Multisite inhibition by phenylacetate of PC-3 cell growth

Joseph J. Bahl[a,b], Richard L. De Armond[c] and Rubin Bressler[a–c]

Phenylacetate (PA) is a reversible inhibitor of tumor cell growth and an inhibitor of mevalonate pyrophosphate decarboxylase (MPD). We hypothesized that MPD inhibition should lower rates of protein accumulation and accretion of cell number in all cell lines regardless of tumorigenic status or origin of the cell lines. PA treatment inhibited growth of MCF-7, NIH-3T3, Detroit 551, UT-2, NCTC-929, COS-1 and PC-3 cell lines. NCTC-929 cells lack cadherins and Cos-1 cells are deficient in PPARα and PPARγ, proteins suggested to be central to the action of PA. Oxidative metabolism was not impeded by PA treatment. One-dimensional and two-dimensional FACS analysis of BrdU incorporation failed to demonstrate a redistribution of nuclei in the cell cycle or that the rate of cells entering S phase had changed. Time-lapse photo-microscopy studies reveal a process that left condensed nuclei with little or no cytoplasm. However, negative TUNEL assay results and failure to block cell loss with z-VAD-fmk suggest this type of cell death is not typical apoptosis, but cell death is responsible for the lower rates of cell and protein accumulation. Supplementation studies with mevalonate pathway intermediates during inhibition of the mevalonate pathway of cholesterol biosynthesis by lovastatin confirmed MPD as a site of PA inhibition of growth, but in the presence of lovastatin with or without farnesyl pyrophosphate plus geranylgeranyl pyrophosphate, additive inhibition by PA revealed additional site(s). The existence of site(s) in addition to MPD suggests effective PA-based agents might be developed that would not inhibit MPD. *Anti-Cancer Drugs* 15:513–523 © 2004 Lippincott Williams & Wilkins.

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Introduction

Phenylacetate (PA) is a naturally occurring deaminated metabolite of phenylalanine found in human serum at low micromolar concentrations. Millimolar concentrations of PA have been reported to cause cell differentiation and inhibition of cell growth *in vitro* in a variety of tumorigenic cell lines including cells derived from prostate cancer, neuroblastoma and melanoma [1–5]. Rapid conjugation of millimolar concentrations of PA with glutamine and subsequent renal excretion provides effective treatment for hyperammonemia in children [6,7]. Sequestration of glutamine necessary for rapid tumor growth was a previously postulated rationale for studying PA as a potential antitumor agent. This mechanism is not, however, an explanation for the effect of PA on growth *in vitro* [8].

The amount of PA that must be infused to maintain any given plasma level of the parent PA molecule is increased by *in vivo* conjugation. While low millimolar concentrations of PA slow the accumulation of protein and cell numbers in culture, clinical trials have shown the therapeutic benefits of PA in the treatment of cancer to be limited [9].

Interest in PA increased with reports of its ability to reversibly induce a greater state of differentiation in cancer cells [3,10–12]. Functionally, this has been defined as an increase in the expression of proteins associated with a more differentiated state, loss of the highly invasive motility and slower cell growth that typically describes the earlier precancerous state. The details of how proteins associated with the more differentiated state accumulate depend on the protein studied. The mechanisms reported include selective increased transcription and translation or decreased protein degradation. The connections in the proteomic pathways that link these proteins remain to be elucidated, but suggest the possibility that the differentiated state of many cell types is maintained by a common mechanism that may be missing in many cancerous cells and at least partially restored by PA.

A number of different mechanisms have been proposed and of course all could be operating simultaneously. These include consequences of PPARα or PPARγ stimulation, increased cadherin levels, elevated $p21^{Waf1}$, inhibition of the mevalonate pathway of cholesterol biosynthesis and accelerated apoptosis.

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Cadherins are cell adhesion molecules that have altered expression in invasive cancerous cells. Different PPAR subtype stimulation is important in regulating fatty acid metabolism and progression to a more differentiated cell type. Previous studies reported PA treatment results in increased levels of these proteins and/or their activity [13,14]. The experimental design of those studies did not look at cells deficient in these proteins and/or cellular activity, leaving it an open question as to whether they play a central role in the action of PA.

Cell proliferation is more or less an ordered process requiring protein synthesis, DNA replication and cell division for both normal tissue and cancer cells. The distribution of nuclei in the phases of the cell cycle and the rate of progression through those phases is in large part the result of necessary protein accumulation and disinhibition of cell-cycle proteins. The details of where a block occurs can suggest which regulators of the cell cycle might be elevated. PA has been reported to increase the percentage of cells in G_0/G_1 [12,15–20], whereas some studies suggest this is a consequence of increased $p21^{Waf1}$ [21–23]. Others have not found any change in the distribution of nuclei in the cell cycle [24,25]. Prior to this study it was not known if active cell proliferation was required to see a PA effect on cell number.

Reports of apoptosis are generally associated with 10 mM or greater concentrations of PA, but there is no consensus on PA induction of apoptosis at lower concentrations [1,16].

Inhibition of the mevalonate pathway for the biosynthesis of cholesterol and other key cellular regulators (see Fig. 1) has been suggested to be central to the effects of PA and inhibition of mevalonate-5-pyrophosphate decarboxylase (MPD) by PA is well documented [26–28]. MPD is a key enzyme in the mevalonate pathway; the decarboxylation is an irreversible reaction which produces the isopentenyl pyrophosphate building block that supplies the carbon backbone for all subsequent pathway intermediates. The great majority of carbon that enters the mevalonate pathway is converted to cholesterol and smaller amounts are converted to other important isopentenyl. Mevalonate pathway intermediates and products such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are involved in the regulation of cellular function. Both FPP and GGPP have key roles in regulating the activity of proteins important for control of cell growth and the cell cycle such as $p21^{ras}$ and $p27^{kip1}$ [29,30]. Ras requires farnesylation with FPP for activation and $p27^{kip1}$ protein levels are negatively regulated by a small rho protein which in turn is regulated by geranylgeranylation. The rank order of the potency of PA analogs to inhibit growth is the same as their rank order for inhibiting $p21^{ras}$. Cell lines with higher levels of $p21^{ras}$ have been reported to be more effectively inhibited by PA [31].

