

α -Lipoic Acid Induces p27^{Kip1}-Dependent Cell Cycle Arrest in Non-Transformed Cell Lines and Apoptosis in Tumor Cell Lines

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α -Lipoic acid is a naturally-occurring co-factor found in a number of multi-enzyme complexes regulating metabolism. We report here that α -lipoic acid induces hyperacetylation of histones *in vivo* and has differential effects on the growth and viability of normal versus transformed cell lines. The human tumor cell lines FaDu and Jurkat, as well as a Ki-v-Ras-transformed Balb/c-3T3 murine mesenchymal cell line, all initiated apoptosis following exposure to α -lipoic acid. In contrast, treatment of non-transformed cell lines with α -lipoic acid resulted only in reversible cell cycle arrest in G₀/G₁. Treatment with butyrate, another short-chain fatty acid, induced a G₀/G₁ arrest in both transformed and non-transformed cell lines. α -Lipoic acid caused a post-translational elevation in the levels of the cyclin-dependent kinase inhibitor p27^{Kip1}. Studies using p27^{Kip1}-deficient MEF cells demonstrated that p27^{Kip1} was required for the α -lipoic acid-mediated cell cycle arrest. The mechanism of apoptosis was independent of Fas-mediated signaling, as α -lipoic acid-treated Jurkat cell mutants deficient in Fas or FADD retained sensitivity to apoptosis. The differential selectivity of the pro-apoptotic effects of α -lipoic acid for transformed cells supports its potential use in the treatment of neoplastic disorders.

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α -Lipoic acid is a naturally-occurring co-factor for vital metabolic multi-enzyme complexes, including pyruvate dehydrogenase and glycine decarboxylase (Biewenga et al., 1997; Fuchs et al., 1997; Packer et al., 1997). Because of its potent anti-oxidant and redox-regulating properties, α -lipoic acid was originally proposed for the treatment of diseases mediated by free radicals, such as heavy metal poisoning, liver disease, radiation poisoning, and diabetes (Biewenga et al., 1997; Fuchs et al., 1997; Packer et al., 1997). α -lipoic acid has been suggested as a therapeutic to reverse the redox disturbance and oxidative stress associated with HIV infection or ischemic tissue damage (Handelman et al., 1994; Fuchs et al., 1997). Other studies have shown that α -lipoic acid can act as a modulator of signal transduction and gene expression through alterations in NF- κ B activation (Suzuki et al., 1992, 1995; Packer et al., 1997).

Originally identified as 1,2-dithiolane-3-pentanoic acid, α -lipoic acid is a lipophilic compound consisting of a heterocyclic carboxylic acid with a five-membered cyclic disulfide ring (Biewenga et al., 1997; Fuchs et al., 1997; Packer et al., 1997). It has a chiral center at position 6 and exists as two enantiomers: R(+) and S(–) α -lipoic acid. The racemic mixture is also referred to as thioctic acid (Biewenga et al., 1997; Fuchs et al., 1997; Packer et al., 1997). α -lipoic acid is reduced in the cell to form 6,8-dithiol-octanoic acid (dihydrolipoic acid) (Biewenga et al., 1997; Fuchs et al., 1997; Packer et al., 1997).

One focus of our laboratory has involved the elucidation of the growth-inhibitory mechanisms of the butyrates (Vaziri et al., 1998; Boosalis et al., 2001) and the discovery of novel, related short-chain fatty acid derivatives (SCFADs). Our approach has been to synthesize or identify orally-bioavailable small molecules that exhibit a differential effect on proliferation in transformed versus non-transformed models of cell growth: in particular, agents that elicit apoptotic responses in transformed models, but not in immortalized, non-transformed models. During the screening of SCFADs for such differential activities, we discovered

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that one compound, α -lipoic acid, induced a reversible G₁/G₀ cell cycle arrest in non-transformed cell lines similar to butyrate, but, in contrast to butyrate, initiated apoptosis in a number of different human tumor cell lines. As the activation of apoptosis may be caused by a number of different molecular mechanisms, including receptor signaling (e.g., through the Fas receptor), loss of substratum adhesion (e.g., anoikis), and perturbations in normal cell cycle progression (e.g., activation of G₁ checkpoints), we examined several of these potential mechanisms in order to elucidate the growth inhibitory or pro-apoptotic effect of α -lipoic acid at the molecular level.

We report here that α -lipoic acid, like butyrate, can inhibit histone deacetylase activity in human tumor cells, but also increases the levels of the cyclin-dependent kinase inhibitor p27^{Kip1}, which in turn is required for α -lipoic acid-induced G₁ arrest.

MATERIALS AND METHODS

Cell culture

The human squamous cell carcinoma cell line, FaDu (Rangan, 1972), was obtained from the American Type Cell Culture (ATCC, Bethesda, MD) and cultured in DMEM (MediaTech, Herndon, VA) supplemented with 10% heat inactivated fetal calf serum (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine and 100 U/ml penicillin, 100 μ g/ml streptomycin, (Gibco BRL, Rockville, MD), referred to as growth media. Human dermal fibroblasts, isolated from a primary culture, were a gift from Dr. N. Prury, Department of Dermatology, Boston University School of Medicine, and cultured in the same growth media. The murine Balb/c-3T3 (ATCC) and the Ki-v-Ras-transformed Balb/c-3T3 cell line, prepared by retroviral infection and described previously (Flyer et al., 1983) were grown in DMEM with penicillin, streptomycin, and L-glutamine, and heat-inactivated 10% donor calf serum (Sigma-Aldrich). The human lymphoblastoid leukemia cell line Jurkat, and the CML/erythroleukemia cell line K562, were obtained from ATCC and cultured in RPMI 1640 media (Gibco BRL), supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, penicillin, and streptomycin. The Fas- and FADD-deficient Jurkat mutants were the gift of J. Blenis (Juo et al., 1999) and were cultured in the same media. All cell lines were cultured in a humidified atmosphere with 5% CO₂ at 37°C. p27^{Kip1}-deficient (Coats et al., 1996; Fero et al., 1996) and p21^{Cip1}-deficient MEF cells (Deng et al., 1995) were the generous gift of Dr. J. Roberts and Dr. P. Leder, respectively. These knockout cell lines, along with wild-type MEF cell lines, were cultured in DMEM with penicillin, streptomycin, and L-glutamine, and heat-inactivated 10% fetal bovine serum (Sigma-Aldrich).

Preparation of short-chain fatty acid derivatives and treatment of cells

α -lipoic acid (DL-6,8-thioctic acid, Sigma-Aldrich): A 20 mM stock solution was prepared in DMEM, using a 75% w/v solution of choline bicarbonate to solubilize and neutralize the compound (final concentration of choline approximately 0.9% w/v). The choline control solution was prepared by adding the same amount of choline bicarbonate to DMEM and adjusting the pH to 7.4 with

HCl. α -lipoamide (DL-6,8-thioctic acid amide, Sigma-Aldrich): a stock solution was prepared by dissolving 25 mg in 0.5 ml of DMSO. n-Octanoic acid (Caprylic acid, Sigma Aldrich): a 300 mM stock solution was prepared in ddH₂O. Arginine butyrate: a 0.1 M stock solution was prepared by adding n-Butyrate (Sigma-Aldrich) to DMEM and a solution of 0.5 M L-Arginine was used to neutralize the solution. 2,2-dimethylbutyric acid: a 0.1 M solution was prepared in DMEM and neutralized with L-arginine. Isobutyramide: a 0.1 M stock solution was prepared in DMEM.

FaDu and human dermal fibroblast cell lines were plated at 1×10^5 cells/ml the day before treatment (for a total of 3×10^5 cells for a 60 mm dish and 1×10^6 for a 100 mm dish). Balb/c-3T3 cells and Ki-v-Ras-transformed Balb/c-3T3 cells were plated on 60 mm dishes at 2×10^5 and 3×10^5 cells, respectively. p27^{Kip1}-deficient MEF cells and corresponding wild-type cells were also plated on 60mm dishes at 1.5×10^5 and 1×10^5 cells, respectively. In addition, the p27^{Kip1}-deficient MEF cells were plated up to 2.5×10^5 cells. The following day, the conditioned media was replaced with fresh growth media, supplemented with the compound to be tested. Jurkat cells (and Fas- and FADD-mutant Jurkat cells) were seeded into a 24-well dish at 5×10^5 cells per well in growth medium, with or without the compounds being tested. K562 cells were seeded into a 60mm dish for a total of 1×10^6 cells per dish (2.5×10^5 cells/ml) in growth media or growth media supplemented with the compound being tested. Growth media alone, or growth media supplemented with choline bicarbonate (equivalent to the concentration of choline in the 5 mM α -lipoic acid solutions) were used as controls. Cells were treated for the indicated time intervals and harvested for analysis.

RNA analysis

Cells were treated for 24 h with or without SCFADs. Total RNA was extracted using guanidine and cesium chloride (Chirgwin et al., 1979). For relative reverse transcription-polymerase chain reaction analysis (RT-PCR), RNA was transcribed and amplified using SuperScript II and Platinum Taq polymerase (Gibco BRL, Bethesda MD). Primers were synthesized by GIBCO BRL. 5'-primer specific for p27 sequence is: 5'-GCGCGGATCCATGTCAAACGTGAGAGTGTGTC-3'. Reverse primer sequence is: 5'-GCGCGAATTCTT-ACGTCTGGCGTGAAGGC-3'. Note that amplification with this primer set introduces a 5' BamHI site and a 3' EcoRI site into the PCR product. Pilot experiments were done to verify that the conditions were carried out in the linear range of the PCR. In addition, the multiplex PCR was done using β -actin primers was kept in the linear range of the p27^{Kip1} reaction by the addition of competing non-productive actin primers (actin competitors from Ambion).

Cell cycle analysis

Propidium iodide staining of nuclear DNA was carried out as previously described (Vaziri et al., 1998). A total of 10,000 cells per sample were collected and analyzed by a FACScan instrument (Becton-Dickinson, San Jose, CA), using the Cell Quest program.

To assess [^3H]-thymidine incorporation, FaDu cells were plated in a 12-well dish and treated as described or left untreated for 24 h. The media was removed, and replaced with 1 ml of growth media containing 1 μCi of [^3H]-thymidine (New England Nuclear, Boston, MA), and incubated for 6 h at 37°C with 5% CO_2 . Thymidine incorporation was quantitated as previously described (Vaziri et al., 1998).

Cell surface immunofluorescence staining

Anti-CD29,-CD104, or-Fas (Pharmingen, San Diego, CA) were used at the indicated dilutions and detected with a fluorescein-conjugated sheep (FAB)2 fragment against mouse IgG (whole molecule) (Cappel Research, Westchester, PA) at a 1:25 dilution and cells were analyzed for cell surface expression of antigens by a FACScan instrument (Becton-Dickinson), using the Cell Quest program.

