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RESEARCH ARTICLE

Vitamin D receptor levels and binding are reduced in aged rat intestinal subcellular fractions

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Abstract The hormonal form of vitamin D, $1\alpha,25(OH)_2$-vitaminD_3 [$1\alpha,25(OH)_2D_3$], stimulates signal transduction pathways in intestinal cells. To gain insight into the relative importance of the vitamin D receptor (VDR) in the rapid hormone responses, the amounts and localization of the VDR were evaluated in young (3 months) and aged (24 months) rat intestinal cells. Immune-fluorescence and Western blot studies showed that VDR levels are diminished in aged enterocytes. Confocal microscopy assays revealed that the VDR and other immune-reactive proteins have mitochondrial, membrane, cytosol and perinuclear localization. Western blot analysis using specific antibodies detected the 60 and 50 kDa bands expected for the VDR in the cytosol and microsomes and, to a lesser extent, in the nucleus and mitochondria. Low molecular weight immune-reactive proteins were also detected in young enterocytes subcellular fractions. Since changes in hormone receptor levels appear to constitute a common manifestation of the ageing process, we also analyzed $1\alpha,25(OH)_2D_3$ binding properties and VDR levels in subcellular fractions from young and aged rats. In competition binding assays, employing [^{3}H]-$1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$, we have detected specific binding in all subcellular fractions, with maximum binding in mitochondrial and nuclear fractions. Both, VDR protein levels and $1\alpha,25(OH)_2D_3$ binding, were diminished with ageing. Age-related declines in VDR may have important consequences for correct receptor/effector coupling in the duodenal tissues and may explain age-related declines in the hormonal regulation of signal transduction pathways that we previously reported.

Keywords Enterocytes · $1\alpha,25(OH)_2D_3$ · Vitamin D receptor · Subcellular fractions · Ageing

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Introduction

Vitamin D receptor (VDR) was cloned and identify as a member of steroid hormone family receptors in chicken (Lu et al. 1967), human (Baker et al. 1988) and rat (Burmester et al. 1988). The VDR presents the classical structure of nuclear receptors; consists of an N-terminal activation domain, a DNA-binding region (DBD) comprising two zinc finger domains, a hinge region and the ligand-binding domain (LBD). Upon ligand binding the receptor dimerizes with the retinoic X receptors (RXR), binds to DNA-response elements in promoters of vitamin D responsive genes and modulates cell and tissue specific gene expression (Jurutka et al. 2001). Despite the fact that nuclear localization of VDR-binding to the hormone

is well established, little is known about the receptor half life in the nuclear compartment and the signal on/off mechanism. Furthermore, unoccupied VDR subcellular distribution has been a theme of discussion for many decades and still controversial. Barsony and col. using BODIPY-calcitriol monitored the subcellular distribution of VDR in living cells and have reported a significant proportion of VDR residing in the cytoplasm, colocalized with the endoplasmic reticulum, the Golgi complex and microtubules and discrete regions of the nucleus and along the nuclear envelope (Barsony et al. 1997).

Like many other steroids, 1α,25-dihydroxy-vitamin D_3 (1α,25$(OH)_2D_3$) exert rapid effects on target cells which occur within seconds and minutes. Generally, they are described as so called non-genomic or membrane-initiated effects (Norman et al. 1992; Boland et al. 1995). In rat intestinal cells (enterocytes), as in other target cells, the hormone elicits responses through nuclear receptor-mediated gene transcription and a fast mechanism independent of new RNA and protein synthesis (Norman et al. 1992; Boland et al. 1995). We have previously reported 1α,25$(OH)_2D_3$-rapid activation of the MAP quinases ERK1/2 and p38 MAPK in enterocytes from young and aged rats, suggesting that interaction with a plasma membrane receptor might be responsible for the initiation of this non-genomic actions (González Pardo and Russo de Boland 2004; González Pardo et al. 2007). The mechanism responsible for these rapid actions has not been completely elucidated and it is not known whether the nuclear receptor itself or a membrane VDR is involved. The presence of membrane binding sites for 1α,25$(OH)_2D_3$ in chick intestinal cells has been described (Nemere et al. 1994). Recently, a duodenal chicken cDNA coding for a multifunctional membrane-associated protein which binds 1,25(OH)2 vitamin D_3 and transduces its stimulatory effects on phosphate uptake was cloned (1,25$(OH)_2$ vitamin D_3 membrane-associated, rapid-response steroid-binding protein [1,25D_3-MARRS]; Nemere et al. 2004). The expression of the mRNA declined during age which resulted in a loss of 1,25$(OH)_2$ vitamin D_3 mediated phosphate uptake. There is still a considerable debate as to whether the classical "nuclear" receptors could specifically mediate the membrane associated effects while being embedded in a membrane associated protein ensemble. Other lines of evidence point out a role of the VDR itself in mediating some of the rapid, non-genomic effects of the hormone (Erben et al. 2002; Buitrago et al. 2001). Furthermore, Huhtakangas reported the presence of VDR and saturable binding for 1α,25$(OH)_2D_3$ in caveolas-enriched plasma membranes (Huhtakangas et al. 2004). Thus, the problem of membrane associated signaling of "nuclear" receptors awaits further elucidation.

