Antioxidant dietary deficiency induces caspase activation in chick skeletal muscle cells

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Abstract

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Apoptosis and necrosis are two distinct forms of cell death that can occur in response to different agents and stress conditions. In order to verify if the oxidative stress induced by dietary selenium and vitamin E deficiencies can lead muscle cells to apoptosis, one-day-old chicks were reared using diets differing in their vitamin E (0 or 10 IU/kg) and selenium (0 or 0.15 ppm) supplementation. Chick skeletal muscle tissue was obtained from 28-day-old animals and used to verify apoptosis occurrence based on caspase activity detection and DNA fragmentation. Antioxidant deficiency significantly increased caspase-like activity assessed by the hydrolysis of fluorogenic peptide substrates (Abz-peptidyl-EDDnp) at $\lambda_{exc} = 320$ nm and $\lambda_{em} = 420$ nm. Proteolytic activation was not accompanied by typical internucleosomal DNA fragmentation detected by field inversion gel electrophoresis. Although the general caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-fmk) (0 to 80 µM) did not block caspase-like activity when pre-incubated for 30 min with muscle homogenates, the hydrolyzed substrates presented the same cleavage profile in HPLC (at the aspartic acid residue) when incubated with the purified recombinant enzyme caspase-3. These data indicate that oxidative stress causes caspase-like activation in muscle cells and suggest that cell death associated with exudative diathesis (dietary deficiency of selenium and vitamin E) can follow the apoptotic pathway.

Cell death can follow two distinct pathways, necrosis or apoptosis. Necrosis appears to be the result of acute cellular dysfunction in response to severe conditions of stress or after exposure to toxic agents. It is characterized morphologically by a dramatic increase in cell volume followed by rupture of the plasma membrane. Apoptosis is a complex process characterized by cell shrinkage, chromatin condensation and internucleosomal DNA fragmentation (1). The apoptotic program is the physiological form of cell death (2) that occurs during the development of multicellular organisms or in immune responses. Stress conditions, toxins, chemicals and physical agents including heat and radiation can also activate apoptosis.

Biochemical studies have shown that caspases, cysteine proteinases with specificity for aspartic acid, participate in programmed cell

death (3). Based on S_4 subsite preferences, caspases have been subdivided into three groups: I - caspase-1, -4 and -5 (YVADases), II - caspase-2, -3 and -7 (DEVDases) and III - caspase-6, -8, -9 and -10 (IETDases), with preference for aromatic residues, aspartic acid and branched apolar residues, respectively, in the P_4 position of substrates (4).

Reactive oxygen species (ROS) and the resulting oxidative stress have been implicated in both necrosis (5) and apoptosis (6), and the mode of cell death depends on various factors including the intensity of the initial stimulus (7). ROS involvement in apoptosis has been considered because high levels of oxidants or stress oxidative markers are present in apoptotic cells or cells undergoing apoptosis.

A recent report showed that pro-oxidant agents such as hydrogen peroxide, etoposide and semiquinones induce apoptosis and, in contrast, antioxidants suppress apoptosis by acting as ROS scavengers (6). These compounds are also involved in the generation of exudative diathesis in chicks, a pathology due to the combined dietary deficiency of the antioxidants selenium and vitamin E, which results in lower growth and survival and degenerative lesions with varying degrees of necrosis in thigh and breast muscles (8-10).

In the present investigation, we used chick skeletal muscle cells in order to determine if apoptotic cell death can also be detected simultaneously with necrosis, and studied some molecular events involved in exudative diathesis.

Caspase activation and the pattern of DNA fragmentation were used as criteria to determine the occurrence of apoptosis and to differentiate between necrosis and apoptosis. Male chicks were obtained from a local hatchery and reared to 4 weeks of age in thermostatically controlled battery brooders. Food and water were provided *ad libitum*. Chicks were fed a semipurified basal diet based on isolated soy protein and cornstarch, according to the guidelines of the National Academy of Sciences, National Research Council (1994). The basal diet contained 17 ng/g diet total selenium and vitamin E at levels of 5.7 μg/g diet. Chicks (N = 150) were assigned randomly to two groups of 75 animals for each treatment (with (+Se/+VE) or without (-Se/-VE) antioxidants). Vitamin E supplementation consisted of 10 μg/g as dl-α-tocopheryl acetate and selenium consisted of 0.15 μg/g diet added as Na_2SeO_3. Animals were assessed daily for signs of exudative diathesis which included observation of the ability to walk, swelling of hock joints and poor feathering.