Detection of apoptosis

For the TUNEL assay, an in situ cell death detection kit (Boehringer Mannheim, CT) was used, as we have previously described (Chen and Faller, 1996). Cells (0.5×10^6) were washed with 1 \times PBS and incubated in 2% paraformaldehyde solution at room temperature. After centrifugation, cells were resuspended in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice, and then stained with TUNEL reaction mixture. Subsequently, cells were analyzed by FACScan, using the Lysis II software program (Becton Dickinson).

For detection of mono- and oligo-nucleosomes, FaDu cells were plated on 12-well dishes in duplicate for 18, 24, and 48 h treatments. To ensure that the same concentrations of cells were present during the treatment intervals, 5×10^4 cells/well were plated for the 18 and 24 h time points, and 1×10^5 cells/well was plated for the 48 h time point, and all samples were harvested simultaneously. 1,000 cells per sample were harvested for analysis of cytoplasmic histone-associated-DNA-fragments (mono- and oligo-nucleosomes), using the Cell Death Detection Elisa Plus kit (Roche Biochemical, Indianapolis, IN) following the protocol supplied by the manufacturer. To determine the background of the immunoassay, the incubation buffer was added alone to sample wells in place of cell lysate and quantitated. This value was subtracted from each sample reading to determine the actual absorbance.

Immunoblot analysis

Acetylated histone H4. Cells were harvested and lysed in a buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, with 0.5 mM DTT, and 1.5 mM PMSF added just before use. Sulfuric acid was then added at a final concentration of 0.4 N and the samples were incubated on ice for 30 min. The cells were then centrifuged at 11,000 g for 10 min at 4°C. The supernatant was saved and dialyzed twice against 0.1 N glacial acetic acid for 1 h each and then 3 times against ddH_2O for 1 h, 2 h, and overnight. Finally the samples were concentrated approximately fivefold in a Speed-Vac centrifuge. Proteins were separated by electrophoresis, transferred to nitrocellulose, and probed with anti-acetylated histone H4 antibody or anti- β -actin

antibody (Upstate Biotechnology, Lake Placid, NY). The ECL Plus Western Blotting Detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ) was applied to the blot and bands were detected using X-ray film.

p27^{Kip1} detection. Immunoblot analysis was carried out using an anti-p27^{Kip1} polyclonal antibody (Pharmingen) and, anti-rabbit IgG HRP-linked F(ab')₂ fragment (from donkey) (Amersham) as the secondary antibody and developed using an ECL Plus Western Blotting Detection reagent.

p27^{Kip1} half-life

FaDu cells, plated on 100 mm dishes, were treated with or without 5 mM α -lipoic acid for 24 h, and then pairs of plates were treated with or without 10 mg cycloheximide/ml for 2, 4, or 6 h. Protein levels of p27^{Kip1} and β -actin were quantitated by immunoblotting.

Analysis of cellular thiol content

Jurkat cells were treated with α -lipoic acid for 24 h, harvested and washed twice with PBS. Cells were also treated in parallel with known thiol-regulating agents as positive controls: treatment with 150 μM buthionine sulfoximine for 24 h to induce GSH depletion, or with 20 mM *N*-acetyl cysteine to increase GSH content. As an additional control, cells were treated with 250 μM *N*-ethyl maleimide for 10 min prior to staining to measure total blocked thiols. Cells were resuspended in 1 ml of PBS. 5 μl of mono-bromo bimane probe (8 mM MBB stock prepared in acetonitrile) (MBB, Molecular Probes, Eugene, OR) was added to the cell suspension, and the samples were incubated in the dark at room temperature. The cells were analyzed using an EPICS Elite (Coulter, Miami, FL) flow cytometer equipment with a 25 mW powered UV argon laser set at 350 nm. Fluorescence emission was recorded using a 450 nm bandpass filter. Data was collected from a total cell population of 10^5 cells for each sample.

RESULTS

Treatment of cell lines with butyrate and certain other short-chain fatty acids typically results in a profound G₁ arrest, easily observable in the cell population as a morphological shift from cycling cells to quiescent ones. In our initial evaluation of SCFADs for potential therapeutic use, we determined the effect of α -lipoic acid on the condition of human tumor cells in culture. Exposure to α -lipoic acid caused a dramatic and dose-dependent change in the morphology of the FaDu cells, a human head-and-neck squamous cell carcinoma-derived cell line. FaDu cultures were exposed to α -lipoic acid, butyrate, or choline (the vehicle control for α -lipoic acid) at final concentrations ranging from 1 to 5 mM. Characteristic photomicrographs of the cultures at 24 or 48 h time-points are shown in Figure 1. As early as 24 h after exposure to 2 or 5 mM α -lipoic acid, the percentage of rounded and detaching FaDu cells increased. By 48 h, similar effects were detectable in the cultures treated with 1 mM α -lipoic acid (data not shown). More than 95% of the FaDu cells had lost adherence by 48 h after exposure to 5 mM α -lipoic acid, or by 72 h following exposure to 2 mM α -lipoic acid (72 h data not shown). The rate at which these toxic effects occurred was dependent

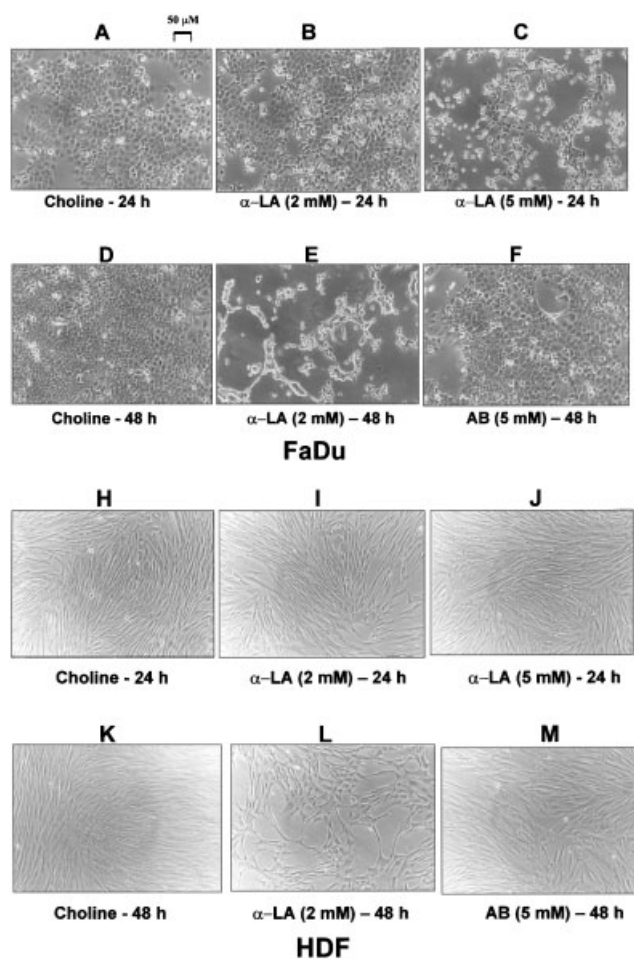


Fig. 1. Morphological and phenotypic changes of cells caused by treatment with α -lipoic acid. FaDu cells were treated for 24 h (A–C) or 48 h (D–F) with α -lipoic acid (α -LA, at concentrations of drug as indicated) or with the choline vehicle (as control). Human dermal fibroblasts (HDF) were treated for 24 h (H–J) or 48 h (K–M) with α -lipoic acid (α -LA, at concentrations of drug as indicated) or with the choline vehicle (as control). Photomicrographs of typical fields are shown. Studies were repeated at least three times.

upon the density of the cells at the time of α -lipoic acid treatment (data not shown). These morphological changes were delayed when cells were treated while subconfluent (less than 60% confluence).

Treatment of FaDu cells with an identical range of concentrations of the arginine salt of butyric acid, a structurally-related compound known to cause reversible G_1 arrest in many cell types, resulted in a decreased number of FaDu cells per plate compared to untreated controls. This decrease was caused by a block in proliferation (see below), however, and was not associated

with morphological changes, toxic effects, or substratum detachment. Furthermore, the primary, non-transformed human dermal fibroblast cell line (HDF) was relatively resistant to the toxic effects of α -lipoic acid. Although there was a profound growth arrest of the HDF lines at the higher concentrations of α -lipoic acid, the compound caused only modest morphological changes and minimal substratum detachment in these primary cell lines (Fig. 1).

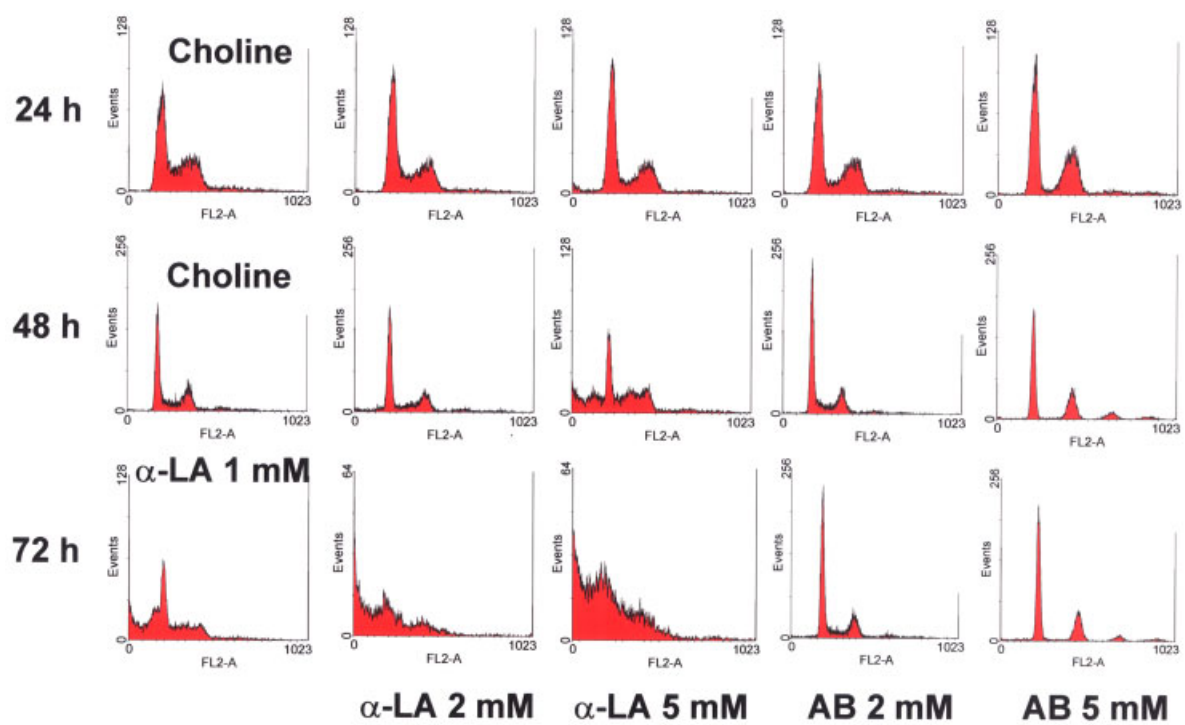
Since we observed potent suppression of proliferation as a result of α -lipoic acid treatment, we next determined whether α -lipoic acid had an effect on cell cycle progression. Cell cycle analysis showed an increase in hypodiploid DNA content (an indicator of apoptosis) for the FaDu cultures exposed to 5 mM α -lipoic acid after 48 h (29% hypodiploid, compared to choline-treated control levels ranging from 4–10%) (Fig. 2A). By 72 h, the apoptotic fraction increased to 36, 46, and 42% for 1, 2, and 5 mM α -lipoic acid, respectively. Interestingly, there was no evidence of a cell cycle arrest in these tumor cells. When the same cells were treated with arginine butyrate, over the same time-course, we detected only cell cycle arrest in G_0/G_1 , with no increase in the hypodiploid fraction. In contrast to FaDu cells, primary cultures of HDF cells appeared to undergo an α -lipoic acid-mediated cell cycle arrest in G_0/G_1 (Fig. 2B). Some apoptosis was induced at the highest concentration of α -lipoic acid (5 mM) at 48 h, and with both 2 and 5 mM concentrations of α -lipoic acid at a later time-point (72 h). At the 1 and 2 mM concentrations of α -lipoic acid, however, HDF cells were relatively resistant to apoptosis, in comparison to the FaDu line. Indeed, 1 mM α -lipoic acid induced less than 10% of the HDF cells to undergo apoptosis at 72 h, compared to choline-treated controls, which ranged from 3–5%. By comparison, the same treatment of FaDu cells induced apoptosis in 36% of the population.

