1α,25(OH)$_2$-Vitamin D$_3$ and 17β-Estradiol: Two Steroid Partners Acting in Skeletal Muscle

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Abstract: 1α,25(OH)$_2$-vitamin D$_3$ and 17β-estradiol regulate skeletal muscle cell proliferation, differentiation, apoptosis and contractility through receptor-mediated transcriptional and non-genomic mechanisms. This review focuses on recent advances on signal transduction pathways activated by both steroid hormones. Data are given on the participation of the VDR and ERs (α and β) in activation of MAPKs in muscle cells. Likewise, we describe novel evidence supporting non-classical localizations of the VDR in the plasma membrane and ER β in mitochondria. 1α,25(OH)$_2$D$_3$ promotes DNA synthesis in skeletal muscle cells implicating c-Src/ERK1/2, whereas 17β-estradiol inhibits apoptosis through ERK2 and p38 MAPKs. This study provides basis for the understanding of vitamin D- and estrogen-dependent myopathies.

Key Words: 1α,25(OH)$_2$-vitamin D$_3$, 17β-estradiol, skeletal muscle, MAPK, VDR, ER, signal transduction.

1. INTRODUCTION

1.1. The Vitamin D$_3$ and 17β-Estradiol Endocrine Systems

In the skin, ultraviolet sunlight converts 7-dehydrocholesterol into the prehormone Vitamin D$_3$. Vitamin D$_3$ is a substrate of D$_3$-25-hydroxylase that generates 25(OH)D$_3$ in the liver and then, in the kidney, this compound is metabolized by 25(OH) D$_3$-1α-hydroxylase to the hormonally active form 1α,25(OH)$_2$D$_3$. Regulation of the vitamin D endocrine system occurs by control of the renal 1α-hydroxylase by 1α,25(OH)$_2$D$_3$ itself, calcium and phosphate serum concentrations and parathyroid hormone action [1, 2].

With respect to 17β-estradiol, this reproductive hormone is mainly produced in granulose cells of the ovaries. Like 1α,25(OH)$_2$D$_3$, 17β-estradiol is derived from cholesterol. After side chain cleavage and utilizing the delta-5 pathway or the delta-4 pathway, androstenedione is the key intermediary. A fraction of the androstenedione is converted to testosterone, which in turn undergoes conversion to 17β-estradiol by the enzyme aromatase. Alternatively, androstenedione is "aromatized" to estrone, which is subsequently converted to 17β-estradiol [3].

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1α,25(OH)$_2$D$_3$ and 17β-estradiol both act through two mechanisms: regulating gene expression by binding to their intracellular receptors (VDR and ERs, respectively) and also triggering rapid, non-transcriptional responses, which involve activation of intracellular signal transduction pathways [4-10] which may be also mediated by the VDR and ERs [11-13]. The participation of both receptors as mediators of the non-genomic effects of the two hormones highlight the activation of signal transduction pathways related to cascades of tyrosine phosphorylation and the Ca^{2+} messenger system [14-16].

1α,25(OH)$_2$D$_3$ is the major regulator of extracellular calcium and phosphorus bone homeostasis [2, 17]. On the other hand, 17β-estradiol promotes the development of female secondary sex characteristics, is involved in the thickening of the endometrial tissue and other aspects of menstrual cycle regulation [18]. 17β-estradiol also modulates cholesterol and lipid profiles [19, 20], glucose oxidation and glycogen degradation [21], NOS expression [22], vasorelaxation and angiogenesis [23]. In addition, both hormones modulate cell proliferation and differentiation as well as apoptosis, muscle growth and contractility, the immune system and neuroprotection events [24-31].

