1,25-Dihydroxyvitamin D_3 Increases the Toxicity of Hydrogen Peroxide in the Human Monocytic Line U937: The Role of Calcium and Heat Shock

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Abstract. 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) increases synthesis of heat shock proteins in monocytes and U937 cells and protects these cells from thermal injury. We examined whether 1,25-$(OH)_2D_3$ would also modulate the susceptibility of U937 cells to H_2O_2-induced oxidative stress. Cell viability was assessed by trypan blue exclusion and [^{3}H]thymidine incorporation into DNA. Prior incubation for 24 h with 1,25-$(OH)_2D_3$ (25 pM or higher) unexpectedly increased H_2O_2 toxicity. Since cellular Ca^{2+} may be a mediator of cell injury we investigated effects of altering extracellular Ca^{2+} ($[Ca^{2+}]_e$) on 1,25-$(OH)_2D_3$-enhanced H_2O_2 toxicity as well as effects of 1,25-$(OH)_2D_3$ and H_2O_2 on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_f$). Basal $[Ca^{2+}]_f$ in medium containing 1.5 mM Ca as determined by fura-2 fluorescence was higher in 1,25-$(OH)_2D_3$-pretreated cells than control cells (137 versus 112 nM, $P < 0.005$). H_2O_2 induced a rapid increase in $[Ca^{2+}]_f$ (to > 300 nM) in both 1,25-$(OH)_2D_3$-treated and control cells, which was prevented by a reduction in $[Ca^{2+}]_e$ to less than basal $[Ca^{2+}]_f$. The 1,25$(OH)_2D_3$-induced increase in H_2O_2 toxicity was also prevented by preincubation with 1,25-$(OH)_2D_3$ in Ca^{2+}-free medium or by exposing the cells to H_2O_2 in the presence of EGTA. Preexposure of cells to 45°C for 20 min, 4 h earlier, partially prevented the toxic effects of H_2O_2 particularly in 1,25-$(OH)_2D_3$-treated cells, even in the presence of physiological levels of $[Ca^{2+}]_e$. Thus 1,25-$(OH)_2D_3$ potentiates H_2O_2-induced injury probably by increasing cellular Ca^{2+} stores. The 1,25-$(OH)_2D_3$-induced amplification of the heat shock response likely represents a mechanism for counteracting the Ca^{2+}-associated enhanced susceptibility to oxidative injury due to 1,25-$(OH)_2D_3$.

ORGANISMS which use molecular oxygen are exposed to the toxic byproducts of oxygen metabolism (20). Several reactive oxygen intermediates may be generated during respiration including hydrogen peroxide (H_2O_2). Some of these reactive oxygen intermediates are used by mononuclear phagocytes for microbiocidal and tumoricidal functions (33, 34); reactive oxygen intermediates, however, may also induce cellular damage (44). Thus, in the context of inflammation, monocytes not only produce reactive oxygen but are also vulnerable to oxidative injury.

The hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)[1] primarily affects cells in intestine, bone, and kidney, where it binds to specific intracellular receptors (6, 35). The 1,25-$(OH)_2D_3$ receptor is homologous in several respects to other steroid receptors (29). The complex of 1,25-$(OH)_2D_3$ bound to its receptor interacts with DNA to facilitate the transcription of several genes such as that which codes for the intestinal calcium-binding protein (17, 19, 25, 47). Recently, significant numbers of receptors of 1,25-$(OH)_2D_3$ have been measured in other normal or neoplastic cells, particularly monocytes (7, 40) and the monocytic line U937 (36). There is evidence that 1,25-$(OH)_2D_3$ acting via these receptors can modulate several functions of monocytes in inflammatory and immunological reactions (1, 4, 8). One of these functions is production of reactive oxygen species. Although 1,25-$(OH)_2D_3$ does not by itself induce the production of superoxide anion by U937 cells, it does increase the production of superoxide anion in response to phorbol ester (41). 1,25-$(OH)_2D_3$ also protects human monocytes (37) and U937 cells (39) from thermal injury.

Exposure of most cells to elevated temperatures or to a variety of other forms of injury including oxidative injury induces a typical physiological reaction, termed the heat shock response (43, 46). This response is characterized by an inhibition of normal protein synthesis and the induction of synthesis of a specific set of proteins, the heat shock proteins (HSPs). Although the precise functions of the HSPs are not known, there is evidence that HSPs are essential for cells to survive exposure to these environmental stresses. In view of our observations that 1,25-$(OH)_2D_3$ protects monocytes from thermal injury we also questioned whether 1,25-

1. *Abbreviations used in this paper*: $[Ca^{2+}]_e$, extracellular Ca^{2+} concentration; $[Ca^{2+}]_f$, cytosolic free Ca^{2+} concentration; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; fura-2AM, acetylmethoxy ester of fura-2; HSPs, heat shock proteins; IMDM, Iscove's modification of Dulbecco's medium.

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$(OH)_2D_3$ would protect these cells from oxidative injury. Since calcium has been implicated as an important mediator of various types of cell injury in many different tissues (12, 30, 42), and calcium potentiates the injury to mitochondria induced by oxygen free radicals (28), we also examined whether H_2O_2 toxicity would be modified by changes in cellular calcium homeostasis.

We found that preincubation with 1,25-$(OH)_2D_3$ at physiological concentrations did not protect against but rather potentiated the toxic effects of H_2O_2 in U937 cells. This enhancement of H_2O_2 toxicity by 1,25-$(OH)_2D_3$ was partially reversed if the cells were exposed to H_2O_2 in calcium-free medium with or without EGTA and was abolished if the preincubation with 1,25-$(OH)_2D_3$ was carried out in calcium-free medium. Whereas exposure to H_2O_2 induced a similar rise in the concentration of cytosolic free calcium ($[Ca^{2+}]_f$) in both 1,25-$(OH)_2D_3$-pretreated and control U937 cells, the basal concentrations of $[Ca^{2+}]_f$ were higher in 1,25-$(OH)_2D_3$-pretreated cells. Finally, preexposure of U937 cells to elevated temperatures (45°C) did not prevent the increase in $[Ca^{2+}]_f$ but did protect the cells from death which resulted from exposure to H_2O_2.

Materials and Methods

Cells and Media

U937 cells were grown in stationary suspension culture in Iscove's modification of Dulbecco's medium (IMDM; Gibco, Grand Island, NY) containing 10% FCS (Bioproducts, Inc., Waranton, OR). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were exposed to H_2O_2 at 37°C either in IMDM with 10% FCS, or in MEM (Gibco) with or without calcium (formula No. 86-0084, with Na pantothenate substituting for Ca pantothenate). Preincubation with 1,25-$(OH)_2D_3$ was also performed in IMDM or McCoy's medium that did not contain calcium; in some of these preincubation experiments, the FCS was first dialyzed for 36 h against three changes of PBS that did not contain calcium.

