1,25-Dihydroxyvitamin D₃ 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 D₃ (1,25-(OH)₂D₃) increases synthesis of heat shock proteins in monocytes and U937 cells and protects these cells from thermal injury. We examined whether 1,25-(OH)₂D₃ would also modulate the susceptibility of U937 cells to H₂O₂-induced oxidative stress. Cell viability was assessed by trypan blue exclusion and [³H]thymidine incorporation into DNA. Prior incubation for 24 h with 1,25-(OH)₂D₃ (25 pM or higher) unexpectedly increased H₂O₂ toxicity. Since cellular Ca²⁺ may be a mediator of cell injury we investigated effects of altering extracellular Ca²⁺ ([Ca²⁺]ₑ) on 1,25-(OH)₂D₃-enhanced H₂O₂ toxicity as well as effects of 1,25-(OH)₂D₃ and H₂O₂ on cytosolic free Ca²⁺ concentration ([Ca²⁺]ᵢ). Basal [Ca²⁺]ᵢ in medium containing 1.5 mM Ca as determined by fura-2 fluorescence was higher in 1,25-(OH)₂D₃-pretreated cells than control cells (137 versus 112 nM, P < 0.005). H₂O₂ induced a rapid increase in [Ca²⁺]ᵢ (to > 300 nM) in both 1,25-(OH)₂D₃-treated and control cells, which was prevented by a reduction in [Ca²⁺]ₑ to less than basal [Ca²⁺]ₑ. The 1,25(OH)₂D₃-induced increase in H₂O₂ toxicity was also prevented by preincubation with 1,25-(OH)₂D₃ in Ca²⁺-free medium or by exposing the cells to H₂O₂ in the presence of EGTA. Preexposure of cells to 45°C for 20 min, 4 h earlier, partially prevented the toxic effects of H₂O₂ particularly in 1,25-(OH)₂D₃-treated cells, even in the presence of physiological levels of [Ca²⁺]ₑ. Thus 1,25-(OH)₂D₃ potentiates H₂O₂-induced injury probably by increasing cellular Ca²⁺ stores. The 1,25-(OH)₂D₃-induced amplification of the heat shock response likely represents a mechanism for counteracting the Ca²⁺-associated enhanced susceptibility to oxidative injury due to 1,25-(OH)₂D₃.

†Abbreviations used in this paper: [Ca²⁺]ₑ, extracellular Ca²⁺ concentration; [Ca²⁺]ᵢ, cytosolic free Ca²⁺ concentration; 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; fura-2AM, acetylmethoxy ester of fura-2; HSPs, heat shock proteins; IMDM, Iscove's modification of Dulbecco's medium.

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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 \( \text{H}_{2}\text{O}_{2} \) toxicity would be modified by changes in cellular calcium homeostasis.

We found that preincubation with 1,25-(OH)\(_2\)D\(_3\) at physiological concentrations did not protect against but rather potentiated the toxic effects of \( \text{H}_{2}\text{O}_{2}\) in U937 cells. This enhancement of \( \text{H}_{2}\text{O}_{2}\) toxicity by 1,25-(OH)\(_2\)D\(_3\) was partially reversed if the cells were exposed to 1,25-(OH)\(_2\)D\(_3\) in calcium-free medium with or without EGTA and was abolished if the preincubation with 1,25-(OH)\(_2\)D\(_3\) was carried out in calcium-free medium. Whereas exposure to \( \text{H}_{2}\text{O}_{2}\) induced a similar rise in the concentration of cytosolic free calcium ([Ca\(^{2+}\)]\(_{c}\)) in both 1,25-(OH)\(_2\)D\(_3\)-pretreated and control U937 cells, the basal concentrations of [Ca\(^{2+}\)]\(_{c}\) were higher in 1,25-(OH)\(_2\)D\(_3\)-pretreated cells. Finally, preexposure of U937 cells to elevated temperatures (45°C) did not prevent the increase in [Ca\(^{2+}\)]\(_{c}\); but did protect the cells from death which resulted from exposure to \( \text{H}_{2}\text{O}_{2}\).