Few studies have examined PA inhibition to see if sites other than MPD might exist or if the effects of PA resulted from buildup of substrate(s) prior to the blockage of MPD rather than depletion of pathway intermediates beyond MPD. The prostate cancer cell line PC-3 was utilized to evaluate the role of MPD in the mechanism of PA. To study the role of MPD, the first committed step in the mevalonate pathway was blocked with the statin drug lovastatin. The effect of inhibition at MPD by PA with and without pathway intermediate supplementation was then studied to examine the possibilities of additional sites of action might be discovered which might provide insight into improving the therapeutic utility of this agent.

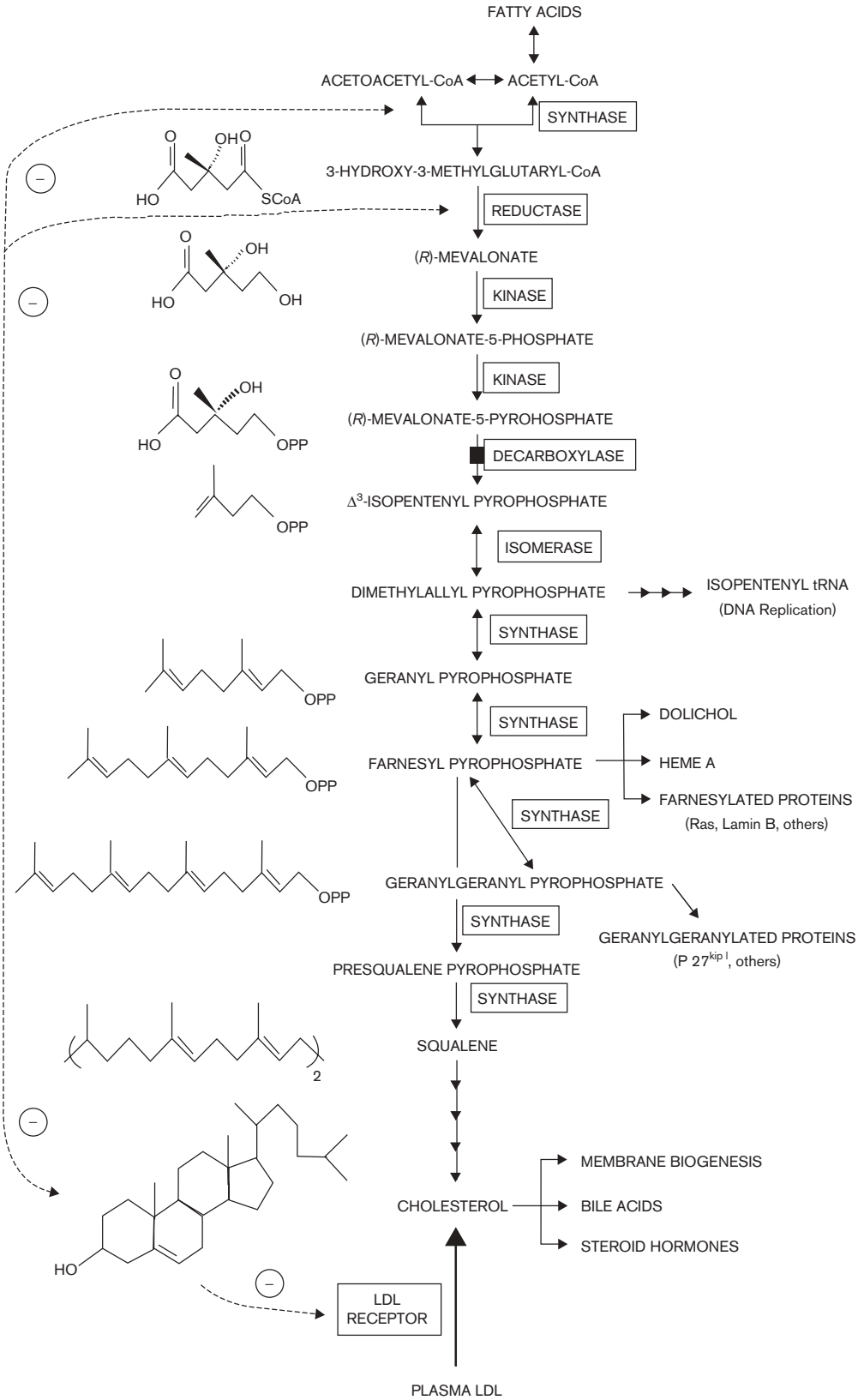
Materials and methods

The cell line UT-2 was a gift from Dr C. J. Waechter (Department of Biochemistry, University of Kentucky). All other cell lines were obtained from ATCC (Manassas, VA). All chemicals were purchased from Sigma (St Louis, MO) unless stated otherwise. RPMI 1640, Ham's F-12, Eagle's MEM, DMEM, 200 mM (100 ×) L-glutamine, 100 × insulin–transferrin–selenium (ITS), 4 × pancreatin (USP grade) and penicillin–streptomycin (P/S) were purchased from Life Technologies (Gaithersburg, MD). Fetal bovine serum (FBS) and horse serum were obtained from Hyclone (Logan, UT). Sodium PA was produced by vortexing 1.0 g of phenylacetic acid (Aldrich, Milwaukee, WI), 0.3 ml distilled water, 0.23 g sodium hydroxide and 10 ml acetonitrile. The solution was evaporated to dryness under a stream of air. Then, 20 ml of fresh acetonitrile was added, the mixture was again vortexed and centrifuged, and the acetonitrile decanted. These steps were repeated a total of 3 times to remove unreacted phenylacetic acid. After the acetonitrile was decanted for the third time, the product was dried under a stream of air. The sodium salt of PA was stored at room temperature prior to stock solutions being prepared. Alternatively, phenylacetic acid was weighed and the pH of the stock solution in cell culture media appropriate for each cell line was adjusted with NaOH to pH 7.38–7.42 at 37°C with an atmosphere of 5% CO_2/95% air and filtered sterilized.