In the present work, we have investigated non classical localization of the VDR in young and aged rat intestinal cells by immunochemical and binding assays to further understand 1α,25$(OH)_2D_3$ signal transduction impairment in rat intestinal cells within aging.

Materials and methods

Materials

1α25$(OH)_2D_3$ was kindly provided by Dr. Jan-Paul van de Velde from Solvay Pharmaceuticals (Weesp, The Netherlands). [^{3}H]1α25$(OH)_2D_3$ (specific activity: 155.4 Ci/mmol) was purchased from NEN Perkin Elmer (Wellesley, MA, USA). Phenylmethanesulphonyl fluoride (PMSF), dithiothreitol (DTT), and Immobilon P (Polyvinylidene difluoride, PVDF) membranes were from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies rat monoclonal anti-VDR (clone 9A7) cat. (MA1-710 and rabbit polyclonal anti-VDR cat. (PA1-711 was from Affinity BioReagents, Inc., (Golden, CO, USA). Secondary antibody anti-rabbit and anti-rat horseradish peroxidase-conjugated IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-biotin was from Pierce Biotechnology, Inc. (Rockford, IL, USA) and streptoavidin-Oregon Green 488-conjugated, secondary goat anti-rabbit IgG Oregon Green 488-conjugated antibody and Mitotracker Red CMX-Ros were from Molecular Probes (Eugene, OR, USA). The Super Signal CL-HRP substrate system for enhanced chemiluminiscence (ECL) was obtained from Amersham Corp. (Arlington Heights, IL, USA). Other chemicals used were of analytical grade.

Animals

Young (3 month-old) and aged (24 month-old) male *Wistar* rats were fed with standard rat food (1.2% calcium; 1.0% phosphorous), given water ad libitum

and maintained on a 12-h light–12-h dark cycle. Animals were killed by cervical dislocation. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals (1996).

Enterocytes isolation

Duodenal cells were isolated as described previously (Massheimer et al. 1995). The method employed yields preparations containing only highly absorptive epithelial cells that are devoid of cells from the upper villus or crypt (Weiser 1973). The duodenum was excised, washed with 0.9% NaCl and trimmed of adhering tissue. The intestine was slit lengthwise and cut into small segments (2 cm length) and placed into solution A containing (in mM): 96 NaCl, 1.5 KCl, 8 KH_2PO_4, 5.6 Na_2HPO_4, 27 Na-citrate, pH 7.3, for 10 min at 37°C. The solution was discarded and replaced with isolation medium containing (in mM): 154 NaCl, 10 NaH_2PO_4, 1.5 EDTA, 0.5 DTT, 5.6 glucose, pH 7.3, for 15 min at 37°C with continuous shaking (50 oscillations/min). The cells were sediment by centrifugation at 750*g* for 10 min, washed twice with 154 mM NaCl, 10 mM NaH_2PO_4, 5.6 mM glucose, pH 7.3, and then resuspended in incubation medium. All the steps mentioned above were performed under an atmosphere of 95% O_2: 5% CO_2. Cell viability was assessed by the Trypan Blue technique. Exclusion of the dye in >90% of the cells were observed for at least 90 min after isolation. Enterocytes isolated by this procedure have been shown to possess functional characteristics of intestinal cells (Weiser 1973). Morphological characterization of preparations was performed by phase-contrast microscopy and revealed no morphological differences between enterocytes isolated from young and old rats.