1.2. 1α,25(OH)$_2$D$_3$ and 17β-Estradiol Receptors

VDR is a monomeric protein with a domain structure homologous to other nuclear steroid receptors [32]. Its 427 amino acids encompass a short N-terminal activation-function 1 domain, a DNA-binding domain containing two Zn^{2+}-fingers, a flexible "hinge" region

1871-5222/09 $55.00+.00

that includes nuclear localization signals and finally the ligand-binding domain whose C-terminal end also has transcriptional activation functions [33]. Classical tissues for VDR expression are bone, kidney and intestine but there are at least thirty other cellular types where the VDR is present [34]. The VDR is also recently known as NR1I1 (nuclear receptor subfamily 1, group I, member 1) belonging to the nuclear receptor family of transcription factors [35]. Upon activation by $1\alpha,25(OH)_2D_3$, the VDR forms a heterodimer with the retinoid-X receptor and binds to hormone response elements on DNA resulting in expression or trans-repression of specific genes. In humans, the VDR is encoded by the *VDR* gene [36]. In recent years, several polymorphisms, such as *BsmI* and *FokI*, have been described in the *VDR* genes that are able to alter the activity of VDR protein [37]. The existence of a novel cell-surface receptor (1,25-MARRS) for $1\alpha,25(OH)_2D_3$ that mediates the nongenomic actions of the hormone has been reported for various target cells [38]. Baran *et al*. published that annexin II, a plasma membrane protein, may also serve as a receptor for rapid effects of $1\alpha,25(OH)_2D_3$ [39].

On the other hand, classical estrogen receptors (ERα and ERβ) are encoded by two distinct genes and expressed in the same and different tissues at varying levels [40, 41]. ERs have six functional domains. The structurally distinct amino terminal A/B domains (17% amino-acid identity) contain a ligand-independent transactivation function (AF1). The central C region is the DNA binding domain (DBD). The hinge region, or D domain, contains a nuclear localization signal and links to the multi-functional carboxyl terminal (E/F) domain which shows 56% amino-acid homology between ERs and is involved in ligand binding, dimerization, and ligand-dependent transactivation function (AF2). In contrast to the VDR, multiple spliced forms of ER α and β have been reported [42, 43 and references therein], whether all are translated to protein and have any biological function is not established. Of relevance, recent studies reveal the existence of a novel 7-transmembrane G protein-coupled receptor, GPR30, which responds to estrogen and tamoxifen stimulation with rapid cellular signaling. GPR30 is becoming recognized as an estrogen receptor, perhaps complementary to the classical estrogen receptors, participating in the non-genomic effects induced by estradiol [44, and references therein].

Classical estrogen targets are the uterus, mammary gland, placenta, central nervous system, cardiovascular system and bone. Non classical target tissues include prostate, testis, ovary, adrenals, pancreas, skin and urinary tract [45].

For $1\alpha,25(OH)_2D_3$, as well as 17β-estradiol, there are coactivators, which appear to serve as links between hormone-receptor complex and the transcription machinery to allow regulation of gene expression [reviewed by 46-48]. Presumably, the capacity to heterodimerize with multiple partners serves in part to allow the fine-tuning of the patterns of gene expression in response to $1\alpha,25(OH)_2D_3$ or 17β-estradiol in various organs and tissues.

Several studies have shown a cross-talk of membrane or cytosolic ERs with nuclear ERs and other receptors localized in the cell membrane (e.g. growth factor receptors) with nuclear ERs, and this can also take place in the absence of sex steroids [49 and references therein]. Like the ERs, the VDR can cross talk with growth factors receptors [50, 51]. Of relevance, it is known that ERβ modulates VDR expression [52].

For ERs, a large pool of cytoplasmic receptors has been also detected at the mitochondrial level [13, 53-59]. These findings clearly show that integration of steroid effects at distinct cellular locations of its receptor leads to important cellular physiological outcomes in both reproductive and non reproductive organs.

2. PHYSIOLOGICAL ROLES OF $1\alpha,25(OH)_2D_3$ AND 17β-ESTRADIOL IN SKELETAL MUSCLE

2.1. VDR and ERs in Skeletal Muscle

The expression of the VDR in skeletal muscle cells (myoblasts/myotubes) and tissue has been reported for avians [60, 61] and rodents [62] as well as humans [63-65]. In addition to its function as a transcriptional factor, new lines of evidence have indicated that it mediates various rapid non genomic responses induced by $1\alpha,25(OH)_2D_3$ in skeletal muscle through a plasma membrane-localized pool of VDR. This dual role may be explained by a $1\alpha,25(OH)_2D_3$ -VDR conformational ensemble model which describes how the hormone can selectively initiate or block either non genomic or genomic biological responses by interacting with two VDR ligand-binding pockets, one kinetically favoured by $1\alpha,25(OH)_2D_3$ and the other thermodynamically favoured [66]. Of relevance, our laboratory reported for the first time that in a target cell, the reverse traffic of the VDR from the nucleus to the plasma membrane was observed in avian myoblasts after hormone exposure [11]. Recent studies using confocal microscopy have confirmed the $1\alpha,25(OH)_2D_3$ –dependent VDR translocation to the plasma membrane using murine C2C12 proliferating muscle cells [Buitrago C. and Boland R. unpublished]. It has been shown that the VDR interacts with the intrinsic channel protein TRPC3 in response to the vitamin D hormone in myoblasts lending further support to receptor associa-