Reagents

1,25-$(OH)_2D_3$ (provided by Dr. M. Uskokovic, Roche Laboratories, Nutley, NJ) was dissolved in ethanol as a stock solution of 1.6 mg/ml and diluted with medium before addition to U937 cells. The ethanol concentration did not exceed 0.01% and appropriate controls were performed to exclude an effect of the ethanol on H_2O_2 toxicity. H_2O_2 and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO). fura-2 and fura-2AM (acetylmethoxy ester of fura-2) were obtained from Molecular Probes, Inc., Junction City, OR.

Cell Viability

Cell viability was assessed using trypan blue uptake and $[^3H]$thymidine incorporation. The number of trypan blue–positive cells were counted among 200 cells in two individual microscopic fields. To avoid potential bias brought about by disappearance of nonviable cells total cell number was sampled at various times during the viability studies. Results presented in the form "% viable" reflect data collected from experiments where the total number of cells did not change. For the $[^3H]$thymidine uptake assay, cells were counted after exposure to H_2O_2, and 200 µl of a cell suspension of ~1.0 × 10^5 cells/ml for each condition were added to each well of 96-well plates (Falcon Labware, Oxnard, CA). After overnight culture, cells were labeled with 1 µCi/well of $[^3H]$thymidine (67 Ci/mmol; New England Nuclear, Boston, MA) for 6 h, then washed and collected with an automated cell harvester on glass filter strips (Mash-II; Flow Laboratories, Inc., McLean, VA), dried, and counted in Aquassure (New England Nuclear).

Evaluation of Cell Swelling

After exposure of U937 cells to various conditions, cells were centrifuged and resuspended in trypan blue. Cells were examined immediately by phase contrast with a Zeiss IM 35 inverted-stage microscope and photomicrographs prepared.

Exposure to Heat

The cells were heated in a waterbath in 15-cm culture dishes (Falcon Labware) for 20 min at 45°C in IMDM with 10% FCS. The temperature of the waterbath was kept constant by a circulating heating unit. The temperature of the culture medium reached that of the waterbath after ~8 min. We previously characterized the time course of induction of synthesis of HSPs and recovery of normal protein synthesis under these conditions in U937 cells (39). After exposure to heat, the cells were allowed to recover in the 37°C incubator for 2–24 h before exposure to H_2O_2.

Determination of Cytosolic Calcium Levels by fura-2 Fluorescence

Cells were loaded with fura-2 using techniques similar to those previously described (10). The culture medium was removed and the cells washed with PBS. The cells were then incubated in buffer containing 120 mM NaCl, 2.7 mM KCl, 1.4 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1.4 mM KH_2PO_4, 25 mM $NaHCO_3$, pH 7.4, 10 mM glucose, 20 mM Hepes, pH 7.4, and 1.5% gelatin. fura-2AM was then added to a concentration of 2.5 µM. Cells were loaded with fura-2 for 30–60 min. Once inside the cell, the acetylmethoxy ester is cleaved and the resultant fura-2 remains trapped within the cell. Other cells were treated in the same manner as the loaded cells and incubated in the same buffer except that dimethyl sulfoxide, the vehicle for fura-2AM, was added alone, without fura-2AM. These cells were used for the determination of autofluorescence. At no time did the dimethyl sulfoxide concentration of the incubation medium exceed 0.5%.

Before the measurement of fluorescence, cells were washed twice by centrifugation and resuspended at 1–2 × 10^6 cells/ml in 2 ml of buffer containing 145 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$, 1 mM Na_2HPO_4, 5 mM glucose, 20 mM Hepes, pH 7.4, and 0.5 mM $CaCl_2$. Fluorescence was measured with an Aminco-Bowman spectrofluorimeter (SLM Instruments, Inc., Urbana, IL) at emission wavelength of 510 nm and excitation wavelengths alternating between 340 and 380 nm. Cells were equilibrated at 37°C in a thermostated cell holder. Fluorescence was determined with care taken to check that there was no quenching with time. To minimize photobleaching, readings were taken intermittently with the incident light interrupted between readings. To ensure that artifacts due to cell sedimentation were minimized, the suspensions were gently mixed by aspirating and expelling the contents of the cuvette tips using wide-bore plastic Pasteur pipettes. This procedure was performed three and six times during each 1–5-min reading period, and the fluorescence determined after each mixing.

To evaluate fura-2AM leakage from the cells, Mn^{2+} (0.1 mM) was added to quench the fluorescence of any extracellular fura-2. Autofluorescence at both wavelengths was subtracted. From the ratio of fluorescence at 340 and 380 nm the $[Ca^{2+}]_f$ was determined using the following expression (21):

$$[Ca^{2+}]_f = K'd\ [(R - R_{min})/(R_{max} - R)]\ [Sf_2/Sb_2],$$

where R is ratio of fluorescence at 340:380 nm at a given time point; R_{max} and R_{min} are ratios measured in reference standard solutions containing 2.5 µM fura-2, 115 mM KCl, 20 mM NaCl, 10 mM 3-(*N*-morpholino)-propanesulfonic acid, 1 mM $MgCl_2$ (pH 7.05) at 37°C with either 1 mM $CaCl_2$ (R_{max}) or 10 mM K_2H_2-EGTA (R_{min}). Sf_2 is the fluorescence of the free dye measured at 380 nm; Sb_2 is the fluorescence of Ca^{2+}-bound dye at 380 nm. K'd is assumed to be 224 nM (21).

Statistics

Groups were compared by paired or unpaired Student's t test as appropriate.

Results

Effects of Preincubation with 1,25-$(OH)_2D_3$ on H_2O_2 Toxicity in U937 Cells

Exposure of the U937 cells to H_2O_2, in the presence of the

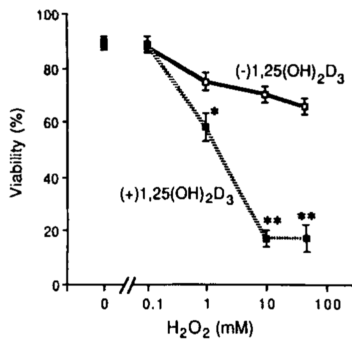

Figure 1. Effects of preincubation with 1,25-$(OH)_2D_3$ on H_2O_2 toxicity in U937 cells. U937 cells were preincubated with or without 25 nM 1,25-$(OH)_2D_3$ for 3 d, then centrifuged and resuspended at 0.3×10^6 cells/ml in 3 ml of IMDM with 10% FCS and the indicated concentrations of H_2O_2. Cells were incubated at 37°C for 60 min, then centrifuged, and viability assessed by trypan blue exclusion as described. Numbers are means of five different experiments, in each of which viability was determined in duplicate. $^*P < 0.05$; $^{**}P < 0.001$. Bars indicate SEM.