SDS-PAGE and Western blot analysis

Proteins were separated by one-dimensional SDS-PAGE (Laemmli 1970). Briefly, samples were mixed with 2× Laemmli sample buffer (250 mM Tris–HCl pH 6.8, 8% SDS, 40% glycerol, 20% 2-β-mercaptoethanol and 0.02% bromophenol blue) and heated for 5 min at 95°C. Proteins (30–35 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide minigels and then transferred to PVDF membranes. The membranes were immersed in blocking solution containing 5% non-fat dry milk and TBS-T buffer (50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 0.1% Tween-20) for 1 h at room temperature. After blocking, the membrane was washed five times using TBS-T buffer. Afterward, the membrane was incubated in primary blocking solution containing TBS-T buffer and 1 μg/ml goat anti-rat IgG-biotin for 2 h at room temperature to block non-specific sites. The membrane was washed three times in TBS-T buffer and incubated overnight at 4°C with primary antibody solution (rat anti-VDR (1:1,000) antibody (clone9A7) or polyclonal anti-VDR (1:100)). The next day, the membranes were washed five times in TBS-T and then incubated in TBS-T 2% non-fat dry milk secondary antibody solution (1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rat IgG or 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG) 1 h at room temperature. After three washes with TBS-T buffer, bands were visualized by using an enhanced chemiluminiscent technique, according to the manufacturer's instructions. Images were obtained with a model GS-700 imaging densitometer from Bio-Rad (Hercules, CA 94547, USA) by scanning at 600 dpi and printing at the same resolution.

Immunocytochemistry

Isolated cells were fixed with 2% paraformaldehyde for 2 h. After two washes with PBS (centrifugation at 200*g* for 1 min), the cells were permeabilized with 0.05% Triton for 10 min, washed with PBS and incubated with primary anti-policlonal VDR (50 μg/ml) antibody in PBS 2% BSA for 2 h at room temperature. The cells were then washed three times with PBS and incubated with the secondary antibody, Oregon-green 488-conjugated goat anti-rabbit IgG, for 1 h at room temperature. Integrity of the nucleus was determined by fluorescence microscopy, using DAPI in the last 20 min of the incubation with the secondary antibody. Analysis was carried out with a Nikon Eclipse 600 camera mounted on a fluorescence microscope equipped with a 60× objective.

Confocal microscopy

Isolated enterocytes from young rats were incubated in presence of mitotracker Red CMXRos for

30 min. Then, they were fixed with methanol for 10 min at −20°C. Afterward, the cells were incubated in blocking solution containing TBS-T buffer 5% BSA and 1 μg/ml anti-rat IgG antibody for 1 h a room temperature to block non-specific sites. After three washes in TBS-T, the cells were incubated with monoclonal anti-VDR (clone 9A7, 5 μg/ml) overnight at 4°C. The next day, the cells were washed as previously described and incubated with anti-biotin–streptoavidin-Oregon Green 488. Control cells were incubated with secondary antibodies alone. Images were taken with an invert Leica DM IRB2 microscope for immunofluorescence and transmitted light. The confocal spectral module SP2 is equipped with Ar laser (458, 476, 488 and 514 nm) and HeNe laser (633 nm). Viewing was carried out with 63× 1.3 NA water-immersion objectives.

Subcellular fractionation

The duodenum from young and aged rats was slit lengthwise, the mucosa was scrapped and homogenized in ice-cold TES buffer (5 ml buffer/1 g tissue) (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 250 mM sucrose) containing proteases inhibitors (1 mM DTT, 0.5 mM PMSF, 20 μg/ml aprotinin and 20 μg/ml leupeptin), using a teflon-glass hand homogenizer. The homogenate was centrifuged at 200*g* for 5 min to eliminate debris; supernatant was further centrifuged at 1,400*g* for 20 min to sediment the nuclear fraction. The supernatant was further centrifuged at 14,000*g* for 20 min to pellet mitochondria. The remaining supernatant was centrifuged for 60 min at 120,000*g* to yield a microsomal pellet and a soluble supernatant (cytosol). Protein concentration from each fraction was estimated by the method of Bradford (1976), using bovine serum albumin as standard. Anti-lamin B antibody was employed for the immunodetection of the nuclear protein marker lamin B in the different fractions. Contamination of nuclear, microsomal and cytosolic fractions with mitochondrial components was assessed by measuring the activity of the mitochondrial marker enzyme cytochrome c oxydase employing the Cytochrome c Oxidase assay kit (Sigma) according to manufactureŕs instructions.

Competition radioligand-binding assays

Hormone binding to subcellular factions was measured by incubating 100 μg protein samples from each fractions in 0.2 ml of TES in the presence of 3 nM [^{3}H]1α,25(OH)$_2$D$_3$ (total binding) and 200-fold excess of unlabeled 1α,25(OH)$_2$D$_3$ (non-specific binding). The samples were incubated at 4°C in dark and with constant shaking for 4 h. Separation of bound and free hormone was accomplished by hydroxylapatite adsorption of the ligand-receptor complex (Wecksler and Norman 1979). Briefly, 200 μl of resin suspension was added to each tube and the mixture was incubated for 15 min at 4°C with vortex mixing every 5 min. Then, the mixture was centrifuged 3 min at 800*g* and the pellet were washed three times with TE buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 2 mM DTT, 0.3 mM PMSF) containing 0.5% Triton X-100, adjusted to reduce non-specific binding.