tion to the plasma membrane. Also, we demonstrated that the VDR mediates rapid changes in muscle protein tyrosine phosphorylation induced by $1\alpha,25(OH)_2D_3$ both in avian and murine skeletal muscle cells (see section 3 below). Regardless of its mechanism of action, the vitamin D receptor appears to play an important role in regulating skeletal muscle physiology. It is well recognized that VDR gene knockdown promotes abnormal skeletal muscle development in the mouse [67]. Moreover, VDR polymorphisms have been described to affect muscle function [68, 69]. Also of importance, sarcopenia in older aged humans has been significantly associated with decreased VDR expression, independent of vitamin D status [65].

Respect to ERα and ERβ, their expression at the mRNA and protein level have been detected in skeletal muscle and myoblasts of different animal species [70-75]. In the C2C12 murine skeletal muscle cell line we described a non classical localization for both ERs [75, 76]. This evidence was obtained by conventional radioligand assays, the use of estrogen agonists and antagonists, blocking with specific monoclonal antibodies, western blot, ligand blot and immunocytochemical assays. ERβ was exclusively detected in mitochondria and ERα in the perinuclear and mitochondrial compartments of C2C12 cells. ERs with non-classical locations were also detected in mouse skeletal muscle tissue [75]. ERβ was also found to a greater extent in mitochondria, compared to ERα which localizes mainly in microsomes, cytosol, mitochondria, and also in the nucleus of muscle tissue. Skeletal mitochondria are key organelles in the apoptotic process. This subcellular location of ERs could be associated with the regulation of apoptosis of muscle cells by the steroid hormone as described below (section 6). Unlike other cell lines [77], the subcellular location of the immunoreactive entities was not modified by treatment with the hormone, reinforcing the hypothesis that one of the two putative ERα localizations detected could be an internal membrane system such as the endoplasmic reticulum or Golgi. Studies employing cells transiently transfected with specific ERα or ERβ siRNA and RT-PCR assays confirmed functional and structural relationships between the classical and the endogenous ERs [75, 76].

2.2. Regulation of Cell Proliferation and Differentiation, Growth and Contractility by Hormones

It is well documented that $1\alpha,25(OH)_2D_3$ is essential for regulation of intracellular calcium levels and growth in skeletal muscle and thereby plays an important role in contractility and myogenesis [24, 78]. The hormone stimulates proliferation of myoblasts and also their differentiation into myotubes [78, 79]. Nakagawa has also reported that vitamin D regulates development and homeostasis of skeletal muscle [80]. Similarly, 17β-estradiol modulates growth of skeletal muscle. In healthy regularly menstruating women the highest level of estrogen during the normal menstrual cycle has been suggested to affect in a helpful manner muscle strength [81, 82]. Furthermore, it is reported that the steroid hormone has an anabolic effect on skeletal muscle, as shown by an increase in muscle strength in postmenopausal women that receive hormone replacement therapy (HRT) [83, 84] and by a protective role from damage during muscle exercise [85].

2.3. Muscle Pathologies Related to Deficiency of $1\alpha,25(OH)_2D_3$ or 17β-Estradiol of Nutritional and Endocrine Origin

$1\alpha,25(OH)_2D_3$ deficiency of nutritional or endocrine origin can cause diverse myopathies (e.g. sarcopenia). Clinical studies have indicated that normal levels of $1\alpha,25(OH)_2D_3$ are positively related to muscle strength and physical performance, diminishing the risk of falling. Vitamin D_3 dietary supplementation in older adults has shown to reduce falls, possibly acting on muscle fiber composition and morphology. As with $1\alpha,25(OH)_2D_3$, several muscle pathologies are caused by a decrease in estrogen levels. Specially, it has been observed that muscle performance diminishes during the postmenopausal years leading to the development of sarcopenia [86]. Therefore, HRT can prevent this myopathy.