Cell lines

We chose to include the cell lines PC-3 and MCF-7 because most of the published studies on PA focused on the effect of PA on these cell lines derived from tumorigenic cells. The PC-3 cell line is a human androgen-independent *ras*-driven prostate cancer cell line. MCF-7 is a hormone-dependent human breast cancer cell line. Immortalized fibroblasts, NIH-3T3 cells and a non-immortalized fibroblast line Detroit 551 were

Fig. 1



The mevalonate pathway of isoprenoid pyrophosphates and cholesterol biosynthesis. The synthesis of mevalonate is the first committed step as well as the rate-limiting step of this pathway. The bulk of the mevalonate produced by this pathway forms cholesterol. Cholesterol is also supplied to the cell exogenously via the receptor-mediated uptake of plasma LDL. Feedback inhibition of cholesterol on the mevalonate pathway is shown to the left of the pathway. Non-sterol isoprenylated products are shown to the right of the pathway. The position of PA's inhibition of MPD is denoted by the solid square.

included in our studies as examples of other replicating cell lines. NTCT-929 cells are fibroblast-like cells isolated from mouse connective tissue that is deficient in cadherins. UT-2 cells are a mutant immortalized CHO cell line deficient in mevalonate production; however, when it became clear that residual mevalonate would complicate interpretation of results, the PC-3 cell line, which can be growth-inhibited by the mevalonate pathway inhibitor lovastatin, was selected for this and many subsequent experiments reported herein. Cos-1 cells are derived from African green monkey kidney cells, and lack functional PPAR α and γ receptors. Rat heart cells in primary culture were studied because of their low potential to proliferate in culture and represent an example of non-dividing, terminally differentiated cells.

Periodically, coverslips of all the cell lines used in this study were stained with Hoecsht reagent 33258 (Kodak, Rochester, NY) and this result was confirmed with a PCR based mycoplasma detection kit (#90-1001K; ATCC). The cell lines PC-3, MCF-7, NIH-3T3 and Detroit 551 were incubated in RMPI 1640 media supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin (P/S), 2 mM L-glutamine and 10% heat-inactivated FBS (30 min at 52°C). The MCF-7 cell line was additionally supplemented with 20 µg/ml of insulin (25.7 U/mg). The NCTC-929 cells were maintained in Eagle's MEM supplemented with P/S, 2 mM L-glutamine and 10% horse serum. The UT-2 cell line was maintained in Ham's F-12 supplemented with 200 µM mevalonic acid, 2 mM L-glutamine, P/S, 2% ITS and 10% FBS. COS-1 cells were incubated in DMEM supplemented with P/S and 10% FBS. The pH of all media ranged from 7.38 to 7.42 at 37°C with 5% CO_2/95% air in a humidified incubator. The cell lines were grown on Falcon 100-mm Petri dishes and split 1:4 when the cell density reached 70–80% confluence. Fresh media was changed 3 times a week as the cells were split, replated or allowed to grow.

Primary cultures of neonatal rat cardiac myocytes

Tissue harvest for primary cultures was conducted under a protocol approved by the Institutional Animal Care and Use Committee of the University of Arizona. Standard techniques of enzymatic digestion were used to disaggregate myocytes from the ventricles of neonatal rat hearts [32]. After initial isolation, the cells were differentially plated to decrease fibroblasts. The cells were counted with a hemocytometer and plated onto 100-mm tissue culture Petri dishes for overnight attachment. At time 0, fresh media with or without 4 mM PA was added, and after 2 days the cells were counted in one set of control and treated plates, and the amount of protein determined on a parallel set of plates.

Drug experiments on cultured cell lines

Previous *in vitro* studies investigating the actions of PA using cell lines have often been conducted at a single concentration around 4 mM [27–33]. At a concentration that was 0.5 log unit lower we found it was difficult to establish a statistically significant difference between control and treated cell protein after 2 days of treatment. At a concentration 0.5 log unit higher, cell loss was readily apparent and would preclude the opportunity to study the PA effect of slowing the rate of growth, thus 4 mM PA was used in these studies. Consistency of the data was improved by verifying that the pH was between pH 7.38 and 7.43 at 37°C under 5% CO_2/95% air for both the control and PA media, and that the degree of confluence at the start of each passage was closely matched.

Growth inhibition experiments in 24-well Falcon tissue culture plates ran for 48 h. Each plate had a control ($n = 4$) containing cells, drug vehicle and media. Forty thousand cells were plated in each well ($n = 4$) with a final volume of 800 µl/well. A sample of the cells transferred to the 24-well plate was tested for viability using the Trypan blue dye exclusion test. Pre-treatment of the cells involving prolonged exposure (more than 2 days) was conducted in 100-mm Petri dishes, with the cells being counted and transferred to 24-well plates for the final 2 days of the experiment. At the end of the experiments each well was rinsed twice with 1.0 ml HBSS and the cells lysed with 600 µl of distilled water, followed by three freeze–thaw cycles (–70°C, + 37°C). Two 100-µl aliquots from each well were analyzed separately on a 96-well plate and the values averaged for each separate sample. Protein measurements were utilized to quantify changes in treated cell growth as a percentage of untreated cell growth. Preliminary experiments comparing DNA, protein and cell number as a measurement of cell growth gave qualitatively similar results (data not shown).