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., Walkerton, OR). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO\(_2\). The cells were exposed to \( \text{H}_{2}\text{O}_{2}\) at 37°C either in IMDM with 10% FCS, or in MEM (Gibco) with or without calcium (formula No. 86-0084, with Na panthotenate substituting for Ca pantothenate). Preincubation with 1,25-(OH)\(_2\)D\(_3\) 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)\(_2\)D\(_3\) (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 \( \text{H}_{2}\text{O}_{2}\) toxicity. \( \text{H}_{2}\text{O}_{2}\) 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 \([\text{H}]\) 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 "$ \% \ \text{viable} $" reflect data collected from experiments where the total number of cells did not change. For the \([\text{H}]\) thymidine uptake assay, cells were counted after exposure to \( \text{H}_{2}\text{O}_{2}\) and 200 µl of a cell suspension of 1.0 x 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 \([\text{H}]\) 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 \( \approx \)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 \( \text{H}_{2}\text{O}_{2}\).

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\(_2\)PO\(_4\), 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 x 10\(^5\) cells/ml in 2 ml of buffer containing 145 mM NaCl, 5 mM KCl, 1.4 mM MgSO\(_4\), 0.5 mM CaCl\(_2\), 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, 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\(^{2+}\)] was determined using the following expression (21):

\[
[\text{Ca}^{2+}] = K_d \frac{(R - R_{\text{max}})(R_{\text{max}} - R)}{[S_f]/[S_b]},
\]

where \( R \) is ratio of fluorescence at 340:380 nm at a given time point; \( R_{\text{max}} \) and \( R_{\text{min}} \) 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\(_2\) or 10 mM K\(_2\)EGTA (Rmax). \( S_f \) is the fluorescence of the free dye measured at 380 nm; \( S_b \) 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)\(_2\)D\(_3\) on \( \text{H}_{2}\text{O}_{2} \) Toxicity in U937 Cells

Exposure of the U937 cells to \( \text{H}_{2}\text{O}_{2} \), in the presence of the
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)₂D₃ were relatively resistant to H₂O₂ toxicity (Fig. 1). Although the viability of cells exposed to 1 mM H₂O₂ in this experiment was decreased significantly, 63% of the cells exposed to a high concentration (50 mM) of H₂O₂ remained viable. Preincubation of cells with 25 nM 1,25-(OH)₂D₃ enhanced significantly the toxicity of H₂O₂ at concentrations of H₂O₂ of 1 mM or higher (Fig. 1). As shown in Fig. 2 the 1,25-(OH)₂D₃-mediated sensitization of U937 cells to the effects of H₂O₂ was observed at concentrations of 1,25-(OH)₂D₃ as low as 25 pM (0.01 ng/ml). When the cells were preincubated with increasing concentrations of 1,25-(OH)₂D₃ the toxic effects of H₂O₂ were proportionately greater (Fig. 2). These effects of 1,25-(OH)₂D₃ were not noted immediately after addition. In a typical experiment, the viability of 1,25-(OH)₂D₃-pretreated cells, exposed to the same concentration of H₂O₂, 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)₂D₃-enhanced H₂O₂ 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)₂D₃ has been reported to increase [Ca²⁺]ᵢ in hepatocytes (3) and neonatal mouse osteoblasts (27) and to affect calcium turnover in mitochondria (11). We therefore hypothesized that 1,25-(OH)₂D₃ potentiated the toxicity of H₂O₂ by altering cellular calcium homeostasis. To test this hypothesis, in additional experiments, cells were preincubated in parallel with or without 1,25-(OH)₂D₃ 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)₂D₃ in medium containing calcium and then exposed to 50 mM H₂O₂ 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)₂D₃ in the absence of calcium, however, there was no increase in toxicity in the 1,25-(OH)₂D₃-pretreated cells exposed to H₂O₂ 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 Ca²⁺ in H₂O₂-mediated cellular injury, U937 cells preincubated in the presence or absence of 25 nM (10 ng/ml) 1,25-(OH)₂D₃ were exposed to H₂O₂ in calcium-free medium in the presence of undialyzed 10% FCS (<10 μM Ca²⁺) or in the presence of EGTA (<10 mM Ca²⁺). Cells exposed to 10 mM H₂O₂ in calcium-free medium were partially protected from the toxicity of H₂O₂ (Table I). The protective effect of the calcium-free medium was particularly dramatic in 1,25-(OH)₂D₃-pretreated U937 cells but was also observed in control cells. The increase in survival of 1,25-(OH)₂D₃-pretreated U937 cells was even more pronounced when cells were exposed to H₂O₂ in the presence of 4 mM EGTA (Fig. 3). The toxicity of H₂O₂ at concentrations as high as 10 mM in 1,25-(OH)₂D₃-pretreated cells was markedly decreased by EGTA;