The mechanisms underlying vitamin D_3 and 17β-estradiol signal transduction in muscle have been studied to a great extent in myoblast/myotube primary cultures and cell lines [24, 78, 86-88]. The information generated is endowed with physiological significance as mature skeletal muscle *in vivo* contains a pool of satellite cells similar to which undergo myogenic differentiation during muscle growth and repair processes as well as in response to endocrine factors [89, 90].

3. SIGNAL TRANSDUCTION PATHWAYS TRIGGERED BY $1\alpha,25(OH)_2D_3$ IN SKELETAL MUSCLE

It has been well established in our laboratory that $1\alpha,25(OH)_2D_3$ stimulates in skeletal muscle cells the adenylyl cyclase/cAMP/PKA and PLC/DAG + IP_3/PKC pathways, the MAPK cascades and calcium messenger system (VDCC influx and intracellular Ca^{2+} release).

P38 MAPK and JNK1/2 are rapidly activated by $1\alpha,25(OH)_2D_3$. The hormone induces in the C2C12 muscle cell line the stimulation of mitogen-activated protein kinase activating protein kinase 2 (MAPKAP-

kinase 2) and subsequent phosphorylation of heat shock protein 27 (HSP27) in a MKK3/6-p38 MAPK activation-dependent manner [91]. Also, in response to 1α,25$(OH)_2D_3$, the ERK1/2 cascade is positively regulated by Ras protein and PKCα through Raf-1 activation and the consequent phosphorylation of MEK1/2 [92], and by Ca^{2+} and CAM kinase II at the level of c-Src [93]. Of relevance, PKC (presumptive δ isoform) and PTPα are involved in c-Src activation [Buitrago and Boland, submitted for publication]. The VDR has been implicated in activation of MAPKs by 1α,25$(OH)_2D_3$ which promotes VDR-c Src association [94].

Through the mechanisms described above, 1α,25 $(OH)_2D_3$ causes translocation of ERK1/2 from the cytoplasm to the nucleus in the active form and induces the expression of the growth-related protein c-myc, the activation of CREB and ELK-1 transcription factors followed by stimulation of muscle cell proliferation [95, 96]. In addition, 1α,25$(OH)_2D_3$ stimulates in myoblasts tyrosine phosphorylation and membrane translocation of PLCγ, events which are mediated by c-Src in cooperation with phosphatidylinositol 3-kinase (PI3-K) [97]. Recent discoveries about upstream steps in the initiation of 1α,25$(OH)_2D_3$ signalling through MAPKs reveal that caveolae and caveolins are implicated in hormone signalling [Buitrago and Boland, unpublished].

The regulation of muscle intracellular Ca^{2+} by 1α,25$(OH)_2D_3$ involves an initial rapid Ca^{2+} mobilization from intracellular stores followed by cation influx through voltage-dependent (VDCC) and store-operated Ca^{2+} (SOCC) channels [98]. Interestingly, it has been shown that protein tyrosine kinases are involved in the mechanism of 1α,25$(OH)_2D_3$-induced muscle SOCC calcium influx [99]. Of relevance, we have proposed that an endogenous TRPC3 protein mediates hormone modulation of calcium capacitative entry (CCE) in skeletal muscle, which seems to implicate VDR-TRPC3 association and the participation of an INAD-like scaffold protein [100].

4. INVOLVEMENT OF PROTEIN PHOSPHORYLATION EVENTS IN 1α,25$(OH)_2D_3$ STIMULATION OF MYOGENESIS

In 1999, Capiati *et al*. reported 1α,25$(OH)_2D_3$ stimulation of myoblast proliferation. These studies showed that selective knock down of PKCα expression inhibited DNA synthesis promoted by the hormone. This suggests that the isozyme may have an important role in mediating 1α,25$(OH)_2D_3$-induced proliferation of muscle cells. On the other hand, an increase in the expression of PKCβ, δ and ε was detected during myogenesis, suggesting that one or more of these isoforms may participate in the differentiation process of myoblasts [79, 101]. This experimental evidence altogether implies that PKC- dependent phosphorylation may play an important role in 1α,25$(OH)_2D_3$ regulation of myogenesis [reviewed in 78]. Activation of tyrosine phosphorylation pathways also mediates the effects of the steroid on muscle cell proliferation. Specifically, we demonstrated that 1α,25$(OH)_2D_3$ promotes DNA synthesis in skeletal muscle and implicated c-Src/ERK1/2 as mediators of this response [95]. Moreover, the hormone up regulates the expression of the early genes *c-myc* and *c-fos*.