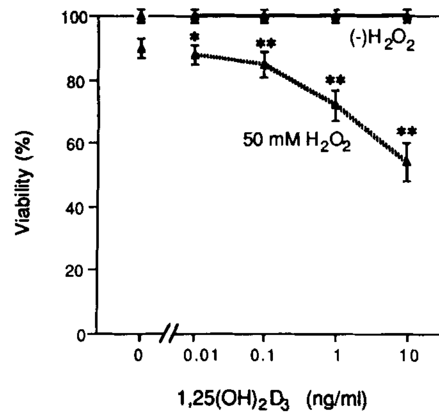

Figure 2. Effects of preincubation with 1,25-$(OH)_2D_3$ on H_2O_2 toxicity in U937 cells as a function of 1,25-$(OH)_2D_3$ concentration. U937 cells were preincubated with or without 1,25-$(OH)_2D_3$ at the indicated concentrations (0.01–10 ng/ml or 25 pM to 25 nM) for 3 d, then exposed to H_2O_2 for 30 min. Viability was assessed by trypan blue exclusion. Numbers are means of six determinations. $(^*)P < 0.05$; $(^{**})P < 0.01$. Bars indicate SEM.

usual concentration of calcium in IMDM (1.5 mM), resulted in loss of viability as measured by uptake of trypan blue. Cells not preincubated with 1,25-$(OH)_2D_3$ were relatively resistant to H_2O_2 toxicity (Fig. 1). Although the viability of cells exposed to 1 mM H_2O_2 in this experiment was decreased significantly, 63% of the cells exposed to a high concentration (50 mM) of H_2O_2 remained viable. Preincubation of cells with 25 nM 1,25-$(OH)_2D_3$ enhanced significantly the toxicity of H_2O_2 at concentrations of H_2O_2 of 1 mM or higher (Fig. 1). As shown in Fig. 2 the 1,25-$(OH)_2D_3$-mediated sensitization of U937 cells to the effects of H_2O_2 was observed at concentrations of 1,25-$(OH)_2D_3$ as low as 25 pM (0.01 ng/ml). When the cells were preincubated with increasing concentrations of 1,25-$(OH)_2D_3$ the toxic effects of H_2O_2 were proportionately greater (Fig. 2). These effects of 1,25-$(OH)_2D_3$ were not noted immediately after addition. In a typical experiment, the viability of 1,25-$(OH)_2D_3$-pretreated cells, exposed to the same concentration of H_2O_2, was 96, 70, and 20% of controls after 1, 6, and 24 h, respectively. The experiments using perturbations described were performed 10 times.

Effects of Calcium on 1,25-$(OH)_2D_3$-enhanced H_2O_2 Toxicity

Calcium which has been implicated in many forms of cellular injury (42) has been shown to sensitize isolated mitochondria to oxygen free radical injury (28). 1,25-$(OH)_2D_3$ has been reported to increase $[Ca^{2+}]_f$ in hepatocytes (3) and neonatal mouse osteoblasts (27) and to affect calcium turnover in mitochondria (11). We therefore hypothesized that 1,25-$(OH)_2D_3$ potentiated the toxicity of H_2O_2 by altering cellular calcium homeostasis. To test this hypothesis, in additional experiments, cells were preincubated in parallel with or without 1,25-$(OH)_2D_3$ in calcium-free or in calcium-containing medium, in the presence of 10% dialyzed FCS. Viability of U937 cells preincubated with or without 1,25-$(OH)_2D_3$ in medium containing calcium and then exposed to 50 mM H_2O_2 in medium containing calcium was 16 ± 1 and 68 ± 6%, respectively ($P < 0.01$, $n = 4$). When U937 cells were preincubated with or without 1,25-$(OH)_2D_3$ in the absence of calcium, however, there was no increase in toxicity in the 1,25-$(OH)_2D_3$-pretreated cells exposed to H_2O_2 in the presence of calcium (viability 70 ± 4.4 and 76 ± 0.6%, respectively, $P > 0.1$, $n = 4$).

To further examine the role of Ca^{2+} in H_2O_2-mediated cellular injury, U937 cells preincubated in the presence or absence of 25 nM (10 ng/ml) 1,25-$(OH)_2D_3$ were exposed to H_2O_2 in calcium-free medium in the presence of undialyzed 10% FCS (<100 μM Ca^{2+}) or in the presence of EGTA (<10 nM Ca^{2+}). Cells exposed to 10 mM H_2O_2 in calcium-free medium were partially protected from the toxicity of H_2O_2 (Table I). The protective effect of the calcium-free medium was particularly dramatic in 1,25-$(OH)_2D_3$-pretreated U937 cells but was also observed in control cells. The increase in survival of 1,25-$(OH)_2D_3$-pretreated U937 cells was even more pronounced when cells were exposed to H_2O_2 in the presence of 4 mM EGTA (Fig. 3). The toxicity of H_2O_2 at concentrations as high as 10 mM in 1,25-$(OH)_2D_3$-pretreated cells was markedly decreased by EGTA;

Table I. Effect of Incubation in Ca^{2+}-free Medium on H_2O_2 Toxicity in U937 Cells

	Viability			
	Control		1,25-$(OH)_2D_3$ pretreatment	
H_2O_2	With Ca^{2+}	Without Ca^{2+}	With Ca^{2+}	Without Ca^{2+}
mM	%	%	%	%
0	97	98	95	98
10	65	91	20	81*

U937 cells were preincubated in IMDM with 10% FCS with or without 25 nM 1,25-$(OH)_2D_3$ for 3 d, then centrifuged, resuspended in MEM with or without added Ca^{++} with 10% FCS, and exposed to H_2O_2 for 1 h. Cell viability was measured by trypan blue uptake. Values indicate means of two different experiments in each of which viability was determined in duplicate tubes.
* $P < 0.05$ compared with 1,25-$(OH)_2D_3$-pretreated cells incubated with H_2O_2 in medium containing calcium.

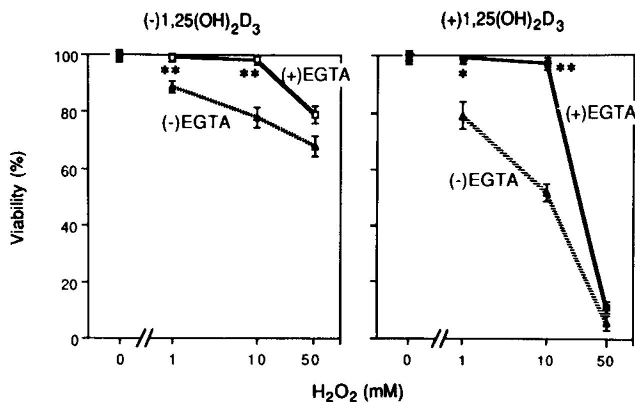

Figure 3. Effects of EGTA on the viability of U937 cells preincubated with or without 1,25-$(OH)_2D_3$ in IMDM with Ca^{2+} and exposed to H_2O_2. U937 cells were preincubated with or without 25 nM 1,25-$(OH)_2D_3$ for 60 h then exposed to H_2O_2 in IMDM in the presence or absence of EGTA (4 mM) for 30 min. Cell viability was assessed by trypan blue exclusion. Numbers are means of six determinations. (*)$P < 0.01$; (**)$P < 0.001$. Bars indicate SEM.

