Biochemical and Biophysical Research Communications 384 (2009) 18–21

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

1,25-Dihydroxyvitamin D_3 induces Ca^{2+}-mediated apoptosis in adipocytes via activation of calpain and caspase-12

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ARTICLE INFO

Article history:
Received 2 April 2009
Available online 23 April 2009

Keywords:
1,25-dixydroxyvitamin D_3
Intracellular Ca^{2+}
Apoptosis
Calpain
Caspase-12
Adipocytes
3T3-L1 cells
Obesity

ABSTRACT

Induction of apoptotic cell death is emerging as a promising strategy for prevention and treatment of obesity because removing of adipocytes via apoptosis may result in reducing body fat and a long-lasting maintenance of weight loss. However, the mechanisms controlling adipocyte apoptosis are unknown and even the ability of adipocytes to undergo apoptosis has not been conclusively demonstrated. We have shown previously that the specific Ca^{2+} signal, sustained increase in intracellular Ca^{2+}, triggers apoptotic cell death via activation of Ca^{2+}-dependent proteases and that the apoptosis-inducing effect of the hormone 1,25-dixydroxyvitamin D_3 ($1,25(OH)_2D_3$) is mediated through Ca^{2+} signaling. Here, we report that $1,25(OH)_2D_3$ induces apoptosis in mature mouse 3T3-L1 adipocytes via activation of Ca^{2+}-dependent calpain and Ca^{2+}/calpain-dependent caspase-12. Treatment of adipocytes with $1,25(OH)_2D_3$ induced, in concentration- and time-dependent fashion, a sustained increase in the basal level of intracellular Ca^{2+}. The increase in Ca^{2+} was associated with induction of apoptosis and activation of μ-calpain and caspase-12. Our results demonstrate that Ca^{2+}-mediated apoptosis can be induced in mature adipocytes and that the apoptotic molecular targets activated by $1,25(OH)_2D_3$ in these cells are Ca^{2+}-dependent calpain and caspase-12. These findings provide rationale for evaluating the role of vitamin D in prevention and treatment of obesity.

Apoptotic cell death in adipose tissues remains a poorly studied phenomenon. However, a better understanding of cellular and molecular events leading to adipocyte apoptosis is critically important for prevention and treatment of obesity and obesity-related disorders because apoptotic cell death may function as a mechanism of reducing adipose tissue mass. The increase in adipose tissue mass is the result of both hypertrophy, an increase in adipocyte size, and hyperplasia, an increase in adipocyte number [1]. Once adipocytes achieve a maximum size, further increases in the adipose tissue mass involve an increase in adipocyte number [2,3]. Therefore, weight loss can be caused not only by a decrease in adipocyte size (i.e., increasing lipolysis with a potential for lipotoxic effects), but also in adipocyte number (e.g., by stimulating apoptosis). Even a small increase in the rate of adipocyte apoptosis will prevent excessive accumulation of adipose tissue and may result in a significant loss of adipose tissue mass over time.

Although mature, differentiated adipocytes are postmitotic and thought to be extremely stable, evidence has been emerging to support the endogenous elimination of adipocytes within adipose tissue. For example, the leptin-induced adipocyte apoptosis has been demonstrated [4–6], and the isoflavone genistein has been shown to reduce body fat in ovariectomized mice due to, at least in part, ablation of fat cells [7].

Ca^{2+} ion, as a key cellular regulator, has been implicated in induction of apoptosis and regulation of apoptotic signaling pathways. We [8–11] and others [12–14] have shown that early and late increases in concentration of intracelluar Ca^{2+} ($[Ca^{2+}]_i$) occur in apoptosis. The critical characteristic of the apoptotic Ca^{2+} signal is a sustained increase in $[Ca^{2+}]_i$, reaching elevated, but not cytotoxic levels [10,11]. Although there is little doubt that such an increase in $[Ca^{2+}]_i$ triggers cell death via apoptosis, interactions of the cellular Ca^{2+} signal with targets in cells undergoing apoptosis have not been identified. Caspases and Ca^{2+}-dependent neutral proteases, the calpains, are considered as the possible Ca^{2+}-activated apoptotic targets [14–19].