Additional methods

Oxidation of ^{14}C-labeled glucose and palmitate was carried out in an apparatus modified for tissue culture from that previously described [34]. Cells were transferred to 24-well plates and allowed to attach overnight before being clamped into the flow through manifold and $^{14}CO_2$ trapped in 1.25 ml of 0.3 N sodium hydroxide with 7.7×10^{-3}% (v/v) Triton X-100. Data was collected after a 60-min period that resulted in linear rates of oxidation following initial thermal and isotopic equilibration in the apparatus. After addition of liquid scintillation fluid, the sample was capped, vortexed and set aside for 3 h to allow for the decay of chemiluminescence prior to liquid scintillation counting.

Protein concentrations were assayed using the Pierce (Rockford, IL) bicinchoninic assay [35]. All data obtained from the 7520 Cambridge Technologies microplate reader

for protein analysis was imported into Sigma Plot (version 8) for analysis and graphing.

FACS analyses were made by the Arizona Cancer Center Flow Cytometry core facility. The method of Dressler *et al.* [36] was followed for one-dimensional (1-D) FACS analysis and for two-dimensional (2-D) FACS analysis of DNA and BrdU incorporation [37]. The TUNEL assay was conducted following the kit instructions provided by the manufacturer (Promega, Madison, WI).

Time-lapse images of heart cells were captured every 2 min for 26 h by NIH Scion 1.6 Image software capturing a field of approximately 150 cells magnified $\times$ 10 on a Nikon inverted microscope while the cells were incubated at 37°C in a humidified plexiglass chamber with CO_2, constantly infused to maintain the atmosphere at 5% CO_2. No cell division was observed in the primary cultured myocytes; however, cell division was frequently observed with the PC-3 cell line. Images were cropped in Adobe Photoshop (version 6) to focus on individual cells. Similarly images of PC-3 cells were collected for 48 h, but because of significant mobility of PC-3 cells the coordinates of the cropped image were changed to follow the cell across time.

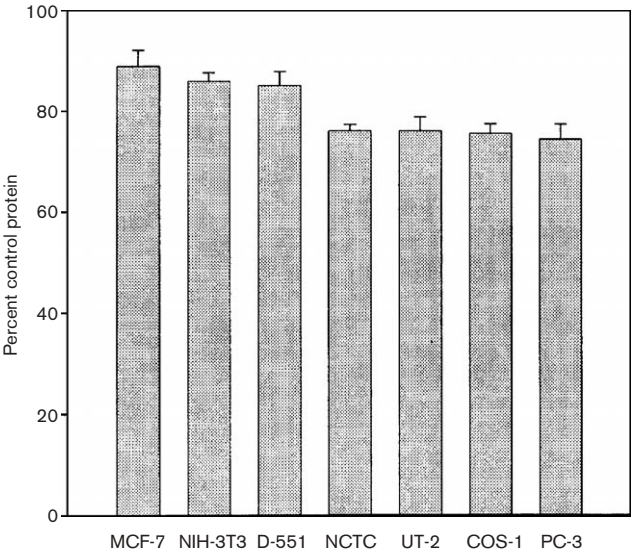
Results

The growth-inhibiting effect of PA was observed in all cell lines studied (Fig. 2). As detailed in the figure legends, the cell lines represent both tumor and non-tumorigenic cell lines. Cos-1 cells have been utilized in studies because they lack functional PPARα and PPARγ receptors [38,39]. However, PA-induced growth inhibition in Cos-1 cells was seen at a level very similar to the other cell lines studied. NCTC-929 cells lack cadherins [40], but showed similarly inhibition of growth. For all cell lines there was no obvious correlation between the magnitude of the PA effect and the rate of growth in cell culture.

The effect of prolonged incubation time with PA was studied in the cell line PC-3 (Fig. 3); however, all of the cell lines tested with prolonged periods of PA incubation showed slower accumulation of cell number than the untreated controls. Upon removal of PA from PC-3 cell media, the rate of cell growth readily returned to that of the control cells (Fig. 3).

Lower rates of cellular growth were not due to inhibition of oxidative metabolism as [^{14}C]glucose oxidation in treated cells was not different than control cells measured at various times up to 15 days (data not shown) and [^{14}C]palmitate oxidation actually increased at longer times in culture (Fig. 4). This increase in labeled palmitate oxidation is consistent with PA being a PPARα agonist [14] and this agent's effect on cellular handling of fatty acids [41].

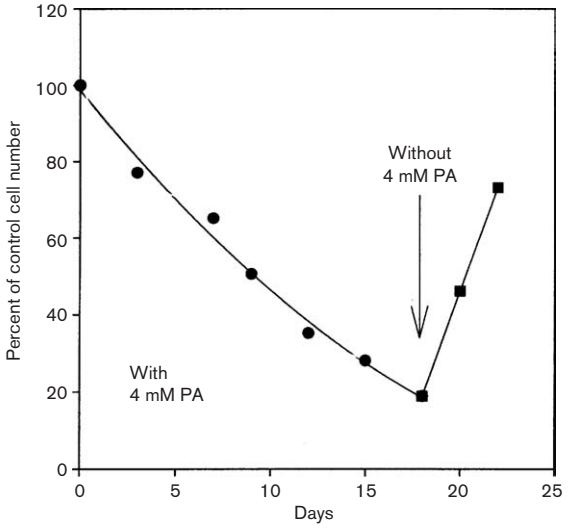
Fig. 2



Inhibition of growth in selected cell lines by 2 days of 4 mM PA incubation. The MCF-7 (hormone-dependent breast cancer cell), NIH-3T3 (immortalized fibroblast), Detroit 551 (a non-immortalized fibroblast), NCTC-929 (isolated from mouse connective tissue, a mouse fibroblast-like cell line deficient in cadherins), UT-2 (mutant immortalized CHO cell line deficient in mevalonate production), COS-1 (green monkey kidney cells deficient in the superfamily of estrogen receptors) and PC-3 (*ras*-driven prostate cancer cell) cells were incubated with and without 4 mM PA for 2 days ($n=4$) at a starting density of 40 000 cells/well in a 24-well tissue culture plate. Protein accumulation was measured in cells grown with or without PA and percent of control protein calculated. All PA-treated cells were significantly different from their respective control groups by 2 days ($p<0.02$, Student's *t*-test). Both the control and PA-treated UT-2 cells were supplemented with 200 μM mevalonate.