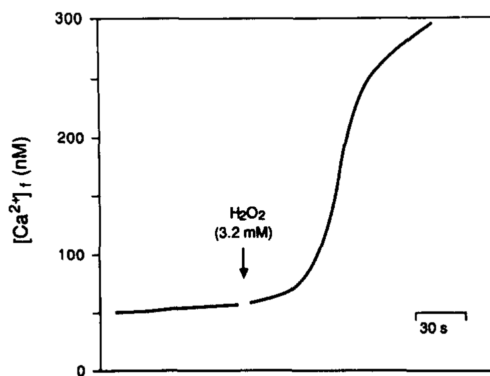

Figure 5. Effects of H_2O_2 on levels of $[Ca^{2+}]_f$ in U937 cells. H_2O_2 induced a rapid increase in $[Ca^{2+}]_f$.

at concentrations of H_2O_2 of 50–100 mM, however, EGTA was not protective.

Toxicity was also assessed by measuring the ability of cells to incorporate [^{3}H]thymidine into DNA after exposure to H_2O_2. As shown in Fig. 4 this was a more sensitive index of cell injury. A significant decrease in [^{3}H]thymidine uptake was observed in control as well as 1,25-$(OH)_2D_3$-pretreated cells after exposure to H_2O_2 at concentrations as low as 1 mM. EGTA partially protected the U937 cells from the effects of H_2O_2. The decrease in basal [^{3}H]thymidine uptake observed in cells preincubated with 1,25-$(OH)_2D_3$ compared with control cells reflects the antiproliferative effects of the hormone previously described (1, 5). Thymidine incorporation after exposure to either 1 or 5 mM H_2O_2 was lower in 1,25-$(OH)_2D_3$-pretreated cells as compared with control nonpretreated cells. The decrease in [^{3}H]thymidine incorporation was observed after exposures to H_2O_2 for times as short as 5 min (data not shown).

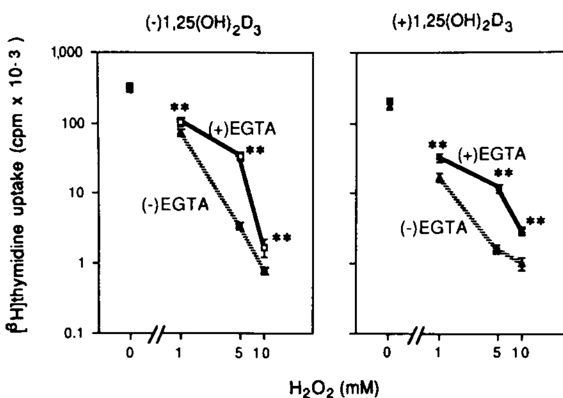

Figure 4. Effects of EGTA on the [^{3}H]thymidine uptake by U937 cells preincubated with or without 1,25-$(OH)_2D_3$ and exposed to H_2O_2. Cells were preincubated with or without 25 nM 1,25-$(OH)_2D_3$ for 60 h, exposed to H_2O_2 in the presence or absence of EGTA (4 mM) for 30 min, centrifuged, washed, and replated in 96-well plates at 1×10^5 cells/ml in 200 µl of IMDM with 10% FCS, in quadruplicate. After overnight culture, cells were labeled with 1 µCi/well of [^{3}H]thymidine for 6 h. (**)$P < 0.001$. Bars indicate SEM. [^{3}H]thymidine incorporation of cells not exposed to H_2O_2 was $336 \pm 8 \times 10^3$ for control and $177 \pm 2 \times 10^3$ for 1,25-$(OH)_2D_3$-pretreated U937 cells ($P < 0.001$, $n = 4$).

Effects of H_2O_2 on Cytosolic $[Ca^{2+}]_f$ in U937 Cells Preincubated with or without 1,25-$(OH)_2D_3$

In view of our observations that a reduction in $[Ca^{2+}]_e$ protected the U937 cells from H_2O_2 toxicity, we examined whether $[Ca^{2+}]_f$ was altered in response to 1,25-$(OH)_2D_3$ or H_2O_2 and whether the protective maneuvers were accompanied by alterations of calcium homeostasis. $[Ca^{2+}]_f$ was therefore measured in cells preincubated in the presence or absence of 1,25-$(OH)_2D_3$ and exposed to H_2O_2 in the presence or absence of EGTA. The mean $[Ca^{2+}]_f \pm$ SEM for control cells was 112 ± 7 nM and for 1,25-$(OH)_2D_3$-pretreated cells, 137 ± 9 nM ($P < 0.005$, $n = 6$ for each group). This increase in $[Ca^{2+}]_f$ was not detected within 1 h of exposure to 1,25-$(OH)_2D_3$ (data not shown) but was evident in measurements made after 24 h exposure to 1,25-$(OH)_2D_3$. When preincubation with or without 1,25-$(OH)_2D_3$ was performed in calcium-free medium, with or without 10% undialyzed FCS, the differences in basal $[Ca^{2+}]_f$ between control and 1,25-$(OH)_2D_3$-pretreated U937 cells were abolished. As shown in Fig. 5, however, when the U937 cells were exposed to H_2O_2 there was a rapid increase in $[Ca^{2+}]_f$ to levels approaching 300 nM. Because of leakage of the fura-2 soon after exposure to H_2O_2, the reversibility of the increase in $[Ca^{2+}]_f$ could not be assessed. This increase in $[Ca^{2+}]_f$ after exposure to H_2O_2 was not affected by preincubation with 1,25-$(OH)_2D_3$ although, as described, the basal levels of $[Ca^{2+}]_f$ were higher in the U937 cells pretreated with 1,25-$(OH)_2D_3$. Reduction of $[Ca^{2+}]_e$ with EGTA completely prevented the rise in $[Ca^{2+}]_f$ induced by concentrations of H_2O_2 as high as 15 mM either in 1,25-$(OH)_2D_3$-pretreated or control cells (Fig. 6). In other experiments, EGTA was also found to prevent the leakage of fura-2 from the cells which followed exposure to H_2O_2 (data not shown). As shown in Fig. 7, EGTA also prevented the cell swelling observed by phase-contrast microscopy when cells were exposed to H_2O_2.

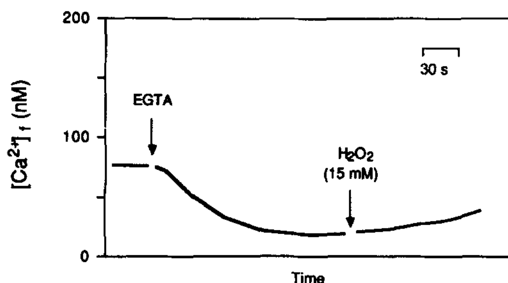

Figure 6. Effects of EGTA on the levels of $[Ca^{2+}]_f$ in U937 cells exposed to H_2O_2. Exposure to 4 mM EGTA resulted in a fall in $[Ca^{2+}]_f$. Exposure to H_2O_2 in the presence of EGTA did not result in the rapid increase in $[Ca^{2+}]_f$ observed in the absence of EGTA as was shown in Fig. 5. Note that the concentration of H_2O_2 was higher in this experiment than that used in the experiment depicted in Fig. 5.