This study was undertaken to determine whether Ca^{2+}-mediated apoptosis can be induced in mature adipocytes. We hypothesized that an increase in $[Ca^{2+}]_i$ would activate Ca^{2+}-dependent apoptotic molecular targets in these cells. We employed the Ca^{2+} regulatory hormone $1,25(OH)_2D_3$ to trigger the apoptotic Ca^{2+} signal in mature mouse 3T3-L1 adipocytes. The results obtained demonstrate that $1,25(OH)_2D_3$ increases basal levels of intracellular Ca^{2+}, activates apoptotic proteases, Ca^{2+}-dependent calpain and Ca^{2+}/calpain-dependent caspase-12, and induces apoptosis in mature adipocytes.

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doi:10.1016/j.bbrc.2009.04.078

Materials and methods

Cell culture. The established, well characterized 3T3-L1 preadipocyte cell line (ATCC) was used in these experiments. Preadipocytes maturation was induced with 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin [20]. 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% adult bovine serum and 1% penicillin-streptomycin (10,000 U/mL penicillin and 10,000 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 10% CO_2 in air. To induce differentiation, 2-day postconfluent preadipocytes were stimulated for 48 h with 0.5 mM IBMX, 0.5 μM dexamethasone, and 10 μg/mL insulin (MDI) added to DMEM/10% fetal bovine serum (FBS) culture medium. After 2-day stimulation, the MDI medium was replaced with DMEM/10% FBS; treatment of mature adipocytes with $1,25(OH)_2D_3$ (12.5–400 nM, Sigma) or vehicle (ethanol, 0.1%) started at this point (day 0) and continued for 1, 3, or 6 days.

Intracellular Ca^{2+}. For $[Ca^{2+}]_i$ measurements [21,22], cells grown in the 96-well, black-wall plates were loaded with 2 μM of fluo-3/AM (Molecular Probes) in Dulbecco's PBS (D-PBS) supplemented with 0.1% DMSO for 40 min at 37 °C. Fluorescence (485 nm excitation, 530 nm emission) was measured in the FLx800 plate reader with KC software (BioTek) and expressed in relative fluorescence units (RFUs) per well. EC_{50} values were calculated using a four parameter logistic dose response model (4PL function, Sigma Plot 11.0, Systat Software).

Apoptosis. Apoptosis was evaluated by the plasma membrane and nuclear changes. Hoesht 33342 was used to detect nuclear fragmentation/condensation. Annexin V assay (Alexa Fluor 488 Annexin V Assay Kit; Molecular Probes) was used for detection of the apoptotic plasma membrane (phosphatidylserine translocation) [18]. Fluorescence (485 nm excitation, 530 nm emission) of the Annexin V-labeled cells grown in 96-well plates was measured in the FLx800 plate reader and expressed in RFUs per well. EC_{50} values were calculated as described above.

Fluorescence microscopy of Annexin V- and Hoechst 33342-labeled cells was employed to visualize cellular apoptotic changes. For fluorescent digital imaging, cells grown on coverslips were labeled with 1 μM of Annexin V-Alexa Fluor 488 (Molecular Probes) and 0.1 μg/mL of Hoesht 33342 in Annexin V binding buffer for 30 min at 37 °C. The images were acquired with cells in the micr-oincubation chamber (37.0 ± 0.2 °C) on a Nikon Eclipse TE-300 inverted microscope equipped for epifluorescent, ratiometric, digital imaging. The images were captured using SuperFluor 40× 1.3 NA oil-immersion objective (Nikon) and CoolSnapFX digital CCD camera (Photometrics), (350 and 490 nm excitation for Hoesht 33342 and Annexin V-Alexa Fluor 488, respectively). Image analysis was performed using MetaFluor 7.0 software (Molecular Devices/Universal Imaging) [23,24].