### Table I. Effect of Incubation in Ca²⁺-free Medium on H₂O₂ Toxicity in U937 Cells

| H₂O₂ | Control | 1,25-(OH)₂D₃ pretreatment |
|------|---------|---------------------------|
|      | Without Ca²⁺ | With Ca²⁺ | Without Ca²⁺ | With Ca²⁺ |
| nM   | %       | %       | %    | %    |
| 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)₂D₃ for 3 d, then centrifuged, resuspended in MEM with or without added Ca²⁺ with 10% FCS, and exposed to H₂O₂ 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)₂D₃-pretreated cells incubated with H₂O₂ in medium containing calcium.
Figure 3. Effects of EGTA on the viability of U937 cells preincubated with or without 1,25-(OH)2D3 in IMDM with Ca2+ and exposed to H2O2. U937 cells were preincubated with or without 25 nM 1,25-(OH)2D3 for 60 h then exposed to H2O2 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.

Effects of 1,25-(OH)2D3 on cell viability at concentrations of H2O2 of 50–100 mM, however, EGTA was not protective.

Toxicity was also assessed by measuring the ability of cells to incorporate [3H]thymidine into DNA after exposure to H2O2. As shown in Fig. 4 this was a more sensitive index of cell injury. A significant decrease in [3H]thymidine uptake was observed in control as well as 1,25-(OH)2D3-pretreated cells after exposure to H2O2 at concentrations as low as 1 mM. EGTA partially protected the U937 cells from the effects of H2O2. The decrease in basal [3H]thymidine uptake observed in cells preincubated with 1,25-(OH)2D3 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 H2O2 was lower in 1,25-(OH)2D3-pretreated cells as compared with control nonpretreated cells. The decrease in [3H]thymidine incorporation was observed after exposures to H2O2 for times as short as 5 min (data not shown).

Effects of H2O2 on Cytosolic [Ca2+]i in U937 Cells Preincubated with or without 1,25-(OH)2D3

In view of our observations that a reduction in [Ca2+]i protected the U937 cells from H2O2 toxicity, we examined whether [Ca2+]i was altered in response to 1,25-(OH)2D3 or H2O2 and whether the protective maneuvers were accompanied by alterations of calcium homeostasis. [Ca2+]i was therefore measured in cells preincubated in the presence or absence of 1,25-(OH)2D3 and exposed to H2O2 in the presence or absence of EGTA. The mean [Ca2+]i ± SEM for control cells was 112 ± 7 nM and for 1,25-(OH)2D3-pretreated cells, 137 ± 9 nM (P < 0.005, n = 6 for each group). This increase in [Ca2+]i was not detected within 1 h of exposure to 1,25-(OH)2D3 (data not shown) but was evident in measurements made after 24 h exposure to 1,25-(OH)2D3. When preincubation with or without 1,25-(OH)2D3 was performed in calcium-free medium, with or without 10% undialyzed FCS, the differences in basal [Ca2+]i between control and 1,25-(OH)2D3-pretreated U937 cells were abolished. As shown in Fig. 5, however, when the U937 cells were exposed to H2O2 there was a rapid increase in [Ca2+]i to levels approaching 300 nM. Because of leakage of the fura-2 soon after exposure to H2O2, the reversibility of the increase in [Ca2+]i could not be assessed. This increase in [Ca2+]i after exposure to H2O2 was not affected by preincubation with 1,25-(OH)2D3 although, as described, the basal levels of [Ca2+]i were higher in the U937 cells pretreated with 1,25-(OH)2D3. Reduction of [Ca2+]i with EGTA completely prevented the rise in [Ca2+]i induced by concentrations of H2O2 as high as 15 mM either in 1,25-(OH)2D3-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 H2O2 (data not shown). As shown in Fig. 7, EGTA also prevented the cell swelling observed by phase-contrast microscopy when cells were exposed to H2O2.
Figure 6. Effects of EGTA on the levels of $[\text{Ca}^{2+}]_\text{r}$ in U937 cells exposed to H$_2$O$_2$. Exposure to 4 mM EGTA resulted in a fall in $[\text{Ca}^{2+}]_\text{r}$. Exposure to H$_2$O$_2$ in the presence of EGTA did not result in the rapid increase in $[\text{Ca}^{2+}]_\text{r}$ observed in the absence of EGTA as was shown in Fig. 5. Note that the concentration of H$_2$O$_2$ 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$_2$O$_2$ in U937 Cells Preincubated with or without 1,25(OH)$_2$D$_3$