In parallel studies, treatment of the human T-lymphoma cell line (Jurkat) with α -lipoic acid also caused profound induction of apoptosis, with a dose-response curve similar to that observed in FaDu cells, albeit with accelerated kinetics of apoptosis induction. In Jurkat cells, a hypodiploid cell population was detectable as early as 24 h after treatment with 1 mM α -lipoic acid (Fig. 3A).

Although these data suggested that transformed (tumor) cells (e.g., FaDu and Jurkat cells) were more sensitive to the apoptotic effects of α -lipoic acid than were untransformed cells (e.g., HDFs), such an interpretation was confounded by the lack of well-matched, untransformed controls for FaDu or Jurkat cells. For an appropriately matched set of untransformed and transformed cells, we utilized Balb/c-3T3 cells, an immortal, but non-transformed, murine mesenchymal cell line, and a paired Balb/c-3T3 cell line transformed by the Ki-Ras oncogene (designated Ki-Ras-Balb) (Flyer et al.,

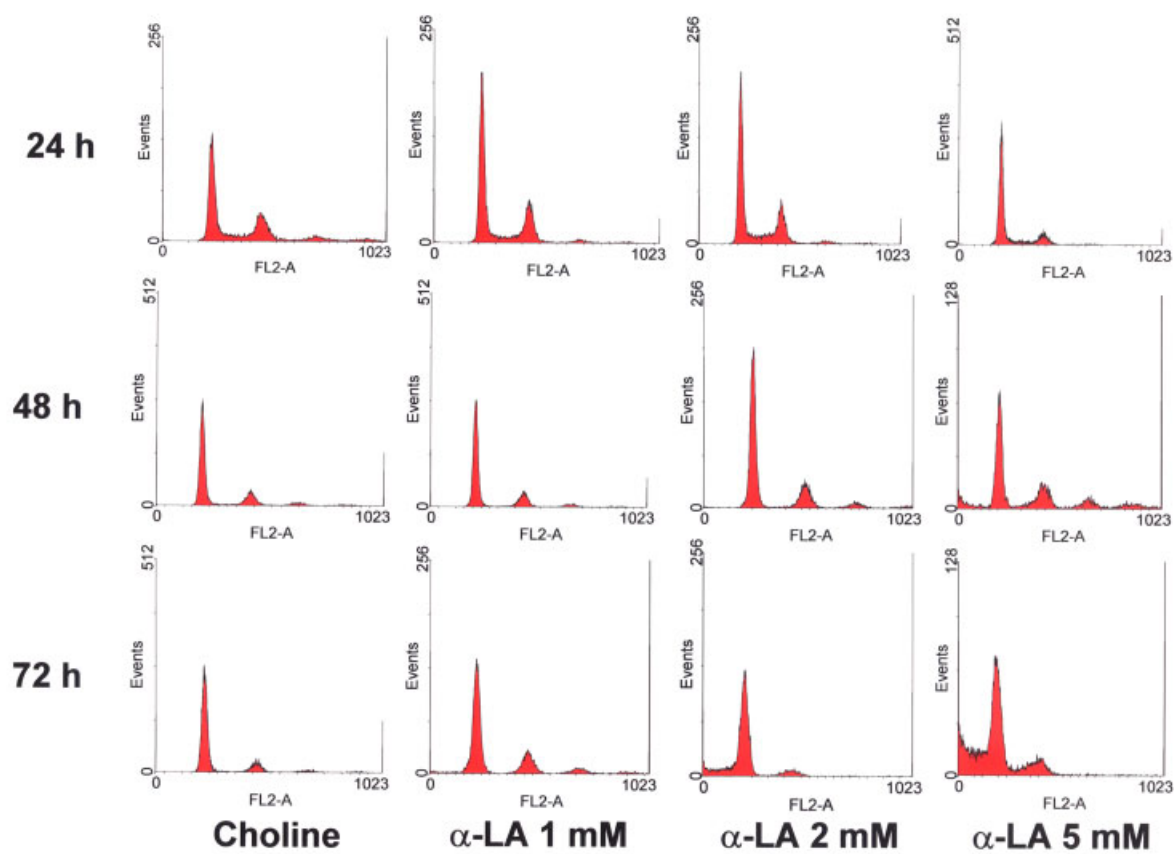
Fig. 2. Cell cycle analysis of transformed and untransformed human cells after treatment with α -lipoic acid or arginine butyrate. FaDu cells (A), or human dermal fibroblasts (HDF cells) (B) were treated with 1, 2, or 5 mM α -lipoic acid (α -LA), or with the choline vehicle (control), for 24, 48, or 72 h, then permeabilized and stained with propidium iodide (see Materials and Methods). In parallel experiments, cells were treated with 2 or 5 mM arginine butyrate, then permeabilized and stained with propidium iodide. Cells were sorted by

FACS using the FL2 detector and cell cycle profiles were acquired and analyzed using the FL2-A parameter. To determine the hypodiploid (sub- G_0/G_1) population, cells from the same samples were acquired and analyzed using the FL2-H parameter. The percentage of hypodiploid cells was calculated based on a total of 10,000 cells analyzed per sample. The histograms shown are representative of independent experiments repeated at least three times.



A

FaDu



B

HfD

Fig. 2

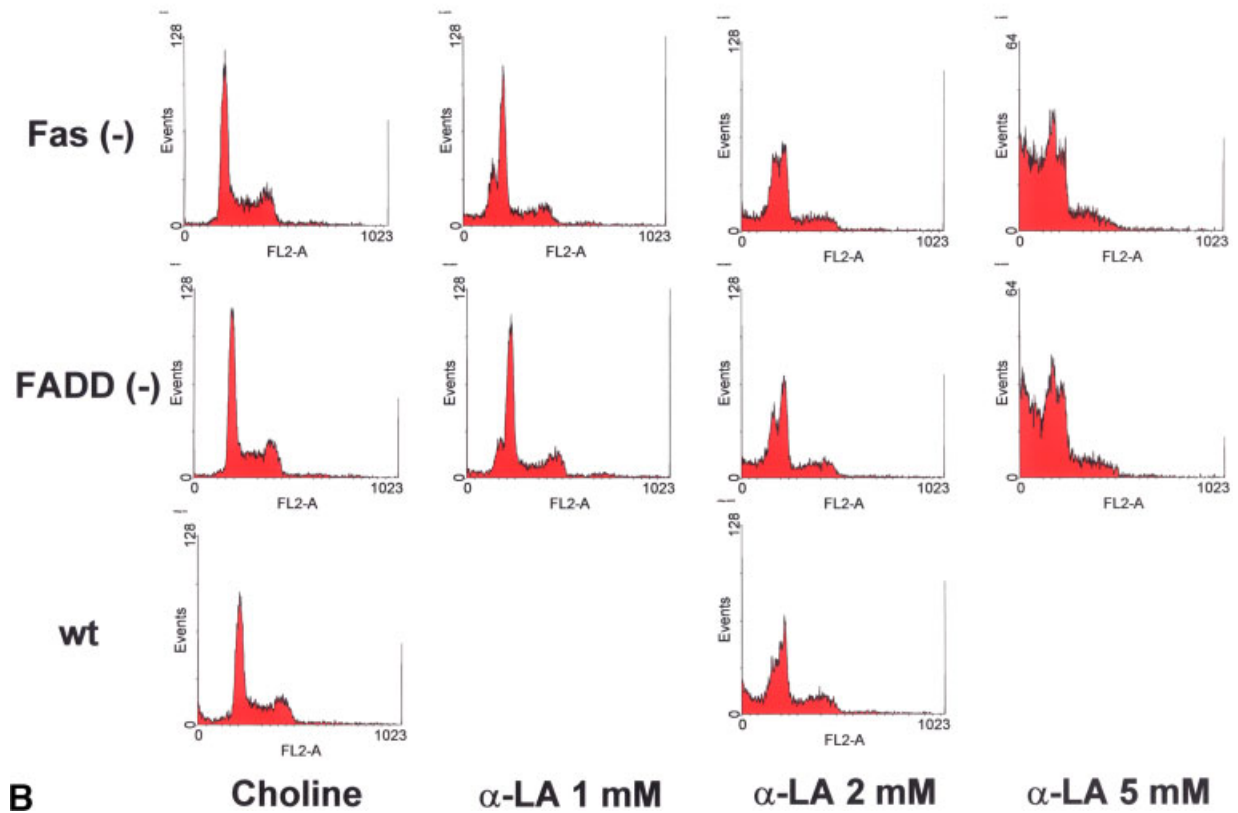
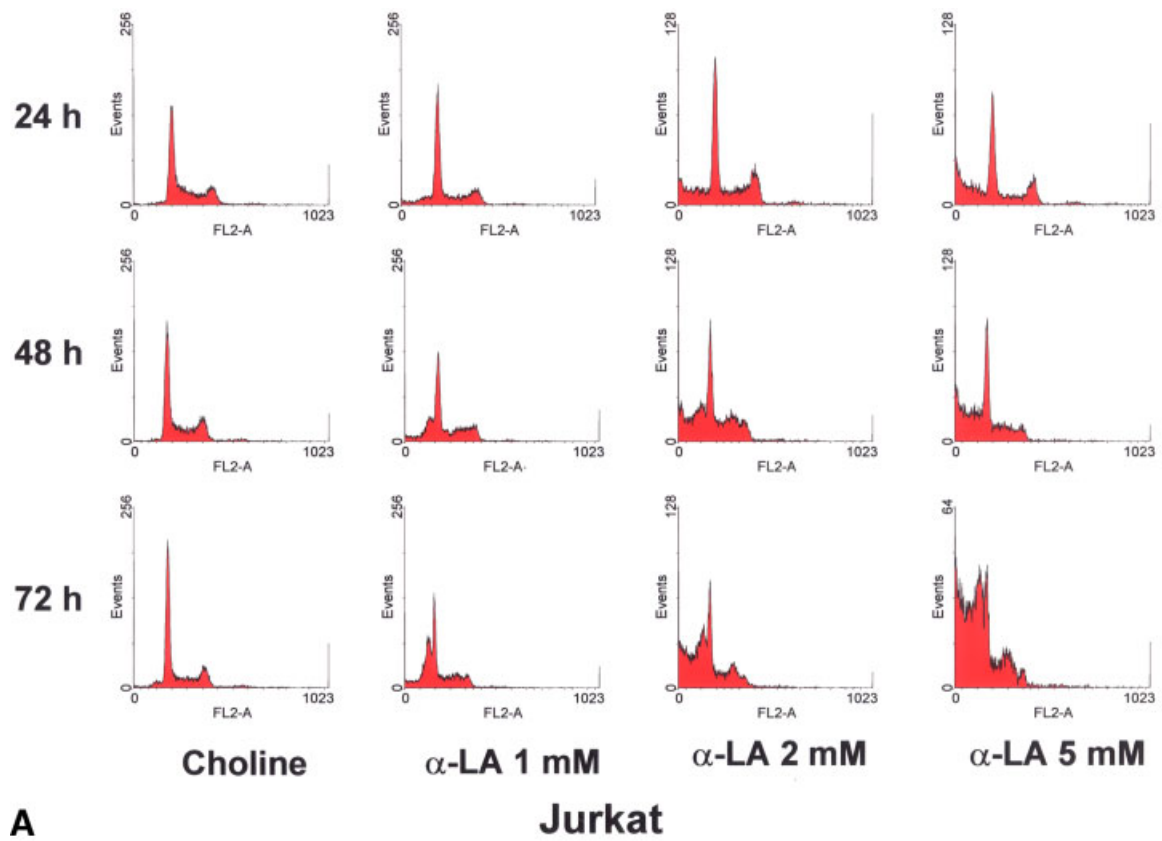