5. REGULATION OF SKELETAL MUSCLE GROWTH-RELATED SIGNAL TRANSDUCTION PATHWAYS BY 17B-ESTRADIOL

The effect of 17β-estradiol on growth-related signaling pathways such as MAPKs has been well documented in many tissues and cellular types. Within these actions exerted by physiological concentrations of the estrogen in muscle cells is the fast activation of ERK1/2 and p38 MAPK in cardiomyocytes [102] and in aortic endothelial cells [103]. As regards skeletal muscle cells, in L6A1 myoblasts it was found that activation of Raf-1 by the hormone resulted in increased phosphorylation of ERK1/2 [104]. In the C2C12 murine skeletal cell line it has also been reported that ERK1/2 is activated by the estrogen [96, 105]. Of relevance, ERK1/2 was predominantly localized in mitochondria [27]. We want to highlight this point and the fact that ERβ was also found in mitochondria, suggesting that estradiol acts, at least in part, modulating an intraorganelle signaling system. In addition, 17β-estradiol-induced phosphorylation of p38 MAPK was demonstrated in C2C12 cells [96]. Hormone activation of these MAPKs in mouse skeletal muscle primary cultures was also observed, demonstrating that the cellular response is not an effect of the muscle cell line [Ronda A., Vasconsuelo A., Boland R. unpublished]. The estrogen receptors have been involved in the fast actions of 17β-estradiol as mentioned above. Thus, it has been reported that the α and β isoforms of the ER are implicated in the activation of the MAPK pathways by 17β-estradiol in endothelial cells [106]. New evidence from our laboratory suggests that in C2C12 muscle cells ERα participates in ERK2 activation whereas p38 MAPK phosphorylation is mediated by an ER-independent mechanism revealing the complexity of the molecular machinery sustaining the estradiol response [Ronda A., Buitrago C., Boland R. unpublished]. On the other hand, recently Hatae *et al*. (2009), reported that 17β-estradiol induces ERα expression *via* ERK1/2 and p38 MAPK, suggesting a positive feedback of the hormone action on skeletal muscle cells [110].

In the C2C12 skeletal muscle cell line it has been reported that 17β-estradiol promotes the fast activation

of CREB and Elk-1 transcription factors involving the ERK1/2 and p38 MAPK pathways [96]. In addition, the hormone is able to induce the early c-Fos oncoprotein expression [107] through ERK1/2 and p38 MAPK activation [96]. New evidence from our laboratory also suggests that c-Jun oncoprotein expression is modulated by the hormone in a similar fashion [Ronda A., Buitrago C., Boland, R. unpublished].