|                     | Control | 1,25-(OH)$_2$D$_3$ pretreatment |
|---------------------|---------|---------------------------------|
|                     | −preexposure to heat | +preexposure to heat | −preexposure to heat | +preexposure to heat |
| H$_2$O$_2$ (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-(OH)$_2$D$_3$ 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$_2$O$_2$ as described. Cell viability was assessed by trypan blue exclusion (n = 4).

* P < 0.01 compared with 1,25-(OH)$_2$D$_3$-pretreated, unheated cells exposed to 10 mM H$_2$O$_2$.

**Effects of Preexposure to Heat or H$_2$O$_2$ on H$_2$O$_2$ Toxicity in U937 Cells**

Since the HSPs may be involved in protecting cells from injury, we examined the effects on H$_2$O$_2$ 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-(OH)$_2$D$_3$. 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$_2$O$_2$ in medium containing calcium. It is demonstrated in Table II that the toxicity of H$_2$O$_2$ was partially prevented by preexposure to 45°C in 1,25-(OH)$_2$D$_3$-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$_2$O$_2$ did not (39). We therefore compared the effects of preexposure to 45°C for 20 min with preexposure to H$_2$O$_2$. Only preexposure to 45°C protected the U937 cells from the toxic effects of subsequent exposure to H$_2$O$_2$ (Table III). The protective effects of preexposure to heat on H$_2$O$_2$ toxicity were observed in each of

Figure 7. Effects of EGTA on morphology of U937 cells exposed to H$_2$O$_2$. U937 cells were exposed to 10 mM H$_2$O$_2$ 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 II. Effects of Preexposure to Heat or H$_2$O$_2$ on Viability of 1,25-(OH)$_2$D$_3$-treated U937 Cells Exposed to H$_2$O$_2$

| Test conditions (cell viability) | Preincubation | 0 mM H$_2$O$_2$ | 1 mM H$_2$O$_2$ | 5 mM H$_2$O$_2$
|---------------------------------|---------------|----------------|----------------|----------------|
| Control                         |               | 98 ± 1         | 68 ± 4         | 46 ± 4         |
| Heat (45°C)                     | 100 ± 1       | 80 ± 1*        | 69 ± 3*        |
| H$_2$O$_2$ (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$_2$O$_2$ (1 mM for 20 min), allowed to recover for 4 h, then exposed to H$_2$O$_2$ 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$_2$O$_2$ pretreated cells exposed to the same concentration of H$_2$O$_2$.