Table II. Effects of Preexposure to Heat on Cell Viability after Exposure to H_2O_2 in U937 Cells Preincubated with or without $1,25(OH)_2D_3$

	Viability			
	Control		$1,25\text{-}(OH)_2D_3$ pretreatment	
H_2O_2	−preexposure to heat	+preexposure to heat	−preexposure to heat	+preexposure to heat
mM	%	%	%	%
0	95 ± 2	91 ± 1	94 ± 2	88 ± 4
10	79 ± 8	79 ± 6	43 ± 5	79 ± 4*

U937 cells were preincubated in IMDM, 10% FCS with or without 25 nM $1,25\text{-}(OH)_2D_3$ for 3 d, then preexposed to heat (45°C for 20 min) or not, allowed to recover at 37°C for 4 h, and exposed to H_2O_2 as described. Cell viability was assessed by trypan blue exclusion ($n = 4$).
* $P < 0.01$ compared with $1,25\text{-}(OH)_2D_3$-pretreated, unheated cells exposed to 10 mM H_2O_2.

Effects of Preexposure to Heat or H_2O_2 on H_2O_2 Toxicity in U937 Cells

Since the HSPs may be involved in protecting cells from injury, we examined the effects on H_2O_2 toxicity of a brief preexposure to elevated temperatures. U937 cells were preincubated for 72 h in the presence or absence of 25 nM $1,25\text{-}(OH)_2D_3$. Some of the dishes containing these cells were heated for 20 min at 45°C. The dishes were then brought back to 37°C, kept in the incubator at 37°C for 4 h, and exposed to 10 mM H_2O_2 in medium containing calcium. It is demonstrated in Table II that the toxicity of H_2O_2 was partially prevented by preexposure to 45°C in $1,25\text{-}(OH)_2D_3$-pretreated cells although preexposure to 45°C by itself induced a slight decrease in cell viability. We previously reported that while elevated temperatures induced the synthesis of HSPs in U937 cells, H_2O_2 did not (39). We therefore compared the effects of preexposure to 45°C for 20 min with preexposure to H_2O_2. Only preexposure to 45°C protected the U937 cells from the toxic effects of subsequent exposure to H_2O_2 (Table III). The protective effects of preexposure to heat on H_2O_2 toxicity were observed in each of

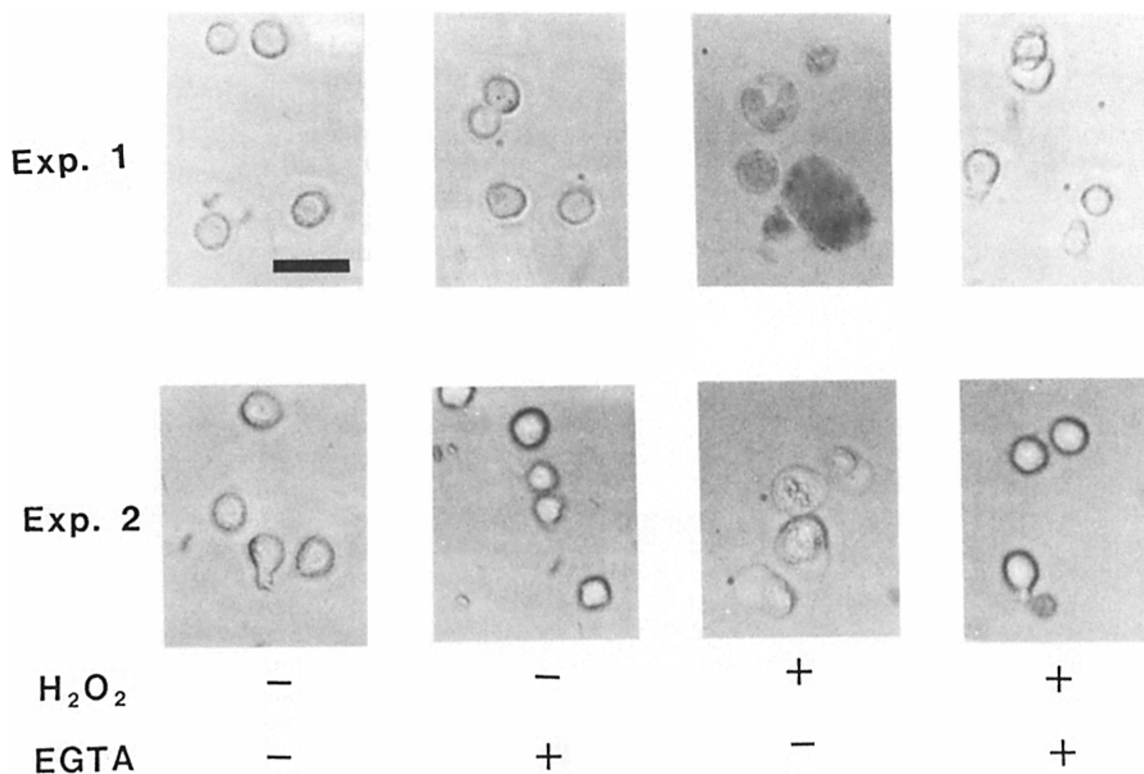

Figure 7. Effects of EGTA on morphology of U937 cells exposed to H_2O_2. U937 cells were exposed to 10 mM H_2O_2 for 30 min in the presence or absence of 4 mM EGTA, centrifuged, and resuspended in trypan blue. Photomicrographs were taken immediately under phase-contrast microscopy using cells from two separate experiments. (*Expt. 1* and *Expt. 2*). Bar, 26 μm.

Table III. Effects of Preexposure to Heat or H_2O_2 on Viability of 1,25-$(OH)_2D_3$-treated U937 Cells Exposed to H_2O_2

Preincubation	Test conditions (cell viability)		
	0 mM H_2O_2	1 mM H_2O_2	5 mM H_2O_2
	%	%	%
Control	98 ± 1	68 ± 4	46 ± 4
Heat (45°C)	100 ± 1	80 ± 1*	69 ± 3*
H_2O_2 (1 mM)	87 ± 3	52 ± 2	37 ± 4

U937 cells were incubated under control conditions, or preexposed to heat (45°C for 20 min) or to H_2O_2 (1 mM for 20 min), allowed to recover for 4 h, then exposed to H_2O_2 for 60 min. Cell viability was assessed by trypan blue uptake in triplicate tubes.
* $P < 0.05$ compared with control cells and $P < 0.01$ compared with H_2O_2 pretreated cells exposed to the same concentration of H_2O_2.