Chicks fed selenium- and vitamin E-deficient diets started to develop exudative diathesis at 17 days of age. The symptoms were those previously described (9,10) and were characterized by a subcutaneous area of edema under the wings, breast and abdominal regions. After four weeks, animals were killed and the pectoralis muscle was removed and immediately frozen in liquid nitrogen for further experiments.

Most events in apoptosis appear to require a caspase-mediated proteolytic step. It was shown that the caspase cascade can be activated or amplified after cytochrome c release from the mitochondria into the cytosol during the early stages of apoptosis (11). Once cytochrome c is released, it leads to a series of protein-protein interactions that culminate in the activation of caspases (12). To determine if the cell death observed in exudative diathesis involves caspase activation, a fluorimetric assay was performed using cell extracts. Fragments (3 g) of the pectoralis muscle were homogenized with a Potter homogenizer (Marconi, Piracicaba, São Paulo, SP, Brazil) in 30 ml PBS, pH 7.4, containing 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 100 μM phenylmethylsulfonyl fluoride (PMSF) on ice. Homogenates were centrifuged at 200 *g* at 4ºC. Cell pellets were washed with the same buffer and submitted to a second centrifugation step at 4ºC. Cell lysis was performed with 25 mM 4-(2-hydroxyethyl)-piperazine-

1-ethanesulfonic acid (HEPES) buffer, pH 7.5, 2 mM EDTA, and 0.5% Triton X-100 with the same inhibitors for 20 min on ice.

Caspase-like activity was determined in cell lysates (100 μg protein) using the intramolecularly quenched substrates: Abz-YVADNQ-EDDnp (I), Abz-DEVDNQ-EDDnp (II), Abz-IETDNQ-EDDnp (III), Abz-DEVDSVQ-EDDnp (IV), Abz-DEVDGVQ-EDDnp (V), Abz-YEVDGVQ-EDDnp (VI), and Abz-VEIDNQ-EDDnp (VII), where Abz is o-aminobenzoic acid and EDDnp is N-2,4-dinitrophenylethylene-diamine. Fluorimetric assays were carried out by the method of Vanags et al. (13) in 25 mM HEPES buffer, pH 7.5, 2 mM EDTA, 10 mM DTT, 10% sucrose, 0.1% 3-([3-chola-midopropyl] dimethylammonio)-1-propane-sulfonate, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 100 μM PMSF (caspase activation buffer). Substrate (10 μM) hydrolysis was monitored for 3 h at 37ºC with a fluorimeter (TD-700, Turner Designs Instrument, Sunnyvale, CA, USA). Enzymatic activity is reported in arbitrary fluorescence units and the results obtained for experimental groups were compared using the Tukey test. $P < 0.05$ was taken as the level of significance. All determinations were done in triplicate in three independent experiments.

Caspase activity was characterized using the general caspase inhibitor N-benzyloxy-carbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-fmk). Samples (75 μg protein) of muscle from both groups (+Se/+VE and -Se/-VE) prepared as described above were incubated with caspase activation buffer for 15 min at 37ºC. Subsequently, increasing amounts of the inhibitor (0 to 80 μM) were added and the mixtures were incubated for 30 min at 37ºC. The enzyme activity was measured on the basis of hydrolysis of the substrate Abz-DEVDNQ-EDDnp (10 μM) at $\lambda_{exc} = 320$ nm and $\lambda_{em} = 420$ nm after 1, 2, 3 and 5 h of incubation. The inhibitory efficiency of Z-VAD-fmk was tested on samples of purified recombinant caspase-3

following the same procedure.