Discussion

We have found in the present study that the hormone 1,25-(OH)$_2$D$_3$ potentiates the toxicity of H$_2$O$_2$ in the human monocytic line U937 by calcium-dependent mechanisms. This increased toxicity of H$_2$O$_2$ 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)$_2$D$_3$. Two different methods were used to measure this toxicity. Effects on [H]$^3$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)$_2$D$_3$ are calcium dependent. (a) Preincubation of U937 cells with 1,25-(OH)$_2$D$_3$ in calcium-free medium prevented the 1,25-(OH)$_2$D$_3$-induced increase in H$_2$O$_2$ toxicity. The preincubation in calcium-free medium was performed in the presence of dialyzed serum. The prevention of 1,25-(OH)$_2$D$_3$-induced H$_2$O$_2$ 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$_2$O$_2$ toxicity. (b) The increased toxicity of H$_2$O$_2$ in 1,25-(OH)$_2$D$_3$-pretreated U937 cells was not observed when cells were exposed to H$_2$O$_2$ in calcium-free medium even in the presence of undialyzed serum or in the presence of EGTA, maneuvers that decreased or eliminated the H$_2$O$_2$-induced increase in [Ca$^{2+}$]. (c) The basal levels of [Ca$^{2+}$] before the addition of H$_2$O$_2$ were higher in cells pretreated with 1,25-(OH)$_2$D$_3$ 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)$_2$D$_3$ has been shown to increase [Ca$^{2+}$] in hepatocytes (3), the myelomonocytic cell line HL-60 (18), and neonatal mouse osteoblasts (27). In GLC$_1$ cells 1,25-(OH)$_2$D$_3$ does not alter basal [Ca$^{2+}$] but does augment the increase in [Ca$^{2+}$] 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)$_2$D$_3$ did not by itself induce an immediately detectable increase in [Ca$^{2+}$]. After 24 h incubation with 1,25-(OH)$_2$D$_3$, however, the basal levels of [Ca$^{2+}$] were higher than in cells not preexposed to 1,25-(OH)$_2$D$_3$, suggesting that the hormone induces a progressive accumulation of calcium within the cells. When the cells were incubated with H$_2$O$_2$, which as described here induced an increase in [Ca$^{2+}$], dependent upon the presence of extracellular calcium, the 1,25-(OH)$_2$D$_3$-pretreated Ca$^{2+}$-laden cells, may have been unable to cope with this additional calcium influx. The causal relationship between the 1,25-(OH)$_2$D$_3$-induced increase in sensitivity to H$_2$O$_2$ toxicity and the 1,25-(OH)$_2$D$_3$-induced increase in [Ca$^{2+}$] is supported by the findings that [Ca$^{2+}$] in cells preincubated with 1,25-(OH)$_2$D$_3$ in calcium-free medium was not increased over control values and these cells were not more sensitive to H$_2$O$_2$ toxicity. It is possible that the 1,25-(OH)$_2$D$_3$-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$^{2+}$] after 1,25-(OH)$_2$D$_3$ treatment may reflect a much larger amount of sequestered Ca$^{2+}$ in the 1,25-(OH)$_2$D$_3$-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$_2$O$_2$ could result from increased permeability of the...
cell membranes. A resultant increase in \([\text{Ca}^{2+}]_i\) could then activate phospholipase and further enhance the permeability of the cell membrane to \(\text{Ca}^{2+}\). Thus an increase in cellular \(\text{Ca}^{2+}\) 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 \(\text{H}_2\text{O}_2\) in the presence of EGTA.

Other factors might also play a role in the increased toxic effects of \(\text{H}_2\text{O}_2\) in cells preexposed to 1,25-(OH)_2D_3. For example, 1,25-(OH)_2D_3 has been shown to activate secretion of \(\text{H}_2\text{O}_2\) by human peripheral blood monocytes (16). In contrast, in U937 cells, 1,25-(OH)_2D_3 alone does not induce production of superoxide anion (41). However, production of \(\text{H}_2\text{O}_2\) was not directly measured in those experiments. 1,25-(OH)_2D_3-mediated enhanced \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\), 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 \(\text{H}_2\text{O}_2\) 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-(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 \(\text{H}_2\text{O}_2\), 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 \(\text{H}_2\text{O}_2\) does induce a heat shock response (39), are indeed protected from exposure to \(\text{H}_2\text{O}_2\) 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-(OH)_2D_3-pretreated cells, consistent with our previous observations on the increased synthesis of HSPs in 1,25-(OH)_2D_3-treated U937 cells (39).

Preexposure to heat, however, did not modify the \(\text{H}_2\text{O}_2\)-induced increase in \([\text{Ca}^{2+}]_i\). Furthermore, the protective effects of preexposure to heat were observed when the U937 cells were exposed to \(\text{H}_2\text{O}_2\) in medium containing calcium. The protective effects of HSPs are therefore not explained simply by a modulation of the \(\text{H}_2\text{O}_2\)-induced increase in \([\text{Ca}^{2+}]_i\). 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-(OH)_2D_3 induces thermal tolerance in human monocytes and U937 cells the changes in \(\text{Ca}^{2+}\) 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-(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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