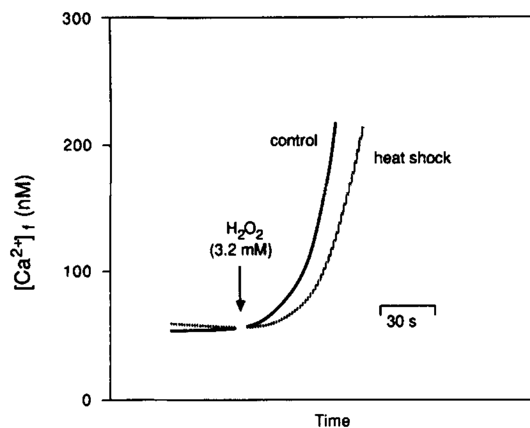

Figure 8. Effects of H_2O_2 on levels of $[Ca^{2+}]_f$ in U937 cells preexposed or not to heat (45°C for 20 min) a temperature which induces the synthesis of HSPs in U937 cells (39).

six separate experiments. In three out of four experiments no differences were found in basal levels of $[Ca^{2+}]_f$ nor in the increases in $[Ca^{2+}]_f$ after exposure to H_2O_2 in U937 cells heated or not heated at 45°C for 20 min 4 h before exposure to H_2O_2. The results of one of these experiments is illustrated in Fig. 8.

Discussion

We have found in the present study that the hormone 1,25-$(OH)_2D_3$ potentiates the toxicity of H_2O_2 in the human monocytic line U937 by calcium-dependent mechanisms. This increased toxicity of H_2O_2 was observed in the range of physiological concentrations of the hormone and appeared between 6 and 24 h after the addition of 1,25-$(OH)_2D_3$. Two different methods were used to measure this toxicity. Effects on [^{3}H]thymidine incorporation into DNA as well as exclusion of trypan blue were assessed since it has been demonstrated that exclusion of trypan blue alone cannot be used as the sole criterion of cell viability (13). Several lines of evidence support the concept that these effects of 1,25-$(OH)_2D_3$ are calcium dependent. (*a*) Preincubation of U937 cells with 1,25-$(OH)_2D_3$ in calcium-free medium prevented the 1,25-$(OH)_2D_3$-induced increase in H_2O_2 toxicity. The preincubation in calcium-free medium was performed in the presence of dialyzed serum. The prevention of 1,25-$(OH)_2D_3$-induced H_2O_2 toxicity was not observed in calcium-containing medium with the dialyzed serum, implicating calcium rather than some other dialyzable component as essential for the H_2O_2 toxicity. (*b*) The increased toxicity of H_2O_2 in 1,25-$(OH)_2D_3$-pretreated U937 cells was not observed when cells were exposed to H_2O_2 in calcium-free medium even in the presence of undialyzed serum or in the presence of EGTA, maneuvers that decreased or eliminated the H_2O_2-induced increase in $[Ca^{2+}]_f$. (*c*) The basal levels of $[Ca^{2+}]_f$ before the addition of H_2O_2 were higher in cells pretreated with 1,25-$(OH)_2D_3$ than in control cells.

Suggestions that vitamin D or its metabolites affect intracellular calcium metabolism were first made nearly 20 years ago when it was found that vitamin D deficiency resulted in depletion of mitochondrial calcium (22). More recently, 1,25-$(OH)_2D_3$ has been shown to increase $[Ca^{2+}]_f$ in hepatocytes (3), the myelomonocytic cell line HL-60 (18), and neonatal mouse osteoblasts (27). In GH_4C_1 cells 1,25-$(OH)_2D_3$ does not alter basal $[Ca^{2+}]_f$ but does augment the increase in $[Ca^{2+}]_f$ induced by thyrotropin-releasing hormone (14). The presumed source of this increase is not from intracellular calcium stores but from enhanced calcium conductance at the level of the plasma membrane. In the U937 cells that we studied here, 1,25-$(OH)_2D_3$ did not by itself induce an immediately detectable increase in $[Ca^{2+}]_f$. After 24 h incubation with 1,25-$(OH)_2D_3$, however, the basal levels of $[Ca^{2+}]_f$ were higher than in cells not preexposed to 1,25-$(OH)_2D_3$, suggesting that the hormone induces a progressive accumulation of calcium within the cells. When the cells were incubated with H_2O_2, which as described here induced an increase in $[Ca^{2+}]_f$ dependent upon the presence of extracellular calcium, the 1,25-$(OH)_2D_3$-pretreated Ca^{2+}-laden cells, may have been unable to cope with this additional calcium influx. The causal relationship between the 1,25-$(OH)_2D_3$-induced increase in sensitivity to H_2O_2 toxicity and the 1,25-$(OH)_2D_3$-induced increase in $[Ca^{2+}]_f$ is supported by the findings that $[Ca^{2+}]_f$ in cells preincubated with 1,25-$(OH)_2D_3$ in calcium-free medium was not increased over control values and these cells were not more sensitive to H_2O_2 toxicity. It is possible that the 1,25-$(OH)_2D_3$-pretreated cells accumulated large amounts of Ca^{2+} which was sequestered into cellular compartments. Since the cytosolic compartment contains only a small fraction of total cell Ca^{2+} the measured increases in levels of basal $[Ca^{2+}]_f$ after 1,25-$(OH)_2D_3$ treatment may reflect a much larger amount of sequestered Ca^{2+} in the 1,25-$(OH)_2D_3$-treated cells. When additional Ca^{2+} was introduced into these Ca^{2+}-loaded cells, most or all of the nonmitochondrial Ca^{2+} storage sites may have been saturated and the added Ca^{2+} entered the mitochondria. This could interfere with ATP production and ultimately result in uncoupling of oxidative phosphorylation (9).

A role for calcium in potentiating injury by reactive oxygen intermediates has been previously demonstrated in an isolated mitochondrial system (28). The mechanism of the potentiation by calcium of injury from reactive oxygen intermediates in mitochondria can possibly be accounted for in part by activation of phospholipase A_2 (24). In our experiments in the U937 cells, an increased calcium influx induced by H_2O_2 could result from increased permeability of the

cell membranes. A resultant increase in $[Ca^{2+}]_f$ could then activate phospholipase and further enhance the permeability of the cell membrane to Ca^{2+}. Thus an increase in cellular Ca^{2+} may initiate a cascade of events leading to cell death; for example, activation of phospholipase with subsequent breakdown of cell membranes, depletion of energy supplies by enhanced use of ATP, and uncoupling of oxidative phosphorylation in mitochondria (9). Under these conditions, inhibition of calcium entry would be expected to be accompanied by a preservation of membrane integrity. In the present study, such preservation of membrane integrity could account for the prevention of swelling and maintenance of viability in U937 cells exposed to H_2O_2 in the presence of EGTA.

Other factors might also play a role in the increased toxic effects of H_2O_2 in cells preexposed to $1,25\text{-}(OH)_2D_3$. For example, $1,25\text{-}(OH)_2D_3$ has been shown to activate secretion of H_2O_2 by human peripheral blood monocytes (16). In contrast, in U937 cells, $1,25\text{-}(OH)_2D_3$ alone does not induce production of superoxide anion (41). However, production of H_2O_2 was not directly measured in those experiments. $1,25\text{-}(OH)_2D_3$-mediated enhanced H_2O_2 toxicity might also be related to induction of differentiation towards the mature macrophage phenotype of the immature U937 cells. Mature peripheral blood monocytes after adherence, however, are not consistently more sensitive to oxidative injury than the undifferentiated U937 cells (Polla, B. S., and S. M. Krane, unpublished data).