Calpain and caspase-12. Calpain activation in adipocytes was measured with the membrane-permeable fluorogenic peptide substrate t-Boc-Leu-Met-CMAC (50 μM; CMAC, 7-amino-4-chloromethyl coumarin; Molecular Probes) [16,21]. Activity of the Ca^{2+}-dependent caspase-12 was measured with the fluorogenic peptide substrate Ala-Thr-Ala-Asp-AFC (50 μM; AFC, 7-amino-4-trifluoromethyl coumarin; Caspase-12 Fluorometric Assay Kit, BioVision) [18,19]. Fluorescence of cells grown in the 96-well microplates

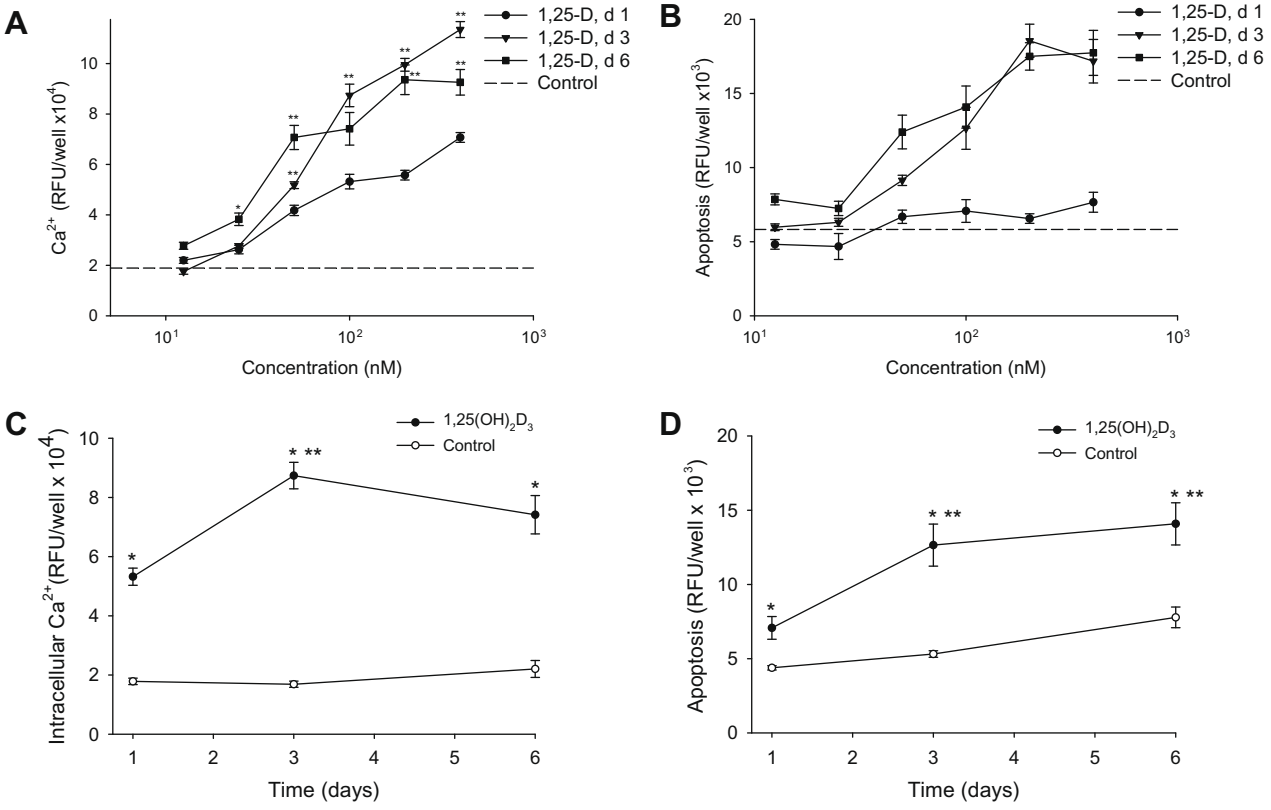

Fig. 1. Ca^{2+}- and apoptosis-inducing activity of $1,25(OH)_2D_3$ in mature adipocytes; (A) and (B), concentration-depended changes in $[Ca^{2+}]_i$ and apoptosis at day 1, 3, and 6 of treatment; (C) and (D), time-course changes in $[Ca^{2+}]_i$ and apoptosis in cells treated with 100 nM of $1,25(OH)_2D_3$. Intracellular Ca^{2+} and apoptosis were measured with fluo-3/AM and Annexin V-Alexa Fluor 488, respectively. Mature adipocytes were treated with $1,25(OH)_2D_3$ (12.5–400 nM) for 1, 3, or 6 days in 96-well microplates; intensity of fluorescence was measured as described in the *Methods* section. Panels A and B, *X* axis is log (common). Results (mean ± SE) are presented as relative fluorescence units (RFUs) of fluo-3 (intracellular Ca^{2+}) or Annexin V-Alexa Fluor 488 (apoptosis) per well. $^{*}p < 0.05$, as compared with the corresponding control; $^{**}p < 0.05$, as compared between day 1 and day 3 or 6 of the treatment with $1,25(OH)_2D_3$. Reference lines for $[Ca^{2+}]_i$ (A) and apoptosis (B) values in controls are average of day 1, 3, and 6. For $[Ca^{2+}]_i$ (A) and apoptosis (B), $p < 0.05$ between day 1, 3, or 6 and control (*) for concentrations of $1,25(OH)_2D_3$ in the range of 50–400 nM. For apoptosis (B), $p < 0.05$ between day 1 and day 3 or 6 (**) for concentrations of $1,25(OH)_2D_3$ in the range of 50–400 nM.

and loaded with the fluorogenic substrates was measured in the FLx800 reader.