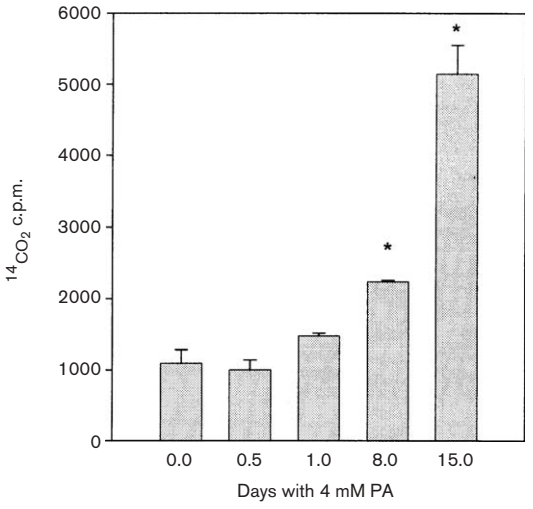
To find out if treated cells accumulated at a slower rate because they took a longer period to transverse the cell cycle, the distribution of nuclei in various phases of the cell cycle was studied using FACS analysis. Lovastatin has been reported to decrease cell-cycle transit by PC-3 cells. PC-3 cells were not allowed to become confluent and were passaged 3 times a week with the final passage always 2 days prior to FACS analysis, and a constant number of treated and untreated cells being cultured for each experiment. In addition, the media was replaced with fresh media with or without PA on the evening before analysis. A specific block of cells in one phase of the cell cycle was not observed and as shown in Figure 5 by standard 1-D FACS analysis, there was no difference between the percentage of cells in S phase treated with or without PA. To eliminate the possibility that all phases of the cell cycle were contributing to a longer generation time, cells were studied with 2-D FACS analysis. PC-3

Fig. 3



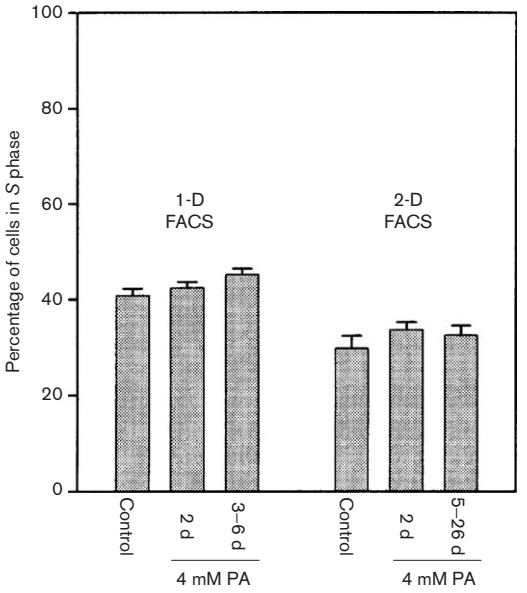
Time course and rebound of PC-3 cells exposed to PA, expressed as a percentage of paired control cell number. Three times a week, including 2 days prior to the data points appearing on the graph, control and treated cells were counted with a hemocytometer and 2×10^6 cells were plated on a 100-mm plate ($n=2$). Treated cells were starting at day 0 and cultured with 4 mM PA. Typically, after 2 days the number of control cells per plate had increased to 5.6×10^6 cells, but actual cell counts for each data point were used in calculating percent. At day 18, cells that had been treated with PA were switched to the standard media and percentages of control cell number calculated for subsequent days of recovery.

Fig. 4



[^{14}C]Palmitate oxidation in PC-3 cells treated with 4 mM PA for various times. Total counts of $^{14}CO_2$ produced by cells metabolizing [^{14}C]palmitate over a 20-min interval. Expressed as c.p.m./20 min/10^6 PC-3 cells. *$p<0.05$ [unpaired Student's t-test compared with control ($n=3$)].

Fig. 5



Comparison of 1- and 2-D FACS analysis of S phase. The values of individual plates of PC-3 cells were averaged (±SEM). No significant difference was found between treated values and untreated control. Values presented combined triplicate sets of cells each containing control and PA treatment at 2 day and beyond 2 days.

cells were labeled by addition of 40 μM of the thymidine analog BrdU for 30 min prior to harvesting. This short period was picked to maximize BrdU incorporation into S phase and minimize BrdU labeling of other cell-cycle phases. With this short period for BrdU incorporation a smaller percentage of cells were found to be in S phase by 2-D FACS compared with 1-D FACS analysis. However, there was no difference between the control and treated values for 1- or 2-D FACS and their respective control groups, suggesting that slower progression through the cell cycle is not responsible for the progressive slowing of cell accumulation. To demonstrate that the effect of PA was not dependent on cells transiting the cell cycle we studied primary cultures of rat heart cells which do not appreciably proliferate in culture. The cardiac myocytes that were treated with PA were found to have a significant reduction in the number of cells in culture after 2 days of treatment (see Table 1).