Detection of increased caspase-like activity (Figure 1A) in the group submitted to a selenium- and vitamin E-deficient diet suggests that nutritionally mediated oxidative stress induces caspase activation. In addition, it appears that caspases may be involved in the cell death observed in exudative diathesis, although the caspase activity detected in the -Se/-VE chick muscle was not reduced by the general caspase inhibitor Z-VAD-fmk even at a high concentration (80 μM) (data not shown). However, there is some evidence that the apoptotic cell death can occur without effector caspases. Foghs-gaard et al. (14) showed cathepsin B-mediated and caspase-independent apoptotic features in WEHI-S fibrosarcoma cells. Interestingly, it was also reported that the cell death process was increased by low but effective concentrations of a caspase inhibitor such as Z-VAD-fmk.

In order to confirm that the peptide substrates were hydrolyzed at the aspartic acid residue, as expected for caspases, and to further characterize the enzyme activity, cleaved bonds in substrates were determined by reverse-phase HPLC. Substrates (5.0 μM) were incubated with recombinant caspase-3 or samples from chick muscle (100 μg) in caspase activation buffer for approximately 2 h at 37ºC. Hydrolysis products were separated and identified using a C_{18} Beckman column (5 μm, 15 x 4.6 mm; Ultrasphere, Palo Alto, CA, USA) developed in 0.1% aqueous trifluoroacetic acid - solvent A - with a linear gradient of acetonitrile - solvent B - for 20 min at a flow rate of 1.0 ml/min (gradient slope of 4% B/min). Elution profiles were obtained by recording absorbance at 365 nm with an absorbance detector (Shimadzu, SPD-6AV, Kyoto, Japan) and fluorescence at $\lambda_{exc} = 320$ nm and $\lambda_{em} = 420$ nm with a fluorescence detector (Shimadzu, RF 535), which allowed the direct identification of EDDnp-containing peptides and Abz-N-terminal sequences, respectively. Controls

were prepared without enzyme or cell lysate under the same conditions.

The retention times of the products were compared to those of the original peptides. The bonds cleaved were the same in the incubation with recombinant caspase-3 or with cell lysates from muscle tissue (-Se/-VE) as identified by HPLC (Figure 1B). The pattern of substrate hydrolysis is an indication that enzyme activity in cell extracts is due to caspases. These enzymes show a high degree of specificity with an absolute requirement for cleavage after an aspartic acid residue and a recognition motif containing at least four amino acid residues N-terminal to the cleavage site. This characteristic, which is exhibited by only one other eukaryotic protease, the serine protease granzyme B, is important for the apoptotic process, since it involves cleavage of a particular group of proteins in an ordered manner rather than indiscriminate proteolysis (3).

A histopathological hallmark of many progressive neuromuscular disorders is muscle fiber loss. While some types of muscular disorders clearly involve variable muscle fiber necrosis, which can be easily identified at the light microscopy level (9,10), apoptotic cell death is much less evident in routine histological preparations. Progress has been made in the identification of apoptotic cells by DNA fragmentation and demonstration of up-regulation of apoptosis-associated factor such as proteins of the bcl-2 family and caspases. Nevertheless, the expression of some individual factor does not confirm the occurrence of apoptosis.

Detection of apoptotic cells in tissue sections currently relies on the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay based on labeling single-strand DNA breaks. However, even DNA fragmentation revealed by TUNEL may not be completely apoptosis-specific (15). Dem-