Trapped radioactivity was measured after a final extraction with ethanol. Specific binding sites were then quantified by subtracting non-specific binding sites from sites bound in presence of [^{3}H]1α,25(OH)$_2$D$_3$ alone (total binding). The radioactivity retained by the resin pellets was determined by liquid scintillation spectrometry.

25OHD$_3$ analysis

Serum 25OHD$_3$ was determined essentially as described (Linbäk et al. 1987). Briefly, 5,000 counts/mm of [26,27-3H]-25-hydroxyvitaminD$_3$ (specific activity 176 kCi/mol), dissolved in 99.5% ethanol, was added to 2 ml of serum, then the sample was extracted with of acetonitrile. After centrifugation the supernatant was apply to a Sep-Pak C38 and was successively eluted with water, methanol/water (7/3 by vol), and acetonitrile. The solvent was evaporated under nitrogen atmosphere and the residue dissolved in methanol/water (9/1 by vol), injected into a Nucleosil C18 column and eluted with methanol/water (9/1 by vol) as mobile phase. After the HPLC step, the absorbance at 254 nm was measured. The amount of 25-hydroxyvitaminD3 in each serum sample was calculated by using a standard curve covering the range 0–100 g/l.

Statistical evaluation

Statistical significance of data was evaluated using Student *t*-test. Quantitative data are expressed as the means ± SD from the indicated set of experiments.

Results

We first evaluated the presence of VDR in isolated enterocytes from young and aged rat by immunochemistry approaches. We used a VDR antibody against aminoacids 395–413 from C-terminus region of the receptor that contains the binding domain (rabbit anti-VDR policlonal). The cells were fixed in paraformaldehyde 2% and followed by permeabilization in 0.05% Triton X-100 and then incubated with anti rabbit VDR polyclonal antibody as was described under "Materials and methods." Figure 1a and d, show that isolated enterocytes from young and aged rat, under light field microscopy, preserved cell morphology after isolation. By epifluorescence microscopy, in both, young and aged cells, VDR and/or VDR immunoreactive proteins showed nuclear, membrane and extended cytoplasmic localization, indicating that VDR and/or related proteins in untreated cells have not only nuclear distribution but also cytoplasm and membrane localization (Fig. 1b and e). Nuclear integrity was evaluated incubating the cells with DAPI (Fig. 1c and f), in either young or aged cells, necrotic or apoptotic cells were not present in most of the observations performed. To quantify VDR levels from young and aged duodenal cells, total homogenate were subjected to SDS-page and then Western blot analysis were carried out using the same anti-VDR antibody (Fig. 2). An immunoreactive band corresponding to VDR molecular weight (~53 kDa) was detected in both young and aged cells. With ageing, VDR levels showed a 70% decreased as comparing to young cells. Moreover, a similar age-related decrease in intestinal VDR were observed in female as in male 24 old month rats (data not shown). Despite an age-related decrease in VDR levels, serum 25-OHD3 concentration did not significantly change with age (34.10 ± 0.98; 36.00 ± 1.10; 45.00 ± 1.25 ng/ml, for 3, 12 and 24 months rats, respectively).

To better characterize subcellular distribution of VDR and/or related proteins, confocal microscopy studies were performed on methanol-fixed young duodenal cells using primary monoclonal VDR (clone 9A7) antibody that recognize DNA binding domain of avian VDR as was described under "Materials and methods." We found extended cytoplasmatic speckles, perinuclear and plasma membrane distribution (50% of cells) of VDR or related proteins (Fig. 3b). Control cells incubated with secondary antibodies alone showed low background fluorescence (Fig. 3a). Because of the speckles observed that resemble mitochondria, we further explore whether VDR or related proteins have mitochondrial localization. For this purpose, isolated intestinal cells were incubated in the presence of mitotracker for 30 min, and then, they were fixed in methanol, and incubated with monoclonal VDR (clone 9A7) antibody as described above (Fig. 4). The VDR or related proteins (green, Fig. 4a) partially colocalize (yellow, Fig. 4c) with mitotracker signal (red, Fig. 4b) suggesting that VDR and related proteins also are located in the mitochondria. In parallel, equivalent protein amounts of subcellular fractions were subjected to SDS-PAGE and Western blot analyses using anti-VDR monoclonal (clone 9A7) antibody. As is shown in Fig. 4d, VDR, that appears as a band of 50 and 60 kDa., was detected in cytosol and microsomes and to lesser extent in nuclear and mitochondrial fractions. The monoclonal antibody also detected lower molecular weight (35–32 kDa) immunoreactive proteins in all fractions.