Results

Intracellular Ca^{2+}

The basal levels of intracellular Ca^{2+} were measured in mature 3T3-L1 adipocytes treated with $1,25(OH)_2D_3$ for 1, 3, or 6 days. $1,25(OH)_2D_3$ induced a sustained increase of $[Ca^{2+}]_i$ in a concentration- and time-dependent fashion (Fig. 1A). Effective concentrations (EC_{50}) of $1,25(OH)_2D_3$ were 69.6 ± 16.6, 62.3 ± 7.16, and 67.0 ± 20.1 nM for day 1, 3, and 6, respectively. These findings demonstrate that $1,25(OH)_2D_3$ increases basal levels of intracellular Ca^{2+} in mature adipocytes in vitro at concentrations which are similarly effective in various cell lines [10]. A rapid (day 1) onset of the $[Ca^{2+}]_i$ increase and its sustainability (day 3 and 6) (Fig. 1C) may indicate involvement of both genomic and non-genomic mechanisms in producing this effect [11].

Apoptosis

Apoptosis in the mature 3T3-L1 adipocytes was evaluated by the plasma membrane changes (loss of membrane asymmetry due to phosphatidylserine translocation) and morphological criteria (nuclear fragmentation/DNA condensation). Mature adipocytes were treated with $1,25(OH)_2D_3$ as described above for $[Ca^{2+}]_i$ measurements. $1,25(OH)_2D_3$ induced apoptosis in these cells in a concentration- and time-dependent fashion similar to that observed for its $[Ca^{2+}]_i$-elevating effect (Figs. 1B and D and 2). EC_{50} concentrations of $1,25(OH)_2D_3$ were similar to those for inducing $[Ca^{2+}]_i$ increase: 77.4 ± 23.2 and 61.7 ± 20.5 nM for day 3 and 6, respectively. $1,25(OH)_2D_3$ was not effective in inducing apoptosis at day 1 of treatment, although $[Ca^{2+}]_i$ was increased. A delayed onset of $1,25(OH)_2D_3$-induced apoptosis in adipocytes correlated with the delayed increase of $[Ca^{2+}]_i$ (compare Fig. 1C and D). It is important to note that the EC_{50} values of $1,25(OH)_2D_3$ for increasing $[Ca^{2+}]_i$ and inducing apoptosis were similar.