To determine if cell loss from apoptosis contributed to the decreased rate of cellular accumulation, control TUNEL-positive nuclei (four out of 673) and PA-treated PC-3 cells (five out of 670) were compared, without a significant difference being observed. The inhibitor z-VAD-fmk blocks the caspase cascade of classical apoptosis [42]. It was added in an attempt to block apoptosis and have more cells accumulate. The effect of

Table 1 Protein and cell numbers of primary cultures of rat cardiomyocytes incubated with and without PA for 2 days

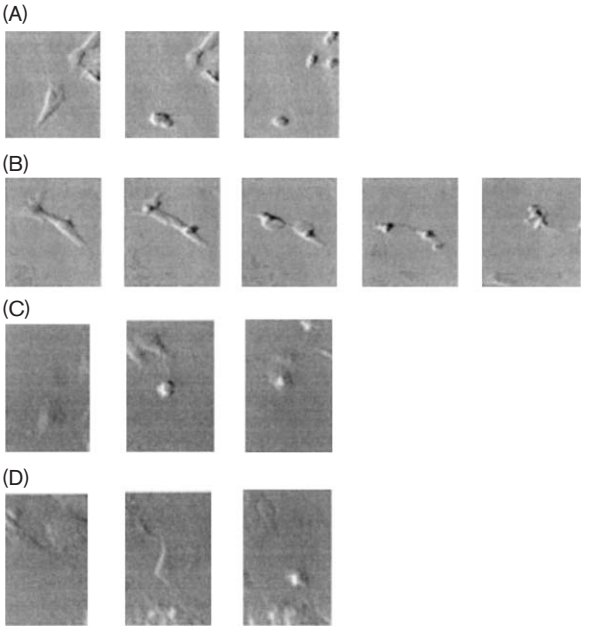
	Protein (μg)	Cell number
No treatment	13.58	988000
4 mM PA treatment	8.88	438000

After being rinsed twice with PBS one set of paired plates was lysed for protein analysis, while the other set was trypsinized, centrifuged and resuspended prior to the cells being counted. Values are the average of two experiments on different days.

PA on the cells, however, was not altered by incubation with the pan-specific inhibitor of the caspase cascade (data not shown). Limited vacuolization was observed in all of the PC-3 cells with or without PA treatment, although occasionally cells appeared to be undergoing an alternative pathway of programmed cell death, in which large clear fluid vacuoles filled the interior of the cell suggesting the process termed parapoptosis [43]. Figure 6 shows selected frames of time-lapse image capture of heart cells in culture treated with PA. While myocytes slowly spread over the surface of the tissue culture plate until contact was established with other myocytes, fibroblasts actively moved over the surface of the plate, and appeared able to move over the top of myocytes and between adherent myocytes and the tissue culture plate, sometimes altering how the cell contacted and attached to the plate. Minimal cell loss was observed in myocytes incubated in control media (data not shown). Cells appeared to loose their cytoplasm, thus leaving condensed nuclei loosely attached or floating on the surface of the plate that likely would be lost during media changes or any procedural rinse steps (Fig. 6A and B). Other cells in the same general proximity remain unchanged. No example of cell loss by non-swollen cells lifting from the plate surface was observed. There did not appear to be a requirement for interaction between fibroblasts and myocytes for the phenomena of cell death to occur. While PC-3 cells are known to display both round and flat morphologies, only flat cells in colonies appeared to remain relatively immobile on the plate. It was necessary to follow single cells images sequentially to assure the identity and fate of individual cells because of the mobility of the PC-3 cells. Although the final result of the process in the two types of treated cells appears similar (Fig. 6), the images of the heart cells showed more pronounced and extensive swelling over protracted times compared to PC-3 cells.

In trying to establish the mechanism of action of PA the role of MPD inhibition by PA was studied. We collected data (Table 2) using the Chinese hamster ovary cell line UT-2 which is deficient in inducible HMGCoA reductase with less than 5% of the basal activity of the parent cell line [44,45]. However, the data in UT-2 cells, while suggestive, could not definitively be interpreted because the residual HMGCoA reductase activity, while small,

Fig. 6



Images at various times after PA treatment. Images of cells magnified × 10 containing approximately 100–200 cells per microscope field were captured every 2 min. Myocytes (A and B) or PC-3 cells (C and D) after 48 h in culture were changed to fresh media containing 4 mM PA. Each panel follows a cell that was unambiguously tracked and where the last image did not change appreciably during the remaining time of data collection (26 h myocytes, 48 h PC-3 cells). Times of images shown in (A): (1) 2 h 28 min, (2) 2 h 40 min, (3) 26 h 40 min; (B): (1) 0 h 2 min, (2) 3 h 8 min, (3) 3 h 20 min, (4) 7 h 24 min, (5) 26 h 40 min; (C): (1) 30 h 48 min, (2) 35 h 54 min, (3) 45 h 30 min; and (D): (1) 35 h 54 min, (2) 40 h 40 min, (3) 45 h 30 min.

was still present. We chose to study the PC-3 cell line treated with a concentration of lovastatin sufficient to completely block entry into the mevalonate pathway at a step prior to MPD. Initial studies were performed to establish the concentrations of mevalonic acid or isoprenoid compounds, including FPP and GGPP which had only a biologically small effect on protein accumulation in control cells (see Table 2). Addition of PA to control cells reduced protein accumulation to 77.6% of the control value, while addition of mevalonate did not significantly alter this number. If the only site of PA action had been inhibition of MPD in the PC-3 cells then either GGPP or FPP supplementation might be expected to restore the levels of protein accumulation in PA-treated cells to values closer to the uninhibited control value. Partial restoration was observed when these intermediates were supplemented in the cell culture, but paradoxically the combination of GGPP and FPP supplementation resulted in PA having a greater inhibitory effect on protein accumulation ($68.7 \pm 1.1\%$ of the uninhibited control, 73.4% of the GGPP + FPP supplemented value).