Since changes in hormone receptor levels appear to constitute a common manifestation of the ageing process, it may be possible that specific binding would be decreased in aged subcellular fractions due to lower levels of VDR. Therefore, we analyzed $1\alpha,25(OH)_2D_3$ binding properties by competition assays and VDR levels in subcellular fractions from young and aged rats. Hormone binding to subcellular factions was measured by incubating 100 μg protein from each fraction in the presence of 3 nM $[^3H]1\alpha,25(OH)_2D_3$ (total binding) and 200-fold excess of unlabeled $1\alpha,25(OH)_2D_3$ (non-specific binding). The samples were incubated at 4°C in darkness with constant shaking for 4 h. Separation of bound and free hormone was accomplished by hydroxylapatite adsorption of ligand-receptor complex (Wecksler and Norman 1979). Specific $1\alpha,25(OH)_2D_3$ binding was determined by the difference between the radioactivity bound in presence of

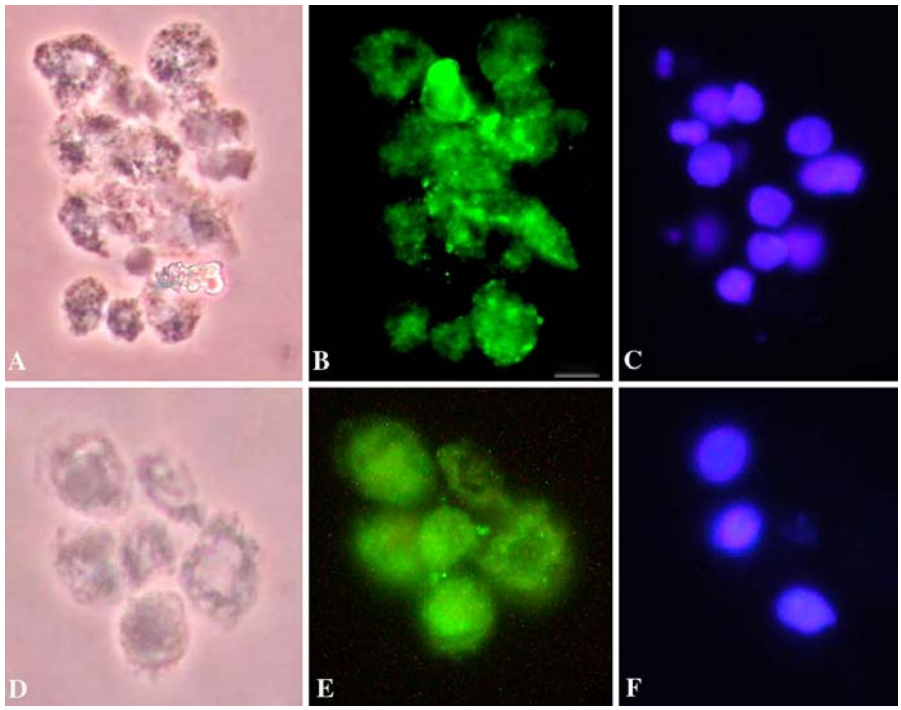

Fig. 1 Cellular distribution of VDR in young and aged rat enterocytes. Enterocytes isolated from young (**a**, **b**, **c**) and aged (**d**, **e**, **f**) rats were fixed with 2% of paraformaldehyde and permeabilized with 0.05% Triton X-100 as described under "Materials and methods." (**a**, **d**) Light microscopy. (**b**, **e**) Cells were incubated with rabbit policlonal antibody against VDR (PAI-711). (**c**, **f**) Cells were stained with DAPI to show nuclear morphology and integrity. The images were obtained by an epi-fluorescence microscopy with a Nikon Eclipse 600 camera. Original magnifications 600×

$[^3H]1\alpha,25(OH)_2D_3$ alone (total binding) and the radioactivity bound in the non-specific tubes. As shown in Fig. 5, specific binding was detected in all subcellular fractions, with maximum binding in mitochondrial and nuclear fractions. With ageing, $1\alpha,25(OH)_2D_3$ specific binding in all subcellular fractions was greatly diminished. In accordance with these results, parallel Western blot analyses of the same subcellular fractions, with anti-VDR polyclonal antibody, revealed decreased VDR protein levels in aged rats (Fig. 5, lower panel). High specific 1,25 $(OH)_2$ D_3 binding to the mitochondrial fraction, in contrast with low VDR protein levels, suggest that other molecular entity might bind to the hormone in a specific manner.

Discussion

The ability to respond to certain hormones is altered during the aging process. Attempts to elucidate the mechanisms involved in such changes have focused primarily on the various molecular components which mediate hormone actions. These include chromatin, the nucleus, cytoplasmic factors, adenylate cyclase and hormone receptors. Age related changes have been observed at all of these levels, and in some cases have been correlated with changes in biological responsiveness to particular hormones.