Calpain and caspase-12

A sustained increase in $[Ca^{2+}]_i$ in mature 3T3-L1 adipocytes treated with $1,25(OH)_2D_3$ (100 nM) for 1, 3 or 6 days was accompanied by activation of the Ca^{2+}-dependent apoptotic proteases, μ-calpain and caspase-12, as evaluated with the specific fluorogenic peptide substrates (Fig. 3). The $1,25(OH)_2D_3$-induced calpain activity reached maximum at day 3 of treatment followed by a statistically significant decrease at day 6, while caspase-12 activity remained elevated at day 6. These findings support our previous observations [11,19] that calpain activation precedes caspase-12 activation in the Ca^{2+}-mediated apoptotic cascade and, thus, may be necessary for caspase-12 activation.

Discussion

Obesity is associated with an increased adipocyte number [1–3] leading to the excessive accumulation of adipose tissue. Apoptosis, a highly regulated form of cell death, is the main mechanism for regulating cell number in most tissues [25]. However, studies on the role of apoptosis in the fat tissue have been limited by the fact that mature adipocytes are extremely stable and not thought to be capable of undergoing apoptosis. As a consequence, our understanding of the signaling pathways by which cell death in adipose tissue is regulated and the mechanisms controlling the adipocyte number are extremely limited.

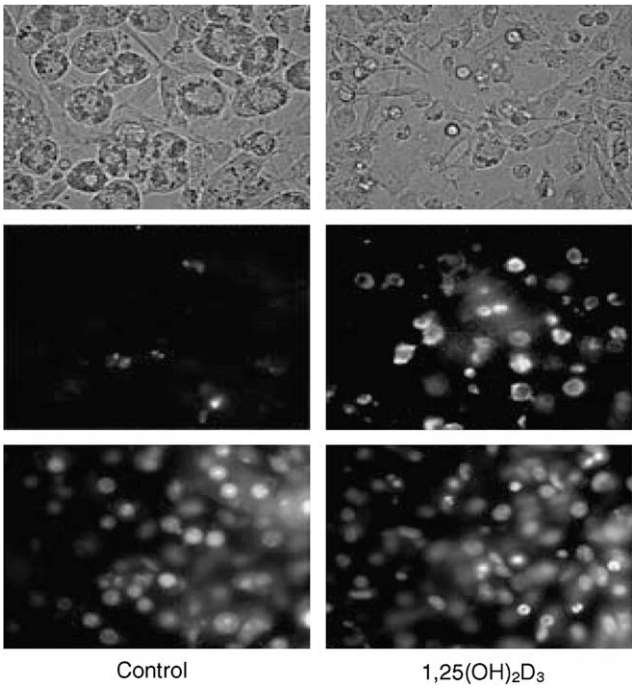

Fig. 2. Apoptosis induced by $1,25(OH)_2D_3$ in mature adipocytes. Upper row, phase-contrast images; middle row, Annexin V-labeled cells (plasma membrane); lower row, Hoesht 33342-labeled cells (DNA). The mature adipocytes were treated with $1,25(OH)_2D_3$ (100 nM) for 3 days. Note membrane labeling of, and nuclear fragmentation/DNA condensation in, the apoptotic (round) cells on fluorescence images; significantly less abundant and smaller lipid droplets in cells treated with $1,25(OH)_2D_3$ than in control cells on phase-contrast images.

Induction of death of adipocytes through apoptosis may emerge as a promising strategy for prevention and treatment of obesity because removal of adipocytes via this mechanism will result in reducing body fat. Importantly, decreasing adiposity through induction of adipocyte apoptosis can result in long-lasting maintenance of weight loss, in contrast to that which occurs after caloric restriction.

Vitamin D deficiency and insufficiency is prevalent in obese adults and children [26,27], and low 25(OH)D concentrations were observed in obese patients [28]. Adiposity and obesity risk may be regulated by dietary calcium [29], although lack of relationship between calcium intake and body size in an obesity-prone population was also reported [30]. These observations implicate a possible role for the hormone $1,25(OH)_2D_3$ in regulation of adiposity.