Fig. 3

1983). The Ras-transformed Balb cells were significantly more sensitive to the pro-apoptotic effects α -lipoic acid, in comparison to their non-transformed counterparts (compare Figs. 4A and B). By 72 h, even the 2 mM α -lipoic acid concentration caused an increase in hypodiploid cells. In contrast, the Balb/c-3T3 cells exhibited a G₀/G₁ cell cycle block within 48 h at all concentrations of α -lipoic acid, but apoptosis did not occur. At the 24 h time-point, there also appeared to be a consistent, but transient, component of G₂/M arrest.

To further confirm that the pattern of hypodiploid DNA content induced by α -lipoic acid in transformed cells was due to apoptosis, TUNEL assays were performed (Fig. 5A). At 24, 48, and 72 h after treatment with α -lipoic acid or control vehicle (choline), HDF, FaDu, Balb/c-3T3, K-Ras-Balb, or Jurkat cells were permeabilized and labeled with tagged dUTP by terminal deoxynucleotidyl transferase (TdT) to detect DNA strand breaks. The percentage of labeled FaDu, K-ras-Balb, and Jurkat cells increased in a time- and dose-dependent fashion during exposure to α -lipoic acid. In contrast, there was no significant increase in the TdT labeling in HDF or Balb/c-3T3 cells treated in a parallel fashion with the choline vehicle control.

To further confirm that α -lipoic acid induced apoptosis in transformed cells by an independent assay, we determined the amount of mono- and oligo-nucleosomes released into the cytoplasmic fraction of cell lysates, quantitated by ELISA. This assay also demonstrated that α -lipoic acid treatment led to formation of cytoplasmic mono- and oligo-nucleosomes (indicative of apoptosis) in FaDu cells, in a dose- and time-dependent fashion (Fig. 5B).

We next examined several potential mechanisms by which α -lipoic acid might mediate the induction of apoptosis in FaDu cultures. In a number of epithelial cell types, detachment from the substratum results in activation of the apoptotic program. This process is termed anoikis (Frisch and Ruoslahti, 1997). Rounding-up and detachment was an early and prominent tumor cell-specific feature that we observed in response to α -lipoic acid treatment [and was also seen in a variety of other tumor cells types, including breast carcinoma cell lines (data not shown), after exposure to α -lipoic acid], raising the possibility that α -lipoic acid could be triggering anoikis. Adhesion of epithelial cells to extracellular matrix is mediated in large part by the integrins. The β_1 (CD29)- and β_4 (CD104)-integrins in particular are known to be expressed on many squamous cell carcinomas and are involved in their adhesion to matrices (Van Waes, 1995; Dowling et al., 1996; DiPersio et al., 1997; Kim et al., 1997). However, our analysis of cell surface antigen expression revealed no change in the levels of the β_1 or β_4 integrin families following α -lipoic acid exposure, despite detachment of > 95% of the cells from the substratum (Fig. 6). Following α -lipoic acid treatment, the detached FaDu cells

were plated onto different extracellular matrices, including laminin, fibronectin, collagen, and vitronectin. Whereas untreated FaDu cells were able to attach to all of these proteins, the α -lipoic acid-treated cells did not attach to any of these matrices (data not shown). The molecular mechanism responsible for α -lipoic acid-induced substratum detachment of FaDu cells remains, therefore, unresolved. In addition, if the detached cells had initiated anoikis, the (apoptotic) suspension population of cells should have a lower clonogenic potential than the adherent cells. We found, instead, that the non-adherent FaDu cells could re-adhere and proliferate if the α -lipoic acid was removed within 24 h (data not shown). Indeed, substrate adherence of FaDu cells is not required for their survival. When FaDu cells were cultured on bacterial-grade plastic dishes for up to 120 h, the cells remained > 95% viable. Similarly, FaDu cells grown in suspension for 48 h before replating did not undergo apoptosis (data not shown). Thus, substratum detachment induced by α -lipoic acid and consequent anoikis was not the cause of apoptosis in these tumor cells. A number of studies have shown that differentiation of keratinocytes and squamous cell tumors is characterized by loss of cell surface expression of β_1 and β_4 integrins, coincident with detachment from the submatrix (Adams and Watt, 1990, 1991; Sugiyama et al., 1993; Hodivala and Watt, 1994). Our finding that there was no effect on integrin levels following α -lipoic acid exposure also therefore argues against induction of differentiation as a trigger for the apoptosis observed.

α -Lipoic acid has been reported to change the redox state of the intracellular environment (Fuchs et al., 1997), and alterations in the cellular redox state can induce apoptosis. To determine whether the levels of α -lipoic acid used in our studies affected intracellular oxidation, we measured the thiol content of cells treated with 2 or 5 mM α -lipoic acid. Cellular thiol levels did not change significantly from control (mean fluorescence values of 41.8 for choline treatment and 44.1 for treatment with 5 mM α -lipoic acid), indicating that the induction of apoptosis by this compound was not caused by fluctuations of the cellular redox state. Cultures exposed to 5 mM octanoic acid (a precursor of α -lipoic acid), however, did show significant thiol depletion (mean fluorescence of 18.5), comparable to the results obtained with exposure to 150 μ M buthionine sulfoximine (mean fluorescence of 19.6), suggesting that treatment with octanoic acid at 5 mM leads to GSH depletion. Treatment with 250 μ M *N*-ethyl maleimide, which blocks thiols, resulted in a mean fluorescence of 0.9.

FaDu and Jurkat cells express the CD95/Fas receptor (Fig. 6) (See also Chen and Faller, 1995, 1999; Chen et al., 1998a, 1998b, 2001). Activation of the Fas receptor is a well-known initiator of apoptosis. To determine whether Fas signaling was involved in α -lipoic acid-mediated apoptosis, we utilized Jurkat cell lines in

Fig. 3. Cell cycle analysis of Jurkat cells after treatment with α -lipoic acid. Wild-type Jurkat cells (A) or Fas-deficient [Fas (-)] or FADD-deficient [FADD (-)] Jurkat mutant cell lines or parental wild-type Jurkat cells (wt) (B) were treated with α -lipoic acid (α -LA), and cell cycle analysis was carried out as described in the legend to Figure 2. The cells used to generate the profiles in (B) were treated for 72 h. The histograms shown are representative of independent experiments repeated at least three times.

