased the levels of glucose 1,6-bisphosphate, which is a potent activator of phosphofructokinase, the rate-limiting enzyme of glycolysis. The decrease in glucose 1,6-bisphosphate leads to a reduction in the activity of phosphofructokinase, as reflected here by the drastic fall in fructose 1,6-bisphosphate, which is the product of phosphofructokinase reaction and also an allosteric activator of this enzyme. Both glucose 1,6-bisphosphate and fructose 1,6-bisphosphate are also activators of pyruvate kinase, another key enzyme in glycolysis (for reviews, see Beitner, 1979, 1985). The clotrimazole induced reduction in glycolysis, which is the primary energy source in malignant cells, leads to the decrease in ATP. A decrease in ATP is known to cause various cell abnormalities, since ATP is required for many energy dependent systems (e.g., ion movement, various translocations, etc.), as well as for various phosphorylation processes, and it is well known that a fall in ATP eventually leads to cell death. This is expressed here by the time-dependent reduction in the viability of CT-26 colon adenocarcinoma and LL/2 Lewis lung carcinoma cells induced by the clotrimazole, which correlated with the fall in ATP content.

The depletion in ATP (Fig. 3) leads to inhibition of energy-dependent pump such as Na^+/K^+-ATPase, which resulted in cellular ionic imbalance, as exhibited by the elevated levels of Na^+ and the low level of K^+ in the cytoplasm (Fig. 6). Another ATP-dependent pump located in the plasma membrane is that of the Ca^{2+}-ATPase, which transports calcium ions out of the cell in order to maintain a stable cytosolic concentration. So ATP depletion could be responsible indirectly for inhibition of pump activity, resulting in an increased cytosolic calcium concentration (Fig. 6B). In addition, this elevation could result from the release of calcium ions from clotrimazole-damaged mitochondria (Fig. 7B,C). Nevertheless, direct damage to the plasma membrane, which results in pump dysfunction, is also known to cause alterations in intracellular ion concentrations. We assume, that the alterations in ion concentrations demonstrated in this study are unlikely to be the result of membranal damage and are related to ATP deletion as a result of damaged glycolysis, since ion concentrations was already significantly altered by clotrimazole before any cytotoxic effect was observed. With more prolonged incubation of LL/2 Lewis lung carcinoma cells we probably would achieve the same results as in CT-26 colon adenocarcinoma cells (Fig. 6B), but it was not possible due to the technique limits, since for the procedure we need coverslips-attached cells. We have shown in our previous study marked cell detachment of LL/2 Lewis lung carcinoma cells (comparatively to the CT-26 colon adenocarcinoma cells) already after 30-min clotrimazole-treatment, due to pronounced cytoskeleton damage that occurs. Our electron microscopic findings (Fig. 7E) supply strong evidence for this statement by demonstrating plasma membrane blebbing, which is known to be due to a deep cytoskeleton rearrangement, causing progressive changes in cell shape. The extensive protrusion or blebbing of the cell surface in LL/2 Lewis lung carcinoma cells (Fig. 7E) must not be confused with that which occurs during apoptosis (Kerr et al., 1995). Blebs can be occasionally also described on the surface of the cells undergoing necrosis, and in this condition they are followed by the rapid appearance of membrane discontinuities, causing water influx followed by cell swelling and disruption. Moreover, it was recently shown that intracellular energy levels and mitochondrial function are rapidly compromised in necrosis, but not in apoptosis (Nicotera et al., 1998) and that intracellular ATP levels determine cell death fate by apoptosis or necrosis (Eguchi et al., 1997). It was suggested that there is existence of two different types of pathways for necrotic cell death, one involves cytoskeleton-dependent surface blebbing, whereas the other does not (Malorni et al., 1993). Our findings strengthen this suggestion. Moreover, the present experiments show that clotrimazole caused two different types of pathways for necrotic cell death in CT-26 colon adenocarcinoma and LL/2 Lewis lung carcinoma cells. The drug induced cell injury and eventual death by modifying plasma membrane integrity and function. However, in LL/2 Lewis lung carcinoma cells (Fig. 6E) this involves cytoskeleton-dependent surface blebbing, whereas in CT-26 colon adenocarcinoma cells (Fig. 7B), it does not.

It has recently become evident that calmodulin antagonists are a new generation of drugs with broad therapeutic applications (for review, see Mannhold and Timmerman, 1992). Calmodulin antagonists were found in our laboratory to be effective drugs in treatment of skin burns and frostbite. They also attenuate skin changes induced by UV radiation (Beitner, 1987) (for review, see Beitner, 1998). Since it is believed that calmodulin plays an important role in the proliferation of lung cancer cells through the mechanism of the promotion of an uncontrolled synthesis of DNA in the cells (Liu et al., 1996), consequently, it is inferred that calmodulin antagonists may be tried as a chemotherapeutic agent for lung cancer. It was shown that in mice inoculated intravenously with MM-RU human melanoma cells, daily subcutaneous injections of clotrimazole induced a significant reduction in the number of lung metastases (Benzaquen et al., 1995). We have recently found that calmodulin antagonists are most effective in treatment of melanoma, acting by reducing glycolysis, which eventually leads to melanoma cell death (Glass-Marmor et al., 1996, 1999; Penso and Beitner, 1998) (for review, see Beitner, 1998).

The present results reveal a novel mechanism of action of clotrimazole in CT-26 colon adenocarcinoma and LL/2 Lewis lung carcinoma cells and provide some new insights on the mode of action of clotrimazole in tumor cells. Since Ca^{2+}/calmodulin plays a critical role in both cell prolifer-

ation and glycolysis, the primary energy source in cancer cell, clotrimazole is most promising agent for treatment of cancer. In addition, the results reported here suggest that a reduction in the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP could be employed to serve as markers for cancer cell dysfunction to evaluate therapeutic action of anti-cancer drugs.

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