In this study, we employed the model of mature mouse 3T3-L1 adipocytes treated with the Ca^{2+} regulatory hormone $1,25(OH)_2D_3$ to investigate the mechanism of Ca^{2+}-mediated apoptosis in these cells. Results obtained demonstrate that $1,25(OH)_2D_3$ is effective in increasing concentration of intracellular Ca^{2+} and inducing Ca^{2+}-mediated apoptosis in mature adipocytes. These observations are important, because they indicate that apoptosis can be induced in mature, differentiated adipocytes and that induction of apoptosis in these cells is associated with a sustained increase in intracellular Ca^{2+}. Although involvement of $1,25(OH)_2D_3$ in increasing $[Ca^{2+}]_i$ and inducing apoptosis has been previously demonstrated in several cell lines [11], the findings presented here demonstrate, for the first time, that apoptosis can be induced in mature adipocytes rather than nondifferentiated preadipocytes, and that Ca^{2+}-mediated signaling can be employed to trigger apoptosis in these cells. Furthermore, our data provide the identification of apoptotic molecular targets in adipocytes, calpain and caspase-12, that are Ca^{2+}-dependent and appear to be capable of executing apoptosis.

Collectively, results reported here support the hypothesis that $1,25(OH)_2D_3$ induces apoptosis in mature adipocytes via activation

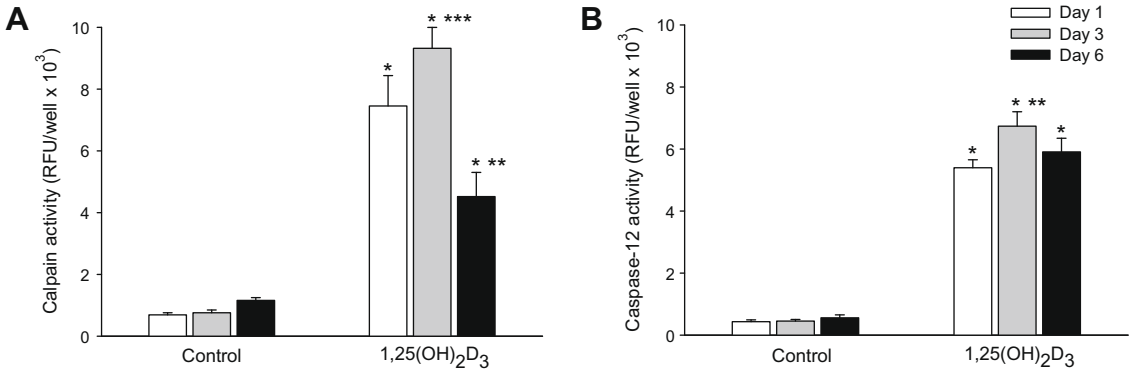

Fig. 3. Calpain and caspase-12 activity in mature adipocytes treated with $1,25(OH)_2D_3$. Adipocytes were treated with $1,25(OH)_2D_3$ (100 nM) or vehicle for 1, 3, or 6 days. Calpain (A) and caspase-12 (B) activities were measured by using the specific fluorogenic peptide substrates, as described in the *Methods* section. Results (mean ± SE) are presented as relative fluorescence units (RFUs) per well. (*), (**) and (***), indicates statistically significant differences ($p < 0.05$) between $1,25(OH)_2D_3$-treated cells and the corresponding control (*), between day 1 and day 3 or 6 (**) or between day 3 and 6 (***) of the treatment with $1,25(OH)_2D_3$.

of the Ca^{2+}/calpain/caspase-dependent pathway and indicate that targeting of Ca^{2+} signaling to induce apoptotic death in adipocytes may represent an effective strategy for prevention and treatment of obesity. Further studies are warranted to explore the potential for targeting Ca^{2+}-mediated apoptosis as a therapeutic approach for induction of adipocyte death.

Acknowledgments

The author thank Julie Colby for her technical assistance. This research was supported by USDA Grants SD00H167-061HG and 2009-35200-05008 to I.N.S.

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