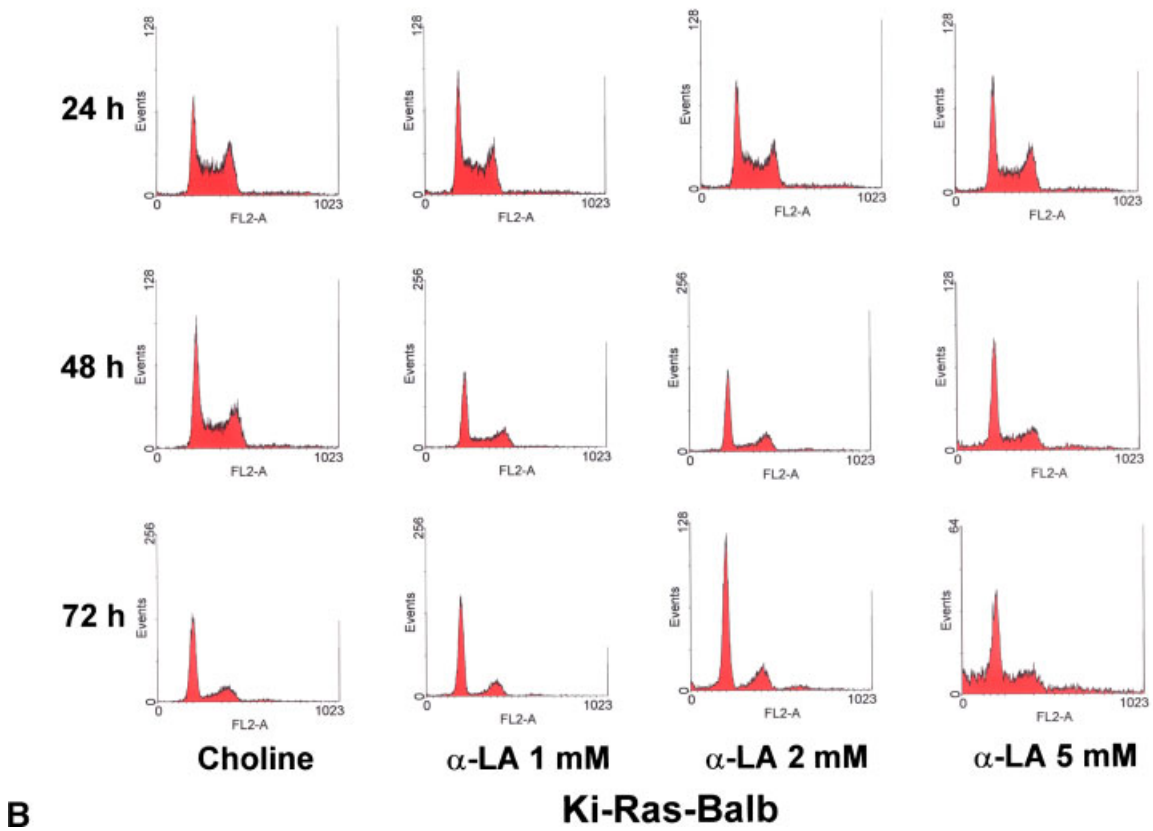
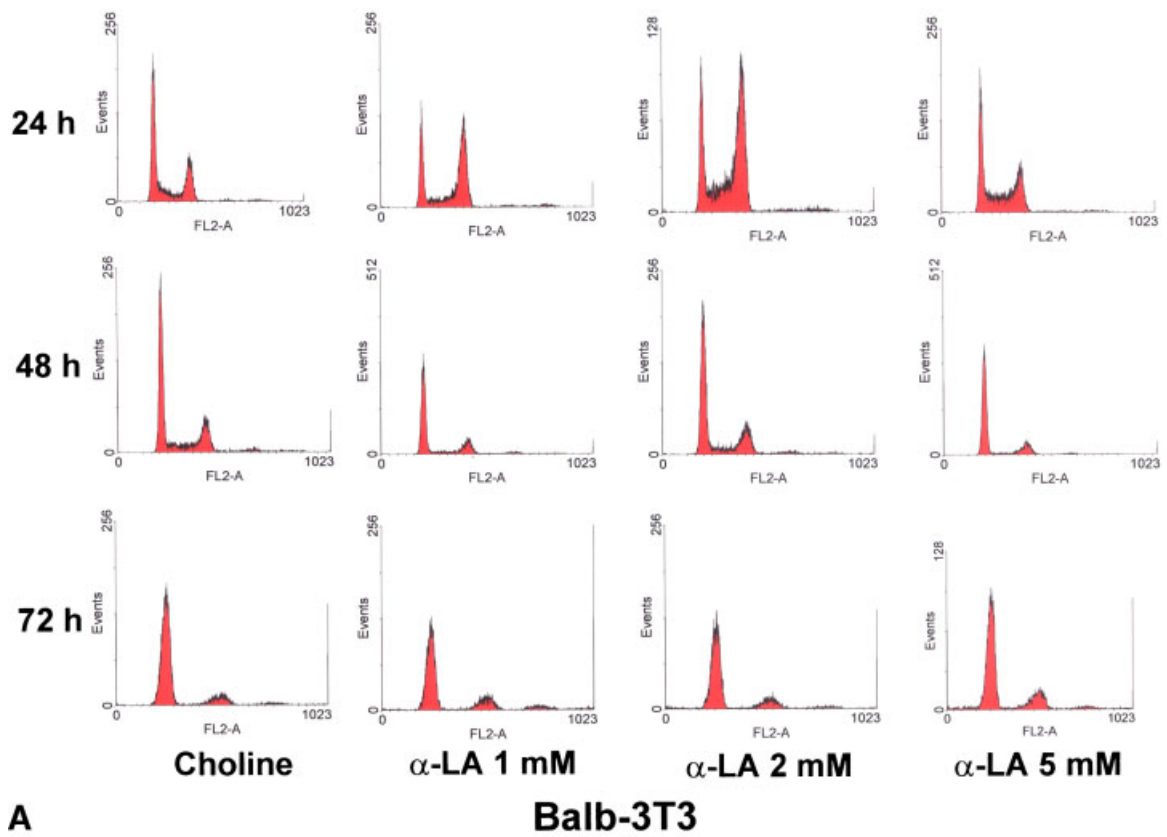


Fig. 4

which the Fas or FADD genes had been disrupted (Juo et al., 1999; Chen et al., 2001). α -Lipoic acid exposure induced apoptosis in the Fas- or FADD-deficient T-cells with identical dose-dependent characteristics compared to parental Jurkat cells (Fig. 3B), thereby excluding Fas signaling as a mechanism for α -lipoic acid-mediated cell death.

We hypothesized that the mechanisms resulting in the striking induction of G₁ cell cycle arrest by α -lipoic acid in normal cells might share common molecular mediators. Butyrate, which is structurally similar to α -lipoic acid, induces G₁ arrest in normal cells and apoptosis in many tumor cell lines, and these effects have been generally ascribed to its actions as a histone deacetylase (HDAC) inhibitor. Assessment of bulk histone (H4) acetylation following treatment of FaDu cells with 5 mM α -lipoic acid demonstrated a marked inhibition of endogenous histone deacetylase activity, greater than that induced by butyrate treatment (Fig. 7). K562, a myeloid tumor cell line which has been extensively studied for its responsiveness to histone deacetylase inhibiting agents (Juo et al., 1999; Chen et al., 2001), also demonstrated marked increases in acetylated H4 after treatment with either butyrate or α -lipoic acid. A number of analogs of butyrate which did not induce cell cycle arrest or apoptosis, including 2,2-dimethylbutyrate, were also tested for HDAC-inhibitory activity, and found to have no inhibitory effect. The finding that only α -lipoic acid and butyrate induced histone hyperacetylation, and only these two compounds caused cell cycle arrest or apoptosis, suggested a common mechanism. Consistent with this possibility, treatment of Jurkat and FaDu cells with 5 mM octanoic acid, a precursor of α -lipoic acid that also possesses HDAC-inhibitory activity in vivo (possibly as a result of metabolism to α -lipoic acid), also induced cell cycle arrest or apoptosis (data not shown). Cell cycle arrest in G₁ can be initiated by the cyclin-dependent kinase inhibitors of the Cip/Kip family. Since previous studies have shown a correlation between cell density and the expression of p27^{Kip1} (Zhang et al., 2000), and the effects of α -lipoic acid were dependent upon cell density, we focused our attention on p27^{Kip1}. Exposure to α -lipoic acid or lipoamide caused a marked elevation of p27^{Kip1} protein levels in FaDu cells (Fig. 8A), consistently and significantly higher than that induced by butyrate. This induction of expression appeared to be predominantly at the level of protein stabilization, with the half-life of the p27^{Kip1} protein increasing from approximately 90 min under control conditions, to greater than 240 min after exposure to α -lipoic acid (Fig. 8B). p27^{Kip1} RNA transcript levels remained unchanged under any condition, as assayed by sensitive relative-RT-PCR assays (Fig. 8C).

To determine if p27^{Kip1} was necessary for the G₁ cell cycle arrest induced by α -lipoic acid, we examined the effects of α -lipoic acid exposure on mouse embryo fibroblasts that were derived from normal animals

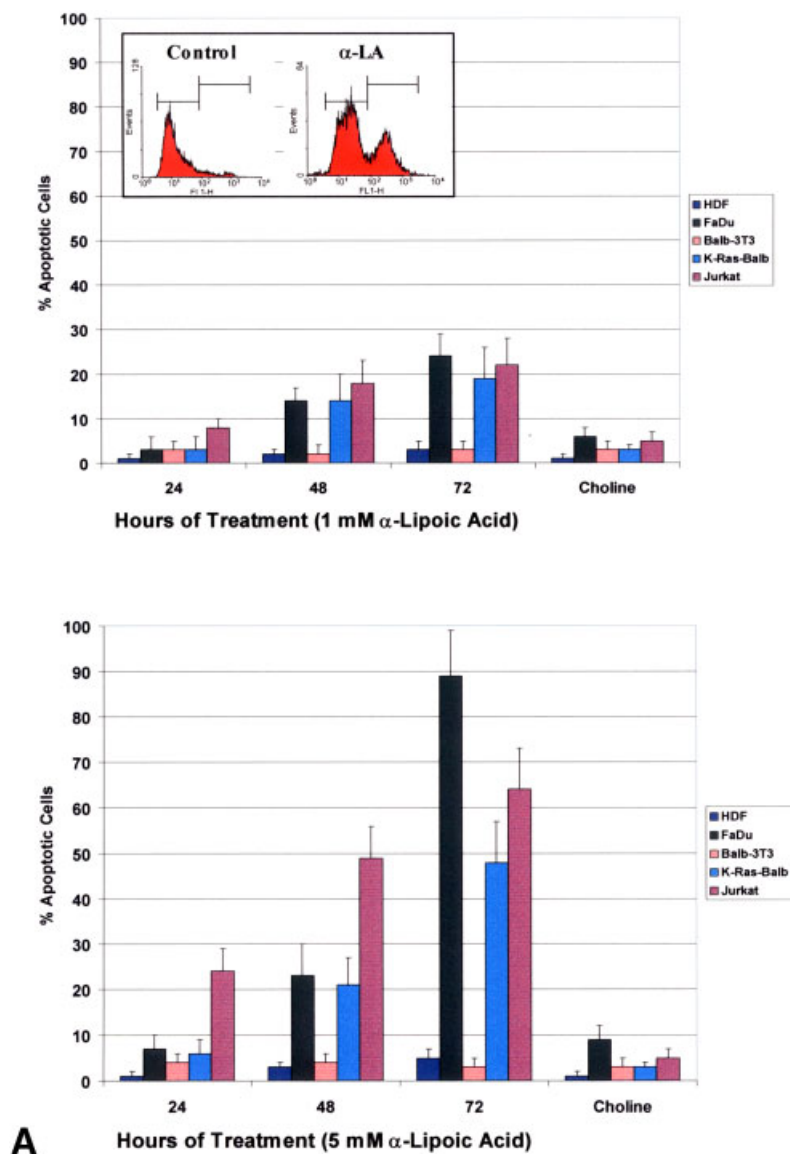
(p27^{+/+}) or animals rendered deficient in p27^{Kip1} through homozygous knock-out of the gene (p27^{-/-}) (Coats et al., 1996; Fero et al., 1996). Cells were serum-starved to synchronize them, and then stimulated with serum. α -lipoic acid at 2 or 5 mM inhibited G₁/S progression in the serum-stimulated wild-type (p27^{+/+}) cells (Fig. 9A). In contrast, cell cycle progression in the p27^{-/-} cells was not affected by α -lipoic acid. It is noteworthy that serum deprivation and confluence did not cause the majority of these p27^{Kip1}-deficient cells to accumulate in G₀/G₁, consistent with previous reports. Similarly, treatment of p27^{Kip1}(^{-/-}) cells in log phase growth with 2 or 5 mM α -lipoic acid for 48 h (Fig. 9B) or 72 h (data not shown) did not induce G₀/G₁ arrest. In contrast, when p21^{Waf/Cip1}-deficient (^{-/-}) MEF cells (Deng et al., 1995) were treated with α -lipoic acid and analyzed by FACS, G₀/G₁ growth arrest was observed, indicating that p21^{Waf/Cip1} is not required for α -lipoic acid-induced cell cycle arrest (Fig. 9C).