Prior exposure to heat protected the U937 cells against oxidative damage. Most organisms react to elevated temperatures by the selective induction of the synthesis of several HSPs. The heat shock response is not induced solely by heat, however, but also by a variety of other forms of injury. Injury produced by reactive oxygen species, such as H_2O_2, induces the synthesis of HSPs in several different prokaryotic cells (15, 22) including human monocytes (39). We have not been able to demonstrate, however, that H_2O_2 induces the synthesis of HSPs in U937 cells (39).

Although the precise functions of HSPs are not yet understood, there is growing evidence for their role in thermotolerance and in a broader sense in protection from other forms of injury (24). RNA splicing, which is interrupted by heat shock, is rescued by HSPs (48). There is also evidence for the concept that the HSPs might be involved in targeting abnormal proteins, such as those that are the products of mutant genes or proteins aggregated as a result of heat or other injury, for proteolytic degradation (2). The 70-kD HSP has also been proposed to act as "a kind of detergent, helping to disrupt aggregates that form after heat shock by binding tightly to hydrophobic surfaces" (26, 31, 32). We have previously reported that $1,25\text{-}(OH)_2D_3$, which increases synthesis of HSPs in human monocytes and U937 cells exposed to heat, also partially protects normal protein synthesis (37, 39). Preexposure to H_2O_2, which does not induce the synthesis of HSPs in U937 cells (39), does not protect these cells. The protective effects therefore appear to be related to the synthesis of HSPs. Monocytes, in which H_2O_2 does induce a heat shock response (39), are indeed protected from exposure to H_2O_2 by preexposure to this same agent (Polla, B. S., and S. M. Krane, unpublished data; 45). In the present study, the protective effects of preexposure to heat were observed in $1,25\text{-}(OH)_2D_3$-pretreated cells, consistent with our previous observations on the increased synthesis of HSPs in $1,25\text{-}(OH)_2D_3$-treated U937 cells (39).

Preexposure to heat, however, did not modify the H_2O_2-induced increase in $[Ca^{2+}]_f$. Furthermore, the protective effects of preexposure to heat were observed when the U937 cells were exposed to H_2O_2 in medium containing calcium. The protective effects of HSPs are therefore not explained simply by a modulation of the H_2O_2-induced increase in $[Ca^{2+}]_f$. The HSPs might therefore act at a point distal to that at which the protective effects of low extracellular calcium concentrations are exerted. Although $1,25\text{-}(OH)_2D_3$ induces thermal tolerance in human monocytes and U937 cells the changes in Ca^{2+} homeostasis brought about by this hormone increase their susceptibility to oxidative injury. Nevertheless, the heat shock response is inhibited in calcium-deprived hepatoma cells (23), supporting the concept that there is a link in some cells between the heat shock response and cellular calcium content (45). Although the precise mechanisms by which HSPs could help protect cells from the consequences of oxidative injury are unknown, they could lead to new approaches to developing therapies. The hormone $1,25\text{-}(OH)_2D_3$, which has many effects on the structure and function of mononuclear phagocytes, also appears to play a complex role in the modulation of their responses to injury.

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References

1. Amento, E. P., A. K. Bhalla, J. T. Kurnick, R. L. Kradin, T. L. Clemens, S. A. Holick, M. F. Holick, and S. M. Krane. 1984. 1α,25-dihydroxyvitamin D_3 induces maturation of the human monocyte cell line U937, and, in association with a factor from human T lymphocytes, augments production of the monokine, mononuclear cell factor. *J. Clin. Invest.* 73:731–739.
2. Ananthan, J., A. L. Goldberg, and R. Voellmy. 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science (Wash. DC)*. 232:522–524.
3. Baran, D. T., and M. L. Milne. 1986. 1,25-dihydroxyvitamin D increases hepatocyte cytosolic calcium levels. A potential regulator of vitamin D-25-hydroxylase. *J. Clin. Invest.* 77:1622–1626.
4. Bar-Shavit, Z., D. Noff, S. Edelstein, M. Meyer, S. Shibolet, and R. Goldman. 1981. 1,25-dihydroxyvitamin D_3 and the regulation of macrophage function. *Calcif. Tissue Int.* 33:673–676.
5. Bar-Shavit, Z., S. L. Teitelbaum, P. Reitsma, A. Hall, L. E. Pegg, J. Trial, and A. J. Kahn. 1983. Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA.* 80:5907–5911.
6. Bell, N. H. 1985. Vitamin D-endocrine system. *J. Clin. Invest.* 76:1–6.
7. Bhalla, A. K., E. P. Amento, T. L. Clemens, M. F. Holick, and S. M. Krane. 1983. Specific high-affinity receptors for 1,25-dihydroxyvitamin D_3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J. Clin. Endocrinol. Metab.* 57:1308–1310.
8. Bhalla, A. K., E. P. Amento, and S. M. Krane. 1986. Differential effects of 1,25-dihydroxyvitamin D_3 on human lymphocytes and monocyte/macrophages: inhibition of interleukin-2 and augmentation of interleukin-1 production. *Cell. Immunol.* 98:311–322.
9. Bonventre, J. V., A. Leaf, and C. D. Malis. 1988. Nature of the cellular insult in ischemic acute renal failure. *In* Acute Renal Failure. B. M. Bren-

ner and J. M. Lazarus, editors. Churchill Livingstone, Inc., New York/London/Melbourne. 3-43.