6. THE SURVIVAL LANGUAGE OF 17B-ESTRADIOL: HORMONE-REGULATED APOPTOSIS IN SKELETAL MUSCLE CELLS

17β-estradiol is able to support cell survival or induce apoptosis depending on the cell context [108-110]. Regarding skeletal muscle, estrogenic regulation of this cell event is important since there is data demonstrating that apoptosis plays a key role in pathophysiological and physiological conditions that lead to skeletal muscle cell loss [111]. Although little is known about the effects of estrogen on apoptosis and the underlying molecular events in skeletal muscle, the evidence indicates that the steroid is associated with survival/beneficial effects in this tissue [90, 112]. We reported that 17β-estradiol inhibits apoptosis in C2C12 skeletal muscle cells through ERs with non classical localization and involving MAPKs [27] and the PI3K/Akt/Bad pathway [113]. In particular, we observed that 17β-estradiol prevents smac/DIABLO and cytochrome c release, caspase-3, PARP cleavage and DNA fragmentation. Also, the hormone blocked the typical apoptotic morphological changes of the nucleus, mitochondria and cell size, and mitochondrial redistribution in C2C12 murine skeletal muscle cells [113] as well as in primary cultures of mouse skeletal muscle. Summarizing, the steroid hormone activates a *"cellular survival or cellular anti-stress state"* in skeletal muscle cells. Moreover, we recently observed that 17β-estradiol increases the expression of HSP27, anti-stress protein, and this effect was directly related to the antiapoptotic action of the estrogen [114]. Although apoptosis may occur via several mechanisms, the mitochondrion is a major regulatory site for this process [115]; also, estrogen actions in mitochondria have been amply reported for a variety of tissues [58, 116, 117]. Accordingly, the data reviewed in this section suggests that estradiol could exert its protective effect on skeletal muscle through mitochondrial signaling. In keeping with this interpretation, as mentioned before, ERβ is mainly associated with mitochondria [75] and it is stabilized in the organelle by interaction with HSP27 in C2C12 cells [114]. Nevertheless, these findings do not explain how 17β-estradiol affects mitochondrial function or homeostasis resulting in apoptosis inhibition in skeletal muscle cells. It remains to be clarified whether the hormone exerts direct and indirect effects on mitochondria that are mediated by genomic and nongenomic activities of both ER isoforms, as has been reported for other systems [reviewed in 117-119].

Given that mitochondria of aged skeletal muscle produce more oxidants, collect calcium, exhibit increased oxidative damage [120] and the levels of estrogens decline upon ageing, the sum of these effects results in apoptosis. Then, myocyte loss *via* apoptosis might represent a mechanism responsible for decline of muscle performance in pathologies such as sarcopenia.

7. POTENTIAL PHARMACOLOGICAL TARGETS AND DRUG DEVELOPMENT IN VITAMIN D- AND ESTROGEN-DEPENDENT MYOPATHIES

The gradual weakness of skeletal muscle associated with certain myopathies, for example sarcopenia, is accompanied by failure of bodily functions and musculoskeletal system alterations. These myopathies result in increasing numbers of people requiring medical care to improve quality of life. It is generally recommended the therapy with anabolic hormones together with exercises.

Understanding the mechanisms that lead to sarcopenia can develop effective prevention, cure and rehabilitation. We reviewed evidence showing that $1\alpha,25(OH)_2D_3$ and 17β-estradiol stimulate proliferation and differentiation and inhibit apoptosis of myoblasts. These hormones then can effectively improve muscle performance, although their secondary effects must be taken into account (e.g. hypercalcemia and breast cancer). The complexity of intracellular signaling mechanisms activated by both hormones, allows us to speculate on more than one potential pharmacological target to overcome this myopathy. An attractive approach is to apply compounds that regulate the enzymatic activity of specific muscle kinases. (e.g. myosin heavy chain kinase) involved in cellular differentiation. Of interest, tamoxifen, an ER modulator used in the treatment of certain types of breast cancers, improves both the structure and function of dystrophic muscles in Mdx mice [121, 122].

8. CONCLUDING REMARKS

Integrating the information reviewed here, we show a schematic model of signalling routes of these steroid hormones in skeletal muscle (Fig. **1**). Skeletal muscle tissue is a target for $1\alpha,25(OH)_2D_3$ and 17β-estradiol. Both steroids act through an intricate signal transduction system which involves MAPKs, PKC and PI3K/Akt resulting in proliferation, differentiation or apoptosis inhibition. These steroid actions involve extranuclear membrane VDR and ER, respectively. Further unraveling of these molecular mechanisms may