DISCUSSION

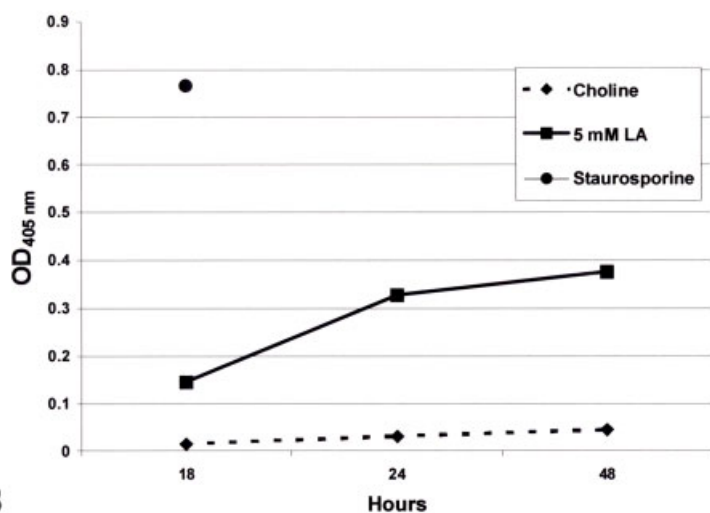
Short-chain fatty acids are small-molecule regulators of gene expression, which have gained acceptance as clinical therapeutics in the treatment of hemoglobinopathies and virus-associated malignancies (Deng et al., 1995). In particular, the butyrates have potent anti-proliferative effects, and are currently in clinical trials for specific cancers (Faller et al., 2001; Mentzer et al., 2001). Interest in the therapeutic potential of α -lipoic acid arose when its redox and anti-oxidant properties were first reported (Fuchs et al., 1997). α -lipoic acid (a disulfide) is reduced to dihydrolipoic acid, a dithiol, by nucleophilic attack through thiolate anions in vivo, or following addition to cell culture medium and uptake by cultured cells (Handelman et al., 1994). Because of their pleiotropic biochemical properties, both α -lipoic acid and dihydrolipoic acid have been found to have therapeutic effects in the treatment of a variety of diseases, including liver cirrhosis, heavy metal intoxication, and diseases caused by free radicals, such as diabetic polyneuropathy (Biewenga et al., 1997; Fuchs et al., 1997; Packer et al., 1997).

α -Lipoic acid and dihydrolipoic acid have been reported to change the redox state of the intracellular environment (Sato et al., 1995; Sen et al., 1997), increasing the cellular glutathione content in Jurkat cells, murine neuroblastoma and melanoma cells in a dose-dependent manner, through promotion of cysteine uptake (Kis et al., 1997; Sen et al., 1997). Octanoic acid induced similar effects in those system, and differentially induced apoptosis in transformed cells in our studies. Apoptosis, a physiological mode of cell death, is closely associated with a pro-oxidant state (Hockenberry et al., 1993; Butke and Sandstrom, 1994; Bustamante et al., 1995). Our analysis of intracellular thiol levels indicated that levels of α -lipoic acid sufficient to induce apoptosis did not alter the cellular redox state, however, and anti-oxidants such as *N*-acetyl

Fig. 4. Cell cycle analysis of transformed and untransformed murine cells after treatment with α -lipoic acid. Balb/c-3T3 cells (A) or K-ras-transformed Balb cells (B) were treated with 1, 2, or 5 mM α -lipoic acid (α -LA), or with the choline vehicle (control), for 24, 48, or 72 h, and cell cycle analysis was carried out as described in the legend to Figure 2. The histograms shown are representative of independent experiments repeated at least three times.



A

Hours of Treatment (5 mM α -Lipoic Acid)

B

Fig. 5. Induction of apoptosis in cells treated with α -lipoic acid. **A:** TUNEL Assay. HDF, FaDu, Balb-3T3, K-Ras-Balb, and Jurkat cells were treated with 1 or 5 mM α -lipoic acid, or with the choline vehicle (as a control), for 24, 48, or 72 h, and TUNEL assay was carried out on the harvested cells, as described in Materials and Methods. Percentage of apoptotic cells was calculated from the flow cytometric profiles (a typical profile for Jurkat cells at 24 h is shown in the inset), and plotted, \pm SEM, for each time point. **B:** Mono- and Oligo-nucleosome ELISA. FaDu cells were treated in duplicate with 5 mM α -lipoic acid, or with the choline vehicle (as a control), for 18, 24, or 48 h. A time point of 18 h was chosen for treatment with 500 nM staurosporine (positive control for apoptosis). A total of 1,000 cells/sample were harvested, lysed, and placed onto a streptavidin-coated microtiter plate. The lysates were incubated with antibodies to histone-biotin and DNA-POD. The amount of released oligo-nucleosomes, quantitated by the POD retained in the immunocomplex, was determined using ABTS as substrate. The samples incubated with ABTS for 45 min and read at 405 nm using a spectrophotometric plate reader. [This figure appears in color in the online version at www.interscience.wiley.com.]

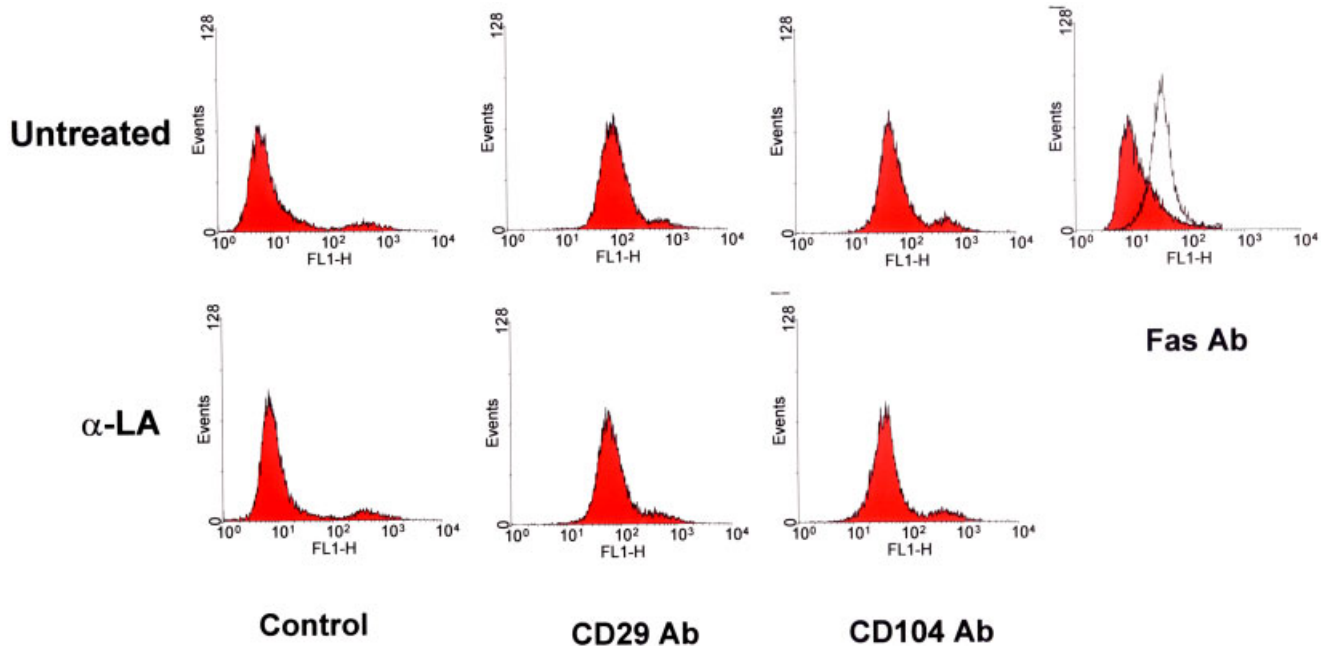


Fig. 6. Cell surface expression of β_1 (CD29) and β_4 (CD104) integrins and Fas antigen on FaDu cells before and after treatment with α -lipoic acid. The cell surface expression of β_1 and β_4 integrins on FaDu cells was quantitated by FACS analysis after treatment with 5 mM α -lipoic acid, or with the choline vehicle (as a control), for 24 h. After treatment, cells were incubated with either mouse anti-human CD29, mouse anti-human CD104, or 10% serum (negative control), and then conjugated to an anti-mouse IgG-FITC antibody. Cells were sorted by

FACS utilizing the FL1 detector. The histogram profiles shown represent the level of expression of CD29 and CD104, compared to cells treated with choline only and stained with second antibody alone. For detection of Fas antigen expression, cells were treated with mouse anti-human Fas antibody, or with 10% serum as a negative control, and then conjugated and analyzed as above. The panel is an overlay of profiles obtained with the anti-Fas antibody and the negative control.