10. Bonventre, J. V., K. L. Skorecki, J. I. Kreisberg, and J. Y. Cheung. 1986. Vasopressin increases cytosolic free calcium concentration in glomerular mesangial cells. *Am. J. Physiol.* 251(Renal Fluid Electrolyte Physiol. 20):F94-F102.
11. Borle, A. B. 1975. Regulation of the mitochondrial control of cellular calcium homeostasis and calcium transport by phosphate, parathyroid hormone, calcitonin, vitamin D and cyclic AMP. *In* Calcium-Regulating Hormones. R. V. Talmage, M. Owen, and J. A. Parsons, editors. Excerpta Medica, Amsterdam. 217-228.
12. Cheung, J. Y., J. V. Bonventre, C. D. Malis, and A. Leaf. 1986. Calcium and ischemic injury. *N. Engl. J. Med.* 314:1670-1676.
13. Cheung, J. Y., A. Leaf, and J. V. Bonventre. 1985. Determination of isolated myocyte viability: staining methods and functional criteria. *Basic Res. Cardiology.* 80:(Suppl.)1:23-30.
14. Chisholm, J. C., S. Kim, and A. H. Tashjian, Jr. 1988. Modulation by 1,25-dihydroxycholecalciferol of the acute change in cytosolic free calcium induced by thyrotropin-releasing hormone in GH_4C_1 pituitary cells. *J. Clin. Invest.* 81:661-668.
15. Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. *Cell.* 41:753-762.
16. Cohen, M. S., D. E. Mesler, R. G. Snipes, and T. K. Gray. 1986. 1,25-dihydroxyvitamin D_3 activates secretion of hydrogen peroxide by human monocytes. *J. Immunol.* 136:1049-1053.
17. Darwish, H. M., J. Krisinger, M. Strom, and H. F. DeLuca. 1987. Molecular cloning of the cDNA and chromosomal gene for vitamin D-dependent calcium-binding protein of rat intestine. *Proc. Natl. Acad. Sci. USA.* 84:6108-6111.
18. Desai, S. S., and D. T. Baran. 1986. Increase in cytosolic calcium by $1,25(OH)_2D_3$ is not necessary for the $1,25(OH)_2D_3$-induced maturation of HL-60 cells. *J. Bone Min. Res.* 1(Suppl. 1):65.
19. Dupret, J.-M., P. Brun, C. Perret, N. Lomri, M. Thomasset, and P. Cuisinier-Gleizes. 1987. Transcriptional and post-transcriptional regulation of vitamin D-dependent calcium-binding protein gene expression in the rat duodenum by 1,25-dihydroxycholecalciferol. *J. Biol. Chem.* 262: 16553-16557.
20. Fridovich, I. 1978. The biology of oxygen radicals. *Science (Wash. DC).* 201:875-880.
21. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440-3450.
22. Kimmich, G. A., and H. Rasmussen. 1969. Regulation of pyruvate carboxylase activity by calcium in intact rat liver mitochondria. *J. Biol. Chem.* 244:190-199.
23. Lamarche, S., P. Chretien, and J. Landry. 1986. Inhibition of the heat shock response and synthesis of glucose-regulated proteins in Ca^{2+}-deprived rat hepatoma cells. *Biochem. Biophys. Res. Commun.* 131:868-876.
24. Lanks, K. W. 1986. Modulators for the eukaryotic heat shock response. *Exp. Cell Res.* 165:1-10.
25. Lee, Y. S., A. N. Taylor, T. J. Reimers, S. Edelstein, C. S. Fullmer, and R. H. Wasserman. 1987. Calbindin-D in peripheral nerve cells is vitamin D and calcium dependent. *Proc. Natl. Acad. Sci. USA.* 84:7344-7348.
26. Lewis, M. J., and H. R. B. Pelham. 1985. Involvement of ATP in the nuclear and nucleolar functions of the 70 kd heat shock protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3137-3143.
27. Lieberherr, M. 1987. Effects of vitamin D_3 metabolites on cytosolic free calcium in confluent mouse osteoblasts. *J. Biol. Chem.* 262:13168-13173.
28. Malis, C. D., and J. V. Bonventre. 1986. Mechanism of calcium potentiation of oxygen free radical injury to renal mitochondria. A model for post-ischemic and toxic mitochondrial damage. *J. Biol. Chem.* 261: 14201-14208.
29. McDonnell, D. P., D. J. Mangelsdorf, J. W. Pike, M. R. Haussler, and B. W. O'Malley. 1987. Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. *Science (Wash. DC).* 235:1214-1217.
30. Morgan, B. P., J. P. Luzio, and A. K. Campbell. 1986. Intracellular Ca^{2+} and cell injury: a paradoxical role of Ca^{2+} in complement membrane attack. *Cell Calcium.* 7:399-411.
31. Munro, S., and H. Pelham. 1985. What turns on heat shock genes? *Nature (Lond.).* 317:477-478.
32. Munro, S., and H. R. B. Pelham. 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell.* 46:291-300.
33. Nathan, C. F., L. H. Brukner, S. C. Silverstein, and Z. A. Cohn. 1979. Extracellular cytolysis by activated macrophages and the granulocytes. I. Pharmacological triggering of effector cells and release of hydrogen peroxide. *J. Exp. Med.* 149:84-99.
34. Nathan, C. F., S. C. Silverstein, L. H. Brukner, and Z. A. Cohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. *J. Exp. Med.* 149:100-113.
35. Norman, A. W., J. Roth, and L. Orci. 1982. The vitamin D endocrine system: steroid metabolism, hormone receptors, and biological response (calcium binding proteins). *Endocr. Rev.* 3:331-366.
36. Peacock, M., S. Jones, T. L. Clemens, E. P. Amento, J. Kurnick, S. M. Krane, and M. F. Holick. 1982. High affinity $1,25(OH)_2$ vitamin D_3 receptors in a human monocyte-like cell line (U937) and in a cloned human T lymphocyte. *In* Vitamin D: Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism. A. W. Norman, K. Schaefer, D. von Herrath, and H.-G. Grigoleit, editors. Walter de Gruyter, Inc., Berlin/New York. 83-85.
37. Polla, B. S., A. M. Healy, E. P. Amento, and S. M. Krane. 1986. 1,25-dihydroxyvitamin D_3 maintains adherence of human monocytes and protects them from thermal injury. *J. Clin. Invest.* 77:1332-1339.
38. Polla, B. S., A. M. Healy, W. C. Wojno, and S. M. Krane. 1986. Analysis of the heat-shock response in human cells; effects of 1,25-dihydroxyvitamin D_3 ($1,25\text{-}[OH]_2D_3$). *J. Bone Min. Res.* 1(Suppl. 1):422.
39. Polla, B. S., A. M. Healy, W. C. Wojno, and S. M. Krane. 1987. Hormone 1α,25-dihydroxyvitamin D_3 modulates heat shock response in monocytes. *Am. J. Physiol.* 252(Cell Physiol. 21):C640-C649.
40. Provvedini, D. M., C. D. Tsoukas, L. J. Deftos, and S. C. Manolagas. 1983. 1,25-dihydroxyvitamin D_3 receptors in human leukocytes. *Science (Wash. DC).* 221:1181-1183.
41. Roux-Lombard, P., A. Cruchaud, and J.-M. Dayer. 1986. Effect of interferon-γ and 1α,25-dihydroxyvitamin D_3 on superoxide anion, prostaglandins E_2, and mononuclear cell factor production by U937 cells. *Cell. Immunol.* 97:286-296.
42. Schanne, F. A. X., A. B. Kane, E. E. Young, and J. L. Farber. 1979. Calcium dependence of toxic cell death: a final common pathway. *Science (Wash. DC).* 206:700-702.
43. Schlesinger, M. J., M. Ashburner, and A. Tissières. 1982. Heat Shock. From Bacteria to Man. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 1-440.
44. Slater, T. F. 1984. Free-radical mechanisms in tissue injury. *Biochem. J.* 222:1-15.
45. Stevenson, M. A., S. K. Calderwood, and G. M. Hahn. 1986. Rapid increases in inositol trisphosphate and intracellular Ca^{++} after heat shock. *Biochem. Biophys. Res. Commun.* 137:826-833.
46. Subjeck, J. R., and T.-T. Shyy. 1986. Stress protein systems of mammalian cells. *Am. J. Physiol.* 250(Cell Physiol. 19):C1-C17.
47. Theofan, G., A. P. Nguyen, and A. W. Norman. 1986. Regulation of calbindin-D_{28k} gene expression by 1,25-dihydroxyvitamin D_3 is correlated to receptor occupancy. *J. Biol. Chem.* 261:16943-16947.
48. Yost, H. J., and S. Lindquist. 1986. RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. *Cell.* 45:185-193.