Table 2 PA's effects on cells supplemented with mevalonate pathway intermediates and blockers

	Conditions	PC-3	*n*	UT-2	*n*
Control	no Mev	100.0 ± 0.7	28	42.6 ± 2.1	16
	200 μM Mev	98.2 ± 2.3	8	100 ± 2.2	32
	20 μM IPP	92.4 ± 3.7	4	43.3 ± 0.5	4
	20 μM GGPP	92.4 ± 1.2	16	79.5 ± 1.1	16
	20 μM FPP	95.3 ± 2.2	20	70.8 ± 3.6	8
	IPP + FPP	103.1 ± 2.1	4	71.3 ± 1.8	4
	GGPP + FPP	92.4 ± 1.8	12	91.7 ± 1.7	4
4 mM PA	No Mev	77.6 ± 1.5	16	20.0 ± 1.6	12
	200 μM Mev	78.7 ± 0.9	4	80.0 ± 2.0	16
	20 μM GGPP	87.5 ± 2.3	4	67.0 ± 4.7	4
	20 μM FPP	82.3 ± 0.8	4	19.2 ± 1.9	4
	GGPP + FPP	68.7 ± 1.1	12	57.5 ± 2.0	4
20 μM Lov	No Mev	42.3 ± 1.9	16	16.8 ± 2.0	20
	200 μM Mev	96.6 ± 1.0	8	103.3 ± 3.3	12
	20 μM GGPP	72.5 ± 1.7	12	74.6 ± 1.0	4
	20 μM FPP	50.3 ± 2.8	8	20.4 ± 2.7	8
	IPP + FPP	61.6 ± 3.1	4	17.2 ± 0.4	4
	GGPP + FPP	81.2 ± 1.4	8	84.0 ± 4.1	4
	4 mM PA	32.4 ± 1.0	8	13.4 ± 1.1	8
	PA + Mev	68.0 ± 3.2	4	66.3 ± 1.6	4
	PA + GGPP	69.7 ± 0.9	4	39.7 ± 0.4	4
	PA + FPP	46.5 ± 1.5	4	28.4 ± 3.5	4
	PA + GGPP + FPP	65.6 ± 1.5	4	48.9 ± 3.0	4

For each condition reported, values represent the calculated average of *n* experiments ± SEM. The amount of protein measured in PC-3 cells without drug or supplementation of mevalonate pathway intermediates harvested on a given day was defined as being 100% of the average growth of cells ($n=4$). Similarly, the growth of UT-2 cells in media supplemented with 200 μM mevalonate (Mev) was defined as control with the average of 100%. Percentages were calculated for each treatment relative to the control value for that day, values for all days were combined and the mean ± SE calculated. Lovastatin (Lov), isopentenyl pyrophosphate (IPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) were added at a concentration of 20 μM each. Phenylacetate when included for the 2 days of incubation was at a final concentration of 4 mM.

Simultaneously, we gathered data in PC-3 cells treated with 20 μM lovastatin. While lovastatin might not be expected to inhibit protein synthesis directly, the lack of mevalonate pathway intermediates limits many biological processes and is known to be an effective blocker of cell cycle progression. Thus the protein accumulation in the lovastatin-treated cells was only 42.3 ± 1.9% of the uninhibited control. While supplementation with mevalonate (96 ± 1.1%) effectively reversed the effect of lovastatin, GGPP (72.5 ± 1.7%) and FPP (50.3 ± 2.8%) were less effective at reversing the inhibition than the combination of GGPP + FPP (81.2 ± 1.4%) (Table 2).

Cells treated with lovastatin were concurrently treated with PA. In cells receiving no supplementation of pathway intermediates, PA had an additive inhibitory effect with lovastatin, reducing the protein to 76.6% of the value seen with lovastatin alone (lovastatin + PA 32.4 ± 1.0%, lovastatin 42.3 ± 1.9%). The PA effect beyond lovastatin alone was observed under conditions when the contribution of MPD to the process should be minimal and suggests at least one additional site of action.

Addition of PA to lovastatin-inhibited PC-3 cells supplemented with either GGPP or FPP resulted in small changes in the observed levels of protein for these conditions from the values without PA (72.5 ± 1.7 to 68.0 ± 3.2% and 50.3 ± 2.8 to 46.5 ± 1.5%, respectively). However, the change in protein accumulation for lovastatin-treated cells supplemented with GGPP plus FPP and treated with PA resulted in a greater change (81.2 ± 1.4% without PA to 65.6 ± 1.5% with PA, the later value being 72.6% of the former). The experimental paradigm is similar to that described for lovastatin + PA (MPD denied substrate by the actions of lovastatin) except that here supplementation of the two pathway products (GGPP + FPP) result in significantly greater growth, while suggesting site(s) of action for PA beyond inhibition of MPD.

Discussion

The normal and neoplastic cell lines studied demonstrated decreased rates of accumulation of protein and/or cell number when cultured with PA, thus the effect is general and not restricted to cell lines derived from tumors. The magnitude of the decrease became greater with prolonged incubation, but this effect was reversed over a few days upon withdrawal of PA.

Previously we reported that hepatic gluconeogenesis was inhibited by 1 mM PA as phenylacetyl CoA, rather than PA itself, and inhibited pyruvate carboxylase without inhibiting oxidative metabolism [46]. At the higher concentration of PA used here, glucose oxidation was not changed in the PC-3 cells, suggesting that energy derived from this substrate was not impeded. At this higher concentration of PA, [^{14}C]palmitate oxidation increased, consistent with PA being a PPARα activator and altering fatty acid metabolism.