Figure 1. A, Detection of caspase-like activity in cell lysates. Fluorimetric assays were performed in caspase buffer as described and substrate hydrolysis (10 µM) was determined at λ_{exc} = 320 nm and λ_{em} = 420 nm after 3 h (I, II and III) or 2 h (IV to VII) of incubation at 37ºC. Enzymatic activity was reported in arbitrary units of fluorescence (AUF) and compared between treatments by the Tukey test. Hydrolysis of all synthetic peptides was significantly higher in cell lysates of the -Se/-VE group ($P < 0.05$). B, Determination of the cleaved bonds. The substrate Abz-DEVDNQ-EDDnp (5.0 µM) was incubated with purified caspase-3 or samples from the -Se/-VE group in caspase buffer. Fragments were resolved by HPLC in a linear acetonitrile gradient. The bonds cleaved (DEVD↓N) were the same in the incubation with caspase-3 or cell extract (-Se/-VE) and are indicated by the arrow.
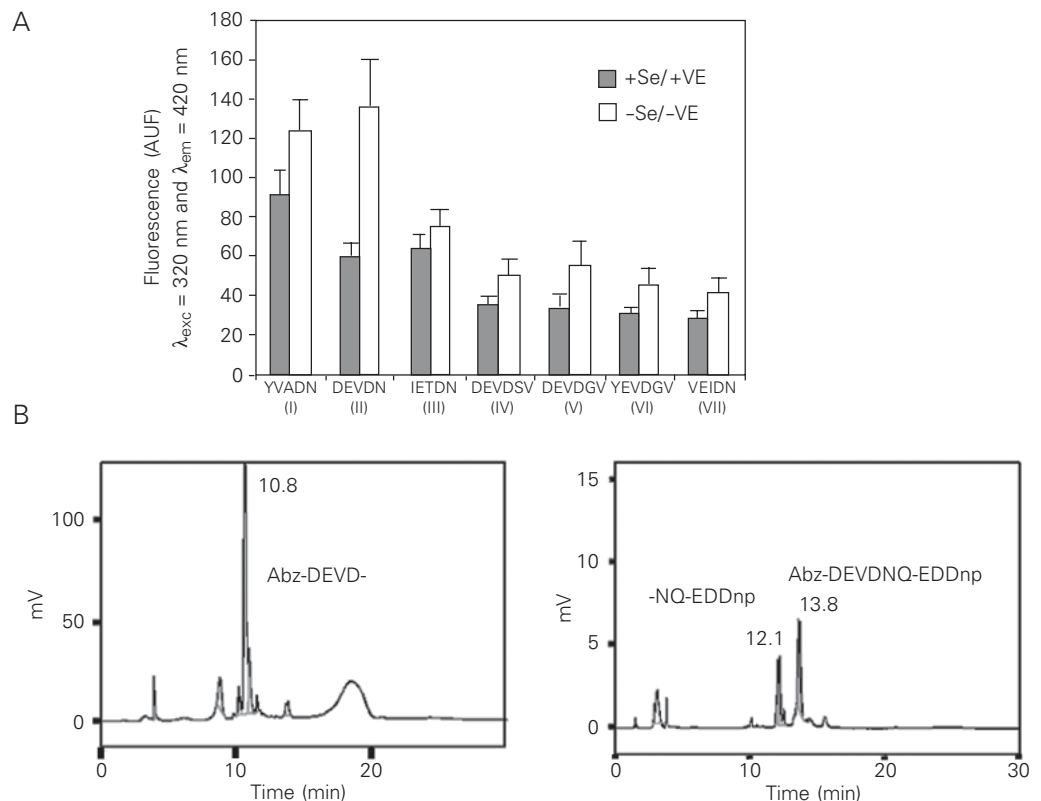
onstration of DNA fragments by electrophoretic DNA laddering is a simple strategy for detecting cells undergoing apoptosis, although it requires DNA isolation.