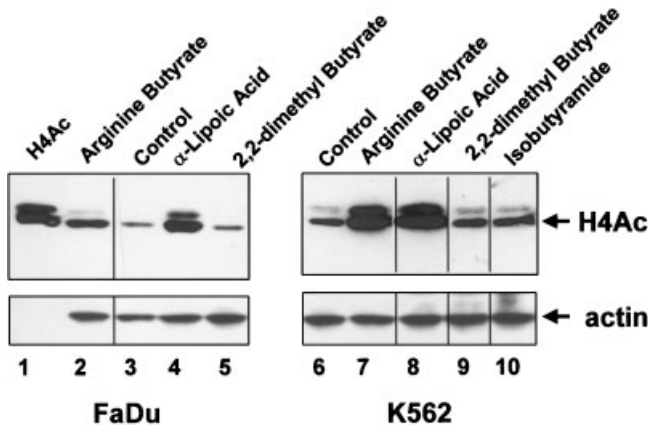


Fig. 7. Histone deacetylase inhibitory activity of short-chain fatty acid derivatives. FaDu or K562 cells were treated for 24 h with either arginine butyrate, α -lipoic acid, 2,2-dimethylbutyrate, or isobutyramide (all at concentrations of 5 mM). For control samples, FaDu cells were treated with media plus choline bicarbonate (the vehicle control for α -lipoic acid). SDS-PAGE (15% polyacrylamide) was used to separate 40 μ g samples of acid-extracted proteins from treated cells. Purified human acetylated histone H4 was run as a control in one lane (H4Ac). The proteins were transferred to nitrocellulose and analyzed by immunoblotting, using an antibody that recognizes acetylated histone H4 (approximately 10 kDa). Lanes: (1) Human acetylated H4; (2) arginine butyrate; (3) media plus choline vehicle; (4) α -lipoic acid; (5) 2,2-dimethylbutyric acid; (6) media plus choline vehicle; (7) arginine butyrate; (8) α -lipoic acid; (9) 2,2-dimethylbutyric acid; (10) isobutyramide. In the lower panel, a parallel membrane immunoblotted for β -actin to verify equivalent protein loading is shown.

cysteine (at 20 mM) had no effect on the induction of apoptosis by α -lipoic acid (unpublished observations), suggesting that alteration in the redox state was not the mechanism of induction of apoptosis by α -lipoic acid. We did, however, observe thiol depletion when cells were treated with octanoic acid, supporting previously reported observations (Sen et al., 1997). Similarly, although dihydrolipoic acid and α -lipoic acid have been reported to modulate NF- κ B activation (Sen et al., 1997), and NF- κ B has been reported to protect cells against apoptosis (Liu et al., 1996; Wu et al., 1996), we found no alterations in the transcription levels of a transiently-transfected NF- κ B-responsive promoter-CAT-reporter vector after exposure of the cells to α -lipoic acid (unpublished data).

Because of the known activity of butyrate as a histone deacetylase inhibitor, this property has been proposed as causing the inhibitory effects on cell cycle progression imposed by certain of the short-chain fatty acids (Yoshida et al., 1990; Vaziri et al., 1998). We found that α -lipoic acid inhibited bulk histone deacetylation *in vivo* with a potency equal to that of butyrate, raising the possibility that HDAC-inhibition is a mechanism mediating cell cycle arrest or apoptosis by α -lipoic acid. Yet, several lines of evidence argue against this mechanism. First, the lethal effects of α -lipoic acid on FaDu cells were in sharp contrast to the reversible cell cycle arrest induced by butyrate in the same cells. Additionally, lipoamide, a metabolite of α -lipoic acid, had differential effects on normal and transformed cells similar to those

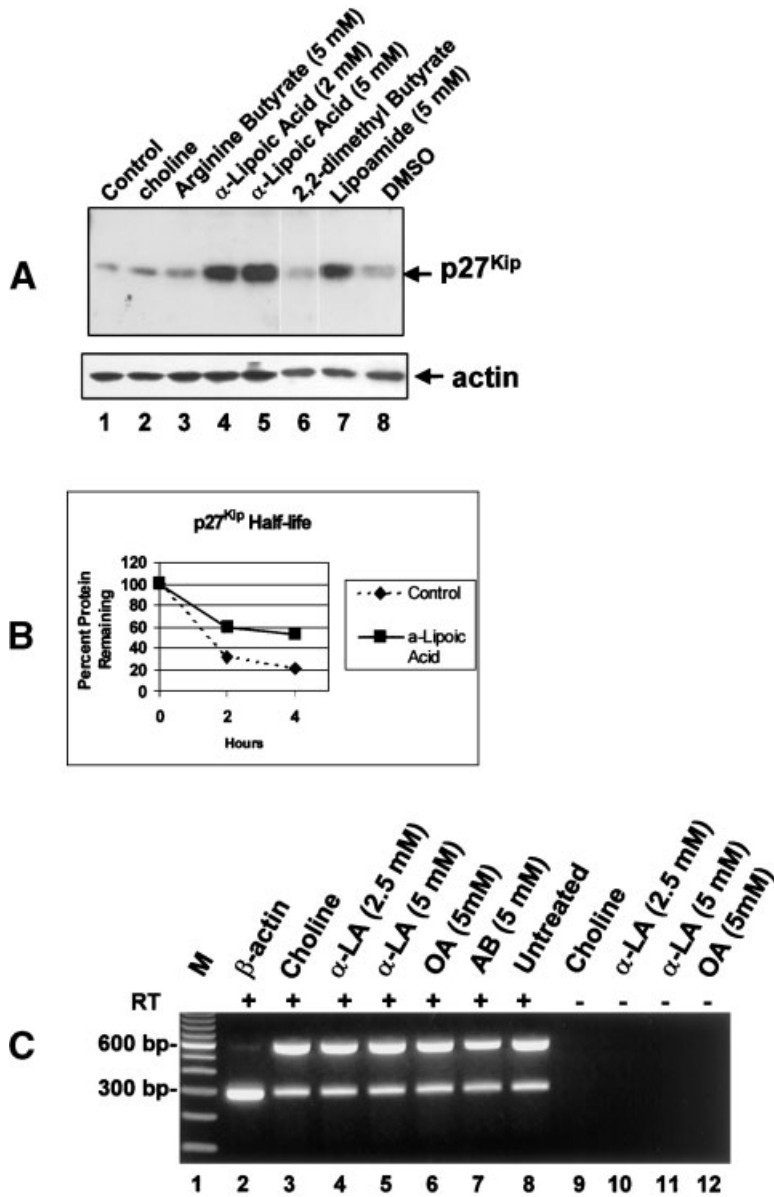


Fig. 8. Expression of p27^{Kip1} protein and transcripts in FaDu cells after treatment with α -lipoic acid and butyrate analogs. **A:** FaDu cells were treated for 24 h with either arginine butyrate, α -lipoic acid, 2,2-dimethylbutyric acid, lipoamide, or left untreated (Control). As vehicle controls, FaDu cells were treated with choline bicarbonate, or 2% DMSO (the vehicle used to solubilize lipoamide). 100 μ g of total protein from treated cells was separated on a 12% polyacrylamide denaturing gel, transferred to PVDF membrane and analyzed by immunoblotting using an anti-human p27^{Kip1} antibody. Lanes: (1) Media alone; (2) choline bicarbonate; (3) arginine butyrate; (4) 2 mM α -lipoic acid; (5) 5 mM α -lipoic acid; (6) 2,2-dimethylbutyric acid; (7) lipoamide; (8) DMSO. In the lower panel, the membrane was stripped and immunoblotted for β -actin to verify equivalent protein loading. **B:** Half-life of p27^{Kip1} protein. FaDu cells were pre-treated with cycloheximide (10 μ g/ml) for 30 min and then exposed to 5 mM α -lipoic acid or the choline vehicle. Cells were harvested immediately after addition of the α -lipoic acid (0 h) and at 2 and 4 h after addition. p27^{Kip1} protein levels were assayed as in (A), quantitated by laser densitometry, normalized to levels of β -actin, and plotted as a function of time. α -lipoic-acid treated cells differ significantly from controls in p27^{Kip1} protein levels at 2 and 4 h ($P < 0.01$). **C:** p27 transcript in BALB/c 3T3 fibroblasts. Total RNA was harvested from Balb/c 3T3 cells after 24 h treatment with or without SCFAs. Relative RT-PCR was performed and products were resolved by gel electrophoresis. Lanes 3–10: multiplex RT-PCR using p27-specific primers and actin-specific primers with RNA from conditions 1–6 listed below; lanes 9–12: RT-minus controls using RNA from conditions 1–4. Lane 1: 100 bp ladder; lane 2: positive control for beta-actin RT-PCR; lane 3: choline control; lane 4: 2.5 mM α -lipoic acid; lane 5: 5 mM α -lipoic acid; lane 6: 5 mM octanoic acid; lane 7: 5 mM arginine butyrate; lane 8; untreated. p27 PCR product is 600 bp, actin product is 300 bp.

of α -lipoic acid, yet had no demonstrable effect on bulk histone acetylation.

There were notable and consistent differences between the sensitivity to α -lipoic acid of true tumor cell lines (FaDu, Jurkat, and MCF7 [not shown]) compared to in vitro-ras-transformed cell lines, in terms of induction of apoptosis. A cell cycle arrest was evident in the Ki-ras-Balb cells following α -lipoic acid treatment, although less dramatic than in Balb-3T3 cells. A prominent component of arrest was not evident in the true tumor cell lines used for the study. The reasons for this difference are not yet clear. Because the tumor cells have likely undergone multiple, sequential mutational events in the course of their natural selection and progression, it is possible that they have lost critical genes or pathways required for the “ α -lipoic acid checkpoint.” Alternatively, the activated *ras* gene used to

generate the Ki-ras-Balb cells is known to activate the anti-apoptotic Akt-PKB pathway (Downward, 1997; Kauffmann-Zeh et al., 1997; Khwaja et al., 1997; Takahashi et al., 1999; Ushio-Fukai et al., 1999),

Fig. 9. p27^{Kip1} protein is required for the cell cycle arrest induced by α -lipoic acid. **A:** Wild-type [p27(+/+)] and knock-out [p27(-/-)] MEF cells at confluence were starved for serum, then stimulated with 10% fetal bovine serum in the presence of 2 or 5 mM α -lipoic acid, or with the choline vehicle for 18 h, then permeabilized and stained with propidium iodide. **B:** p27(-/-) MEF cells in log phase of growth were treated choline (control) or with 2 or 5 mM α -lipoic acid, or with the choline vehicle, for 48 h, then permeabilized and stained with propidium iodide. **C:** p21(-/-) MEF cells in log phase of growth were treated choline (control) or with 2 or 5 mM α -lipoic acid, or with the choline vehicle, for 48 h, then permeabilized and stained with propidium iodide. Cells were analyzed by FACS using the FL2 detector and cell cycle profiles were acquired and analyzed using the FL2-A parameter. Typical profiles are shown.