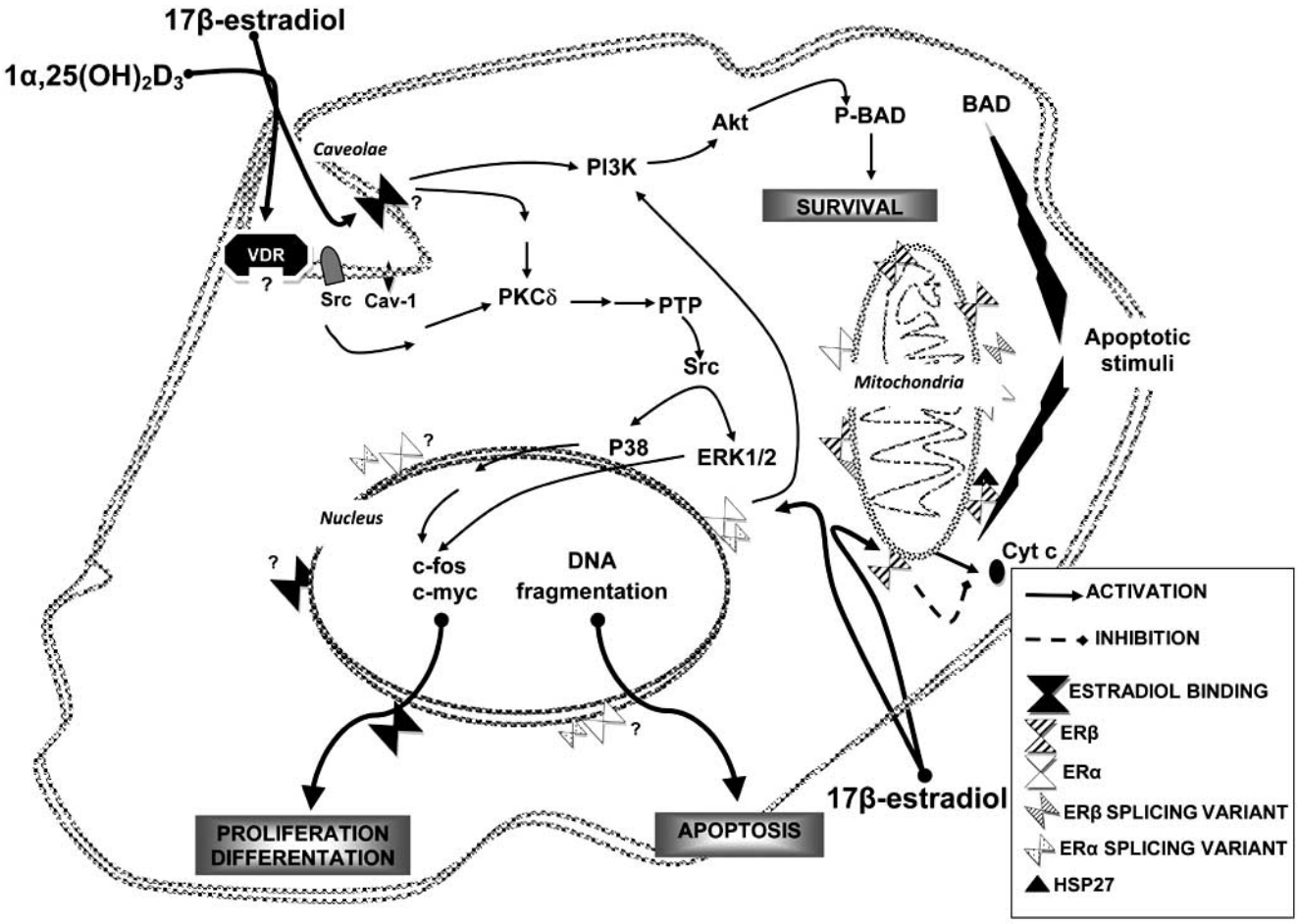

Fig. (1). Schematic diagram showing $1\alpha,25(OH)_2D_3$ and 17β-estradiol intracellular signaling pathways in skeletal muscle cells. The diagram depicts both steroid hormones interacting with specifics receptors to activate intracellular signaling routes. $1\alpha,25(OH)_2D_3$ disrupts cav-1/ Src association and may bind to a VDR pool in caveolae. This hormone induces PKCδ and PTP (Protein Tyrosine Phosphatase)–dependent Src activation with subsequent p38 and ERK1/2 phosphorylation. MAPKs stimulate expression of proto-oncogenes *c-fos* and *c-myc* leading to cell proliferation and differentiation. On the other hand, 17β-estradiol can interact with estradiol binding proteins localized in membrane (caveolae?) and mitochondria promoting activation of the PI3K/Akt/P-BAD survival cascade. Furthermore, this hormone acts to prevent DNA fragmentation and cellular apoptosis.

pave the way for clinical improvements to overcome vitamin D- and estrogen-related myophaties.

ACKNOWLEDGMENTS

This research was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and Universidad Nacional del SUR (UNS), Argentina.

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Received: August 13, 2009 Revised: August 20, 2009 Accepted: August 26, 2009