For DNA fragment detection, samples obtained from muscle tissue (1 g) were prepared and analyzed as described (16), with the following modifications: cells were lysed in 10 mM Tris buffer, pH 8.0, 2 mM EDTA, 100 μM PMSF and 2% SDS for 1 h at 42ºC, and centrifuged at 14,000 rpm for 20 min. The supernatant was precipitated with 40 μl of 2.5 M acetate buffer, pH 5.2, and 2 volumes of absolute ethanol at -20ºC. Samples were incubated at -20ºC for 2 h and centrifuged at 2000 rpm. Pellets were added to 400 μl of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5) containing 0.5% sodium lauroyl sarcosine and 20 μg RNAse. Samples were incubated for 1 h at 65ºC, 150 μg proteinase K was added and the preparation was incubated again at 37ºC. DNA was extracted with phenol:chloroform (3:1) and the mixtures were centrifuged at 6000 rpm. The aqueous phase was transferred to another tube and precipitated again with 2.5 M acetate buffer, pH 5.2, and 2 volumes of absolute ethanol, as previously described. Samples were incubated overnight at -20ºC, centrifuged and the pellets were resuspended in TE buffer. DNA (5 μg) was submitted to electrophoresis in 0.8% agarose gels, 0.5 μg/ml ethidium bromide in 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, and 1.25 mM EDTA, pH 8.0) at 40 V for 6 h. DNA bands were visualized with a UV transilluminator. Samples for the detection of high molecular weight DNA fragments were prepared as described (17). Field inversion gel electrophoresis was run at 180 V in 1% agarose gels in 0.5x TBE buffer at 12ºC, with the ramping rate changing from 0.8 s to 30 s during a 24-h period, and a 3:1 forward to reverse ratio being applied. DNA was stained with ethidium bromide (0.5 μg/ml), visualized at 305 nm by UV light and photographed with a scanner (Fuji, Tokyo, Japan).

Oligonucleosomal DNA fragments were undetectable in both the -Se/-VE and +Se/+VE groups, and apparently high molecular weight fragments either remained in the well or migrated slowly into the gel as a single band by conventional electrophoresis. These fragments (approximately 50 kb in size) were barely resolved by field inversion gel electrophoresis, as shown in Figure 2.

Although the formation of the DNA ladder has been used as a biochemical indicator of apoptosis, it is, in fact, only the end point of DNA degradation and does not reflect the full pattern of DNA fragmentation occurring during apoptosis. Indeed, many cells do not degrade their DNA to this extent. A recent report has shown DNA degradation during apoptosis to be a much more complex process that begins with the generation of high molecular weight DNA fragments, which
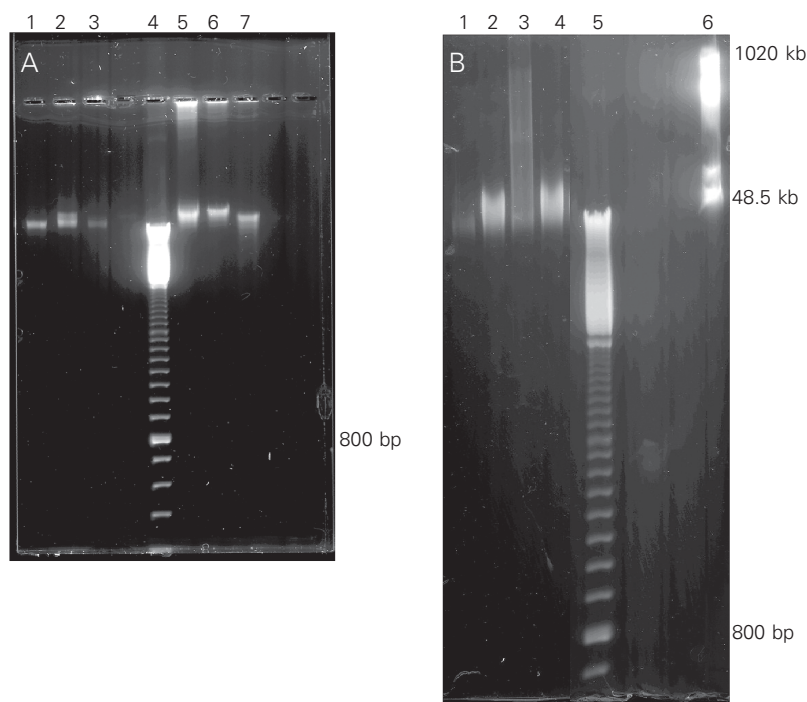

Figure 2. Separation of muscle cell. *A*, Conventional agarose gel electrophoresis was performed in 0.8% agarose gels at 40 V for 6 h. *Lanes 1, 2* and *3*: sample +Se/+VE; *lane 4*: 100-bp ladder (molecular mass