In this work we described the presence and subcellular localization of VDR and VDR related proteins in enterocytes from young and aged rats. We have demonstrated that VDR levels are diminished in aged enterocytes by immune-fluorescence and Western blot studies employing antibodies against the classic VDR. Expanding this view, the existence of immunoreactive proteins at membrane, perinuclear, cytosol and mitochondria by confocal microscopy assays was shown. In addition, by Western blot analysis, using specific antibodies, we have detected the 60 and 50 kDa bands expected for the VDR in the cytosol and microsomes and, to a lesser extent, in the

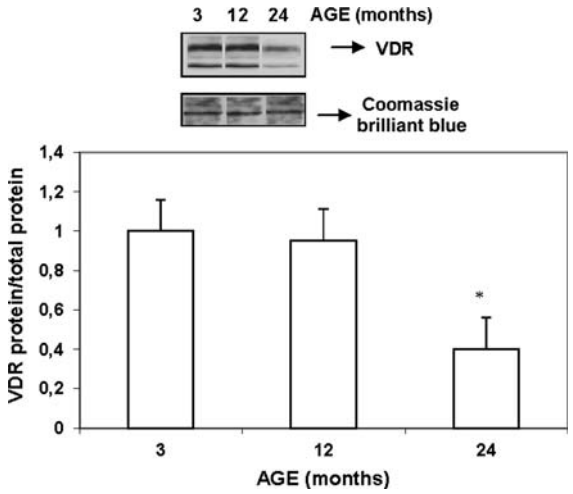

Fig. 2 Vitamin D receptor protein levels. Cells lysates from 3, 12 and 24 month old male rats' duodenum (three each, individually processed) were subject to SDS-PAGE electrophoresis and blotted with rabbit policlonal anti-VDR antibody. To measure the amount of total protein loaded, the membrane was stained with coomassie brilliant blue. Representative immunoblot and quantification by scanning densitometry of three independent experiments are shown; means ± SD are given. $^{*}P < 0.01$

nucleus and mitochondria. Low molecular weight immune-reactive proteins were also detected in young enterocytes subcellular fractions. We have also analyzed $1\alpha,25(OH)_2D_3$ binding properties and VDR levels in subcellular fractions from young and aged rats. In competition binding assays, employing $[^3H]$-$1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$, we have detected specific binding in all subcellular fractions, with maximum binding in mitochondrial and nuclear fractions, where low amount of VDR was detected. An explanation for this finding could be the existence of vitamin D binding proteins with high affinity and capacity unlike classic VDR. In this regard, the first evidence of an intracellular group of proteins that binds 25-hydroxylated vitamin D metabolites in vitamin D-resistant New World primate was reported by Adams. He found that these intracellular vitamin D binding proteins (IDBPs) are homologous to proteins in the heat shock protein-70 family and directly or indirectly, through a series of protein interactions, interact with hydroxylated vitamin D metabolites and facilitate their intracellular targeting (Adams et al. 2004). With ageing, both, VDR protein levels and $1\alpha,25(OH)_2D_3$ binding, were diminished. Furthermore, in humans, similarly to rats, an age-related decrease in intestinal VDR (Ebeling et al. 1992) occurs, and also in elderly osteoporotic patients, a decrease in the renal production of $1\alpha,25(OH)_2D_3$ has been reported (Slovik et al. 1981).

Although it has been shown that serum hormone concentrations are lower in adult (3–4 months) than in growing (<3 months) rats, there are discrepancies on whether its levels change during postmaturational aging (3–24 months) (Armbrecht et al. 1984; Wada