If PA were directly decreasing the rate of PC-3 cell growth it should either have changed the distribution of nuclei in the cell cycle or all of the phases of the cell cycle should become elongated. The 1- and 2-D FACS analysis of BrdU incorporation did not reveal such a pattern of change. Minor changes observed in the BrdU incorporation day to day were not significant when PA treatment was analyzed across time. The reason why previous investigators have observed PA-treated cells accumulate in the G_1 phase, while other investigators found no such effect is not obvious. Increasing levels of $p21^{Waf1}$ often repress apoptosis while inhibiting progression in the cell cycle [47]. Treatment with the PA analog phenylbutryate has been reported to require $p21^{Waf1}$ for apoptosis to occur in MCF-7 cells [48]. This suggests that the role of $p21^{Waf1}$ in repressing or promoting apoptosis may be dependent on the treatment given. Our studies do not address the role of $p21^{Waf1}$ in this process or how that role might change with altered levels of mevalonate pathway intermediates. It is interesting to note that the effect of PA was additive to that of the statin drugs which inhibit

the cell cycle in G_0/G_1. However, a report of PA treatment in conjunction with statin treatment of human malignant glioma cells suggests that the effect of treatment is the result of apoptosis and not an effect on growth *per se* [1]. Additionally, the rat cardiomyocytes in primary culture, which do not proliferate in culture, decreased in number with PA treatment. Taken together, the PA effect appears to be independent of the rate of cell proliferation and suggests that cell loss is responsible for the lower rate of accumulation.

The TUNEL assay results, however, did not provide evidence of a differential rate of apoptosis in treated and untreated cells, nor did the caspase cascade inhibitor Z-VAD-fmk block PA action. While it is clear that cell loss occurred, classical apoptosis was not established. We hypothesized that cells might be de-attaching from the tissue culture plastic at a constant rate and, then, rapidly undergoing apoptosis. However, time-lapse photography did not support this theory. Instead it appeared that the cells lose their cytoplasm and end up with condensed nuclei. With the lack of effect in the caspase inhibitor experiment and the results of the TUNEL assay we are unable to claim classical apoptosis as the mechanism by which this occurs. However, the loss of cytoplasm and observation of condensed nuclei suggests that PA has its effect via an apoptotic process nonetheless.

The effect of PA on cellular accumulation may or may not be separate and distinct from its action as an agent capable of inducing differentiation, an effect not studied in this report. The PPAR family of receptors might represent one possible way that the effects of PA might be linked. However, Cos-1 cells which lack PPAR activity showed the typical PA inhibitory effect, thus the PPAR receptor itself is not likely to be central to the mechanism by which PA limits the rate of cell accumulation. NCTC-929 cells, which lack cadherins, also showed a PA effect, again suggesting that the mechanism of PA activity does not require this protein, but PPAR- and cadherin-regulated pathways may participate in altering the susceptibility to apoptosis and such downstream regulatory roles were not ruled out by the current work.

The first step in the mevalonic acid pathway of cholesterol biosynthesis (Fig. 1) is inhibited by statin drugs resulting in decreased levels of isoprenoids in the cell. Because many proteins require farnesylation or geranylgeranylation to distribute in the cell and function in signaling and other cellular activities, progression in the cell cycle and cell growth are inhibited by this class of drugs. Similarly, inhibition of MPD in the mevalonate pathway by PA has been shown to decrease growth. Cuthbert and Lipsky reported that inhibition of MPD by 6-fluoromevalonate decreased the growth rate of human B lymphoblastoid cells *in vitro* and concluded that the build-up of mevalonate or one of the mevalonate phosphates was key to inhibiting growth [49]. The addition of 200 μM mevalonate, while adequate to maintain growth in the presence of lovastatin, did not augment the growth inhibition caused by 4 mM PA ($77.6 \pm 1.5\%$ no mevalonate, $78.7 \pm 0.9\%$ plus mevalonate). Additionally the inhibition by lovastatin ($42.3 \pm 1.9\%$) resulted in a smaller amount of protein accumulation in the presence of PA ($32.4 \pm 1.0\%$). This suggests that PA inhibition of MPD in PC-3 cells is independent of a build-up of mevalonate and its pyrophosphates. The later results also suggest the possibility that MPD is not the only site of PA action as MPD would lack substrate in cells where the mevalonate pathway was blocked by lovastatin and yet the action of PA was still additive. That same conclusion can be deduced from the experiment with PC-3 cells inhibited with both lovastatin and PA with supplementation of GGPP + FPP showing greater inhibition than cells inhibited with only lovastatin and similarly supplemented. It is not known if these results taken together reveal multiple sites or just one additional site.

Danesi *et al.* [31] reported studies in an androgen-specific LNCaP-based cell line overexpressing $p21^{ras}$ with supplementation of 3 μM FPP, 3 μM GGPP and the combination of these pyrophosphates partially reversed the effect of PA on growth. Their results, however, are clearly different from this study, as their culture conditions of 30 mM lovastatin with 2–10 μM PA failed to show any additive growth inhibition effects. They concluded that inhibition of protein isoprenylation and specifically $p21^{ras}$ farnesylation by PA increased chemosensitivity to growth inhibition and apoptosis. Similar to our findings, synergistic interaction between lovastatin and sodium PA has previously been reported [27].

In our PC-3 cells treated with lovastatin, an additive inhibitory effect of PA was not observed in cells supplemented with either FPP or GGPP alone. This would be expected if MPD was the sole site of inhibition. However, the additive effect of PA to lovastatin inhibited cells with or without GGPP + FPP suggests that PA inhibition occurs at an additional site or sites other than MPD and that the magnitude of the inhibition may be greatest under these conditions. We therefore hypothesize that maximal growth inhibition by a PA-like agent might be obtained with an agent that did not inhibit MPD and production of mevalonate pathway products. It is not known if the effect of PA seen when GGPP + FPP was supplemented in lovastatin-treated cells is at the same site of inhibition seen when cells were not supplemented. All such sites, however, represent potential opportunities for developing more selective and effective pharmacological agents.

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