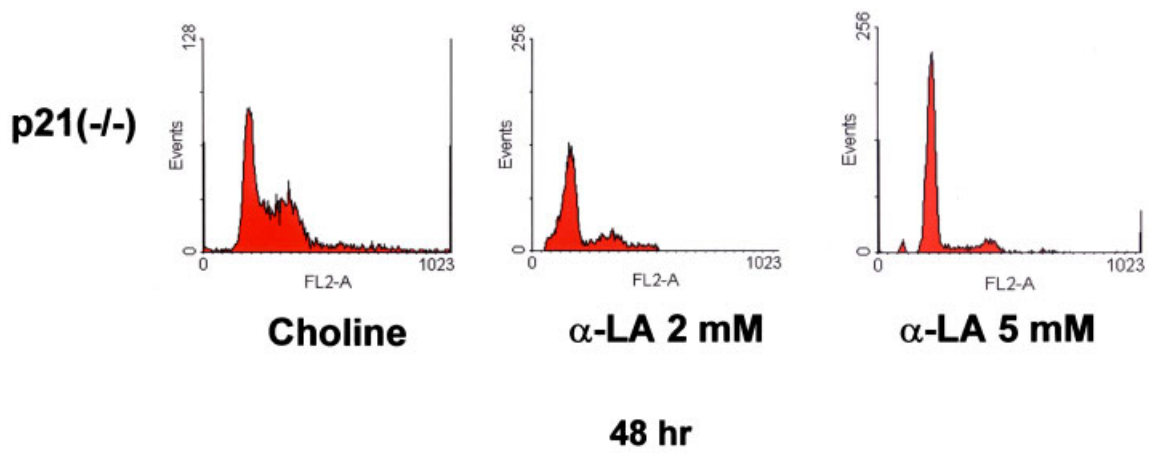
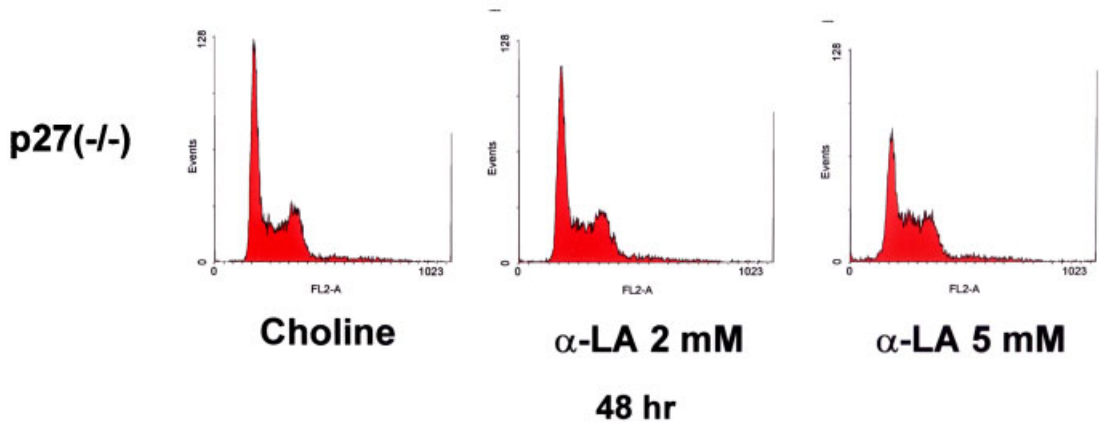
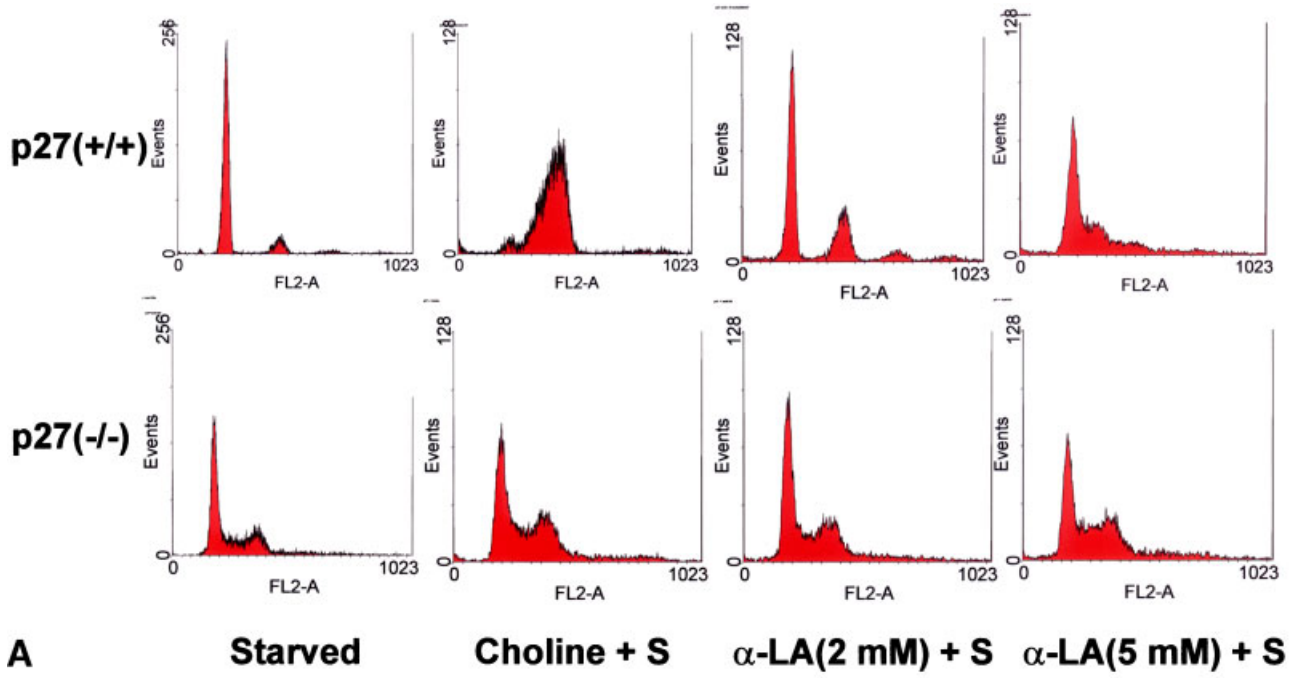


Fig. 9

whereas neither FaDu, Jurkat, nor MCF-7 cells contain a mutated, activated ras allele (Chen and Faller, 1995, 1996).

A number of chemotherapeutic agents, toxins, oxidants, and reducing agents are thought to induce apoptosis through the CD95-Fas/FasL pathway (Friesen et al., 1999; Eichhorst et al., 2000; Engels et al., 2000; Ferreira et al., 2000; Kaufmann and Earnshaw, 2000; Muraki et al., 2000; Nagarkatti, 2000; Ramp et al., 2000; Eichhorst et al., 2001). Fas is a cell-surface receptor, which, when engaged by FasL (Fas ligand), recruits a number of cytoplasmic factors, including FADD, and initiates a rapid and irreversible apoptotic pathway (Eischen and Leibson, 1997; Chen and Faller, 1999; Newell and Desbarats, 1999). Exposure to α -lipoic acid or other related short-chain fatty acids, like butyrate, have been suggested to induce apoptosis through Fas (Eischen and Leibson, 1997; Chen and Faller, 1999; Newell and Desbarats, 1999; Sen et al., 1999; Hara et al., 2000). Additionally, there are novel FADD-dependent, but Fas-independent, apoptotic pathways operative in some transformed cells (Chen et al., 2001). Our finding of efficient α -lipoic acid-mediated apoptosis in both Fas- and FADD-deficient cells, however, suggests that the pro-apoptotic action of α -lipoic acid on transformed cells is not mediated through the Fas-FADD pathway.

Rather than apoptosis, a reversible G₁ cell cycle arrest was the characteristic response of normal, untransformed cells after exposure to α -lipoic acid, lipoamide, or octanoic acid. Cyclin D-Cdk4/6 complexes are critical in regulating G₁-S transition and inhibition of cyclin-Cdk complexes occurs through the action of cyclin-dependent-kinase inhibitors (CDKIs). We hypothesized that a CDKI could be the effector of the growth arrest of untransformed cells induced by short-chain fatty acid derivatives. Indeed, CDKIs such as p21^{Cip} and p27^{Kip1} have been shown to mediate a G₁ arrest in response to an array of stimuli, particularly DNA damage or mitogen withdrawal (Polyak et al., 1994; Toyoshima and Hunter, 1994). We have shown previously that the CDKI p21^{Cip} is not necessary for G₁ arrest induced by the prototypic short-chain fatty acid butyrate, despite being strongly induced by butyrate (Vaziri et al., 1998). p27^{Kip1} is another member of the universal CDKI family, whose members inhibit both CDK4/6 as well as CDK1/2 kinases (Fero et al., 1996). We have recently discovered that elevation of p27^{Kip1} is necessary and sufficient for the cell cycle arrest induced by histone deacetylase inhibitors (J. Chen and D. Faller, submitted). Our findings herein suggest that induction of p27^{Kip1} is necessary for the G₀/G₁ arrest induced by α -lipoic acid. We found that p27^{Kip1}-deficient cell lines did not arrest in response to α -lipoic acid, whereas p21^{Cip}-deficient cell lines did arrest. Half-life studies indicated that the induction of p27^{Kip1} by α -lipoic acid occurs at the level of protein stability. Stabilization of p27^{Kip1} protein has been a common feature in a number of experimental systems in which p27^{Kip1} is functionally regulated (Pagano et al., 1995; Eymin et al., 1999; Borriello et al., 2000; Chiarle et al., 2000).

Whether p27^{Kip1} induction mediates α -lipoic acid-induced apoptosis in transformed cells, however, is not yet clear. In normal cells, the presence of p27^{Kip1} seems

to protect against programmed cell death, but its induction or overexpression has been reported to induce apoptosis in a number of tumor cell types, including oral and oropharyngeal squamous-cell carcinomas, breast cancer cells, and B lymphoma cells (Katayose et al., 1997; Fujieda et al., 1999; Wu et al., 1999). Our data is consistent with such a mechanism, in that p27^{Kip1} was strongly induced in the tumor cell lines we studied, although the tumor cells did not undergo a G₁ arrest. Instead, these cells continued to progress through cell cycle and initiated apoptosis, raising the possibility that this failure to arrest in the face of high levels of p27^{Kip1} may be causally related to the induction of programmed cell death, although this connection has not been proven. In other recent studies, we have reported that α -lipoic acid induces differentiation in a variety of squamous cell carcinomas, as measured by the strong induction of involucrin expression (Krishna et al., 2002). This induction of differentiation was independent of histone deacetylase inhibition, appeared to be dependent upon p27^{Kip1} induction, and was specific to transformed or tumor cells. Although we have not yet causally linked the differentiation process to the initiation of apoptosis by α -lipoic acid in the system studied in this report, terminal differentiation and programmed cell death have been linked in a number of other systems.

Regardless of the mechanism underlying α -lipoic acid induction of apoptosis in tumor cells, the striking specificity of the pro-apoptotic effects of α -lipoic acid for transformed cells, as demonstrated using naturally-occurring tumors as well as paired cell lines, advances the possibility of its therapeutic application in cancer. α -Lipoic acid is already used in the treatment of a variety of pathologies, including liver and neurological disorders, where enhanced free radical peroxidation of membrane phospholipids have been shown (Dikalov and Khramtsov, 1997; Fuchs et al., 1997; Hermann and Niebch, 1997; Zimmer, 1997). In addition, plasma levels of α -lipoic acid in the millimolar range after oral dosing have been safely reached in pharmacokinetic studies. Based on the anti-tumor activity we report here, and the safety of this compound, we propose that in vivo studies of α -lipoic acid as a potential anti-neoplastic agent are warranted.

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