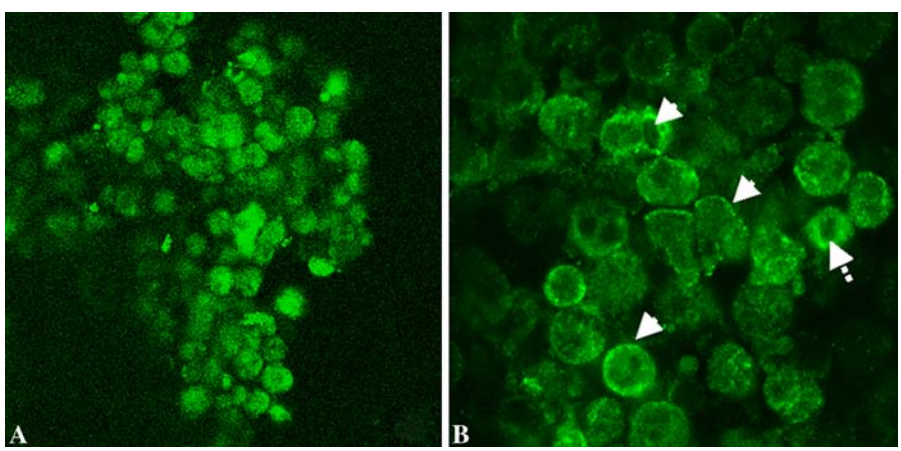

Fig. 3 Subcellular distribution of vitamin D receptor in rat enterocytes by confocal microscopy. Enterocytes isolated from young rats were fixed with methanol as described under "Materials and methods." (**a**) Control cells. At first, cells were incubated with anti-rat antibody to block unspecific sites. Next, they were incubated with anti-biotin–streptoavidin-Oregon Green 488. (**b**) Cells were incubated with monoclonal anti-VDR (clone 9A7) antibody. Fluorescence was detected at cytosol (doted narrow), perinuclear and membrane region (full narrow). Images were obtained from a confocal microscope Leica DM IRB2. Original magnifications 630×

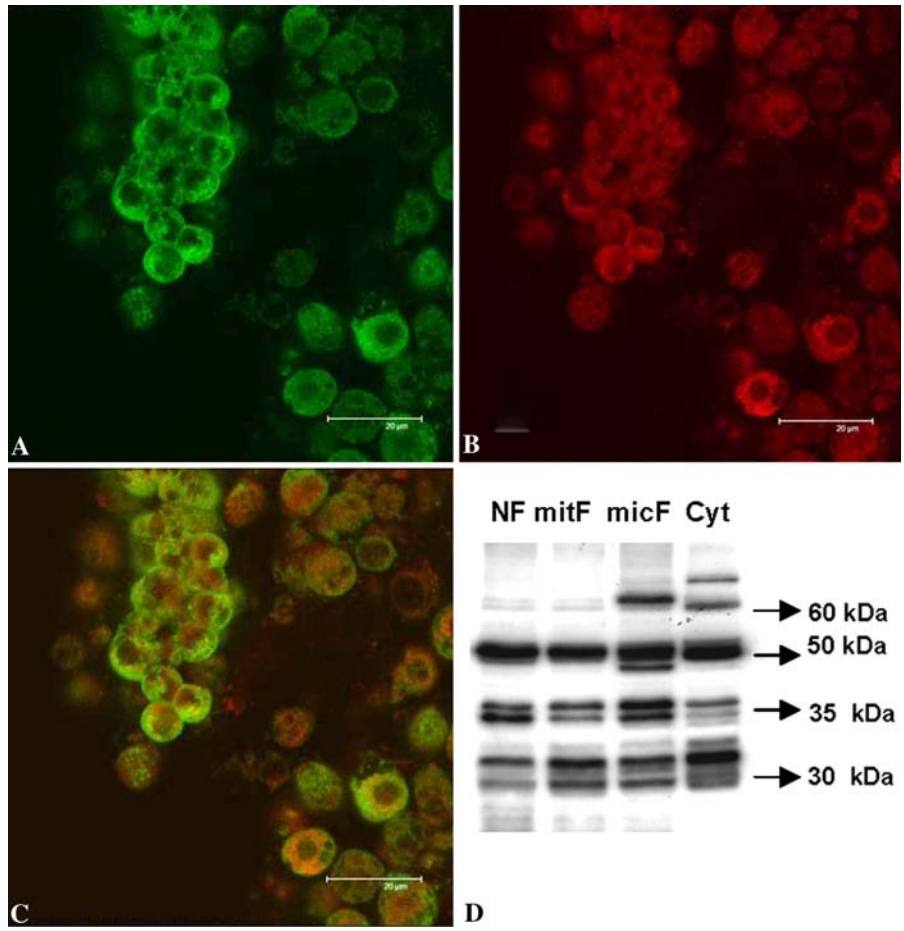

Fig. 4 Localization of VDR and immune-reactive proteins at mitochondria and subcellular fractions. Enterocytes isolated from young rats were incubated with mitotracker for 30 min and fixed in methanol. (**a**) Cell incubated with anti-VDR monoclonal (clone 9A7) antibody (green). (**b**) Mitochondria (red). (**c**) Co-localization (yellow). Images were obtained from a Leica confocal microscope. (**d**) Western blot with anti-VDR monoclonal (clone 9A7) antibody were applied to the following subcellular fractions: nuclear (NF), mitochondrial (mitF), microsomal (micF) y cytosolic (Cit)

et al. 1992; Halloran and Portale 2005). Because $1,25(OH)_2D_3$ up regulates VDR in the intestine (Strom et al. 1989), the possibility that age-related modifications in circulating amounts of $1,25(OH)_2D_3$ contribute in part to the alteration in VDR levels as a function of aging in the enterocytes cannot be excluded. In agreement with our findings, reduced total and unoccupied receptor sites in duodenum from old rats were found (Takamoto et al. 1990). In addition, decreased expression of membrane-associated rapid response binding protein (MARRS) in aged isolated basal lateral chicken membranes has been reported (Larsson and Nemere 2003). Alterations in mRNA expression of duodenal VDR in aged rats also were observed (Liang et al. 1994). Thus, diminishment VDR levels detected in aged rats could be correlated with impairment in fast, non-genomic biological responsiveness to $1\alpha,25(OH)_2D_3$ (González Pardo and Russo de Boland (2004); González Pardo et al. 2007).

Besides the role of $1,25\text{-}(OH)_2D_3$ in the regulation of gene transcription (Minghetti and Norman 1988), a number of cellular and tissue responses to vitamin D metabolites have been described that occur within seconds to minutes (Nemere et al. 1984; Revelli et al. 1998). These rapid, non-genomic actions of $1,25\text{-}(OH)_2D_3$ include activation of the phospholipase C pathway (Civitelli et al. 1990), activation of the adenylate cyclase pathway (Massheimer et al. 1994), opening of L-type, voltage-gated Ca^{2+} channels in the plasma membrane (Caffrey and Farach-Carson 1989; de Boland et al. 1990), Ca^{2+} mobilization from the endoplasmic reticulum (Le Mellay et al. 1997) or activation of MAP kinases (Gniadecki 1996; González Pardo and Russo de Boland 2004; González Pardo et al. 2007) in different cell types. The controversies in this field are whether these rapid effects are mediated by the nuclear VDR. There has been much written on whether the non-genomic effects of $1,25\text{-}(OH)_2D_3$ are mediated by membrane-

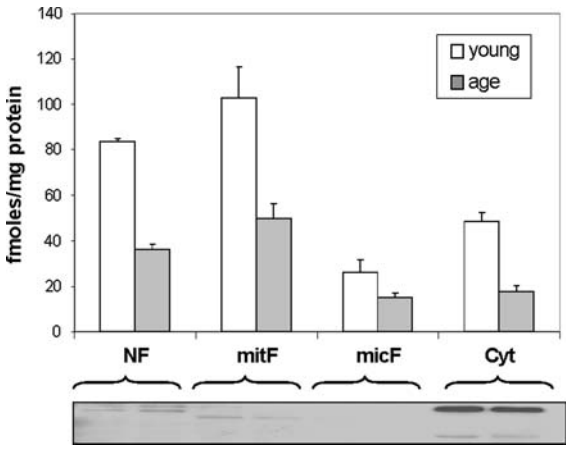

Fig. 5 1α,25(OH)$_2$D$_3$ specific binding to subcellular fractions. Subcellular fractions obtained (nuclear (NF), mitochondrial (mitF), microsomal (micF) and cytosol (Cit)) from duodenal mucosa of young and age male rats (three each, individually processed) were incubated with [^{3}H]-1α,25(OH)$_2$D$_3$ or unlabeled 1α,25(OH)$_2$D$_3$ as described under "Materials and methods." In parallel, protein aliquots from each fraction were subjected to Western blot assays with anti- VDR policlonal antibody. Specific binding is expressed as fmol/mg protein. No differences were observed between individual animals, therefore, the average of three independent experiments preformed by triplicate ± SD and a representative Western blot is shown

bound hormone receptors (VDRm) (Revelli et al. 1998; Norman 1998) and whether or not these receptors are identical to the nuclear VDR.

Rapid activation of extracellular signal-regulated kinases, ERK1/ERK2 in NB4 promyelocytic leukemia cells can be induced not only by 1,25-(OH)$_2$D$_3$, but also by analogs that are unable to activate VDR, suggesting the possibility of a separate receptor (Song et al. 1998). In contrast, Gniadecki has described activation of ERK through 1,25-(OH)$_2$D$_3$-induced activation of Raf as a result of interactions between VDR and the adaptor protein Shc (Gniadecki 1996). Furthermore, it has been reported that deletion of the VDR DNA binding domain also eliminates non-genomic responses (Erben et al. 2002). Thus, depending on the cell type, some of the rapid actions of 1α,25(OH)$_2$D$_3$ may be dependent upon nuclear VDR, whereas others are not.

Age-related decline in VDR may have important consequences for correct receptor/effector coupling in the duodenal tissues. It may explain age-related decline in the hormonal regulation of signal transduction pathways leading to MAPKs activation, c-Fos expression and intestinal cell proliferation that we previously reported (González Pardo et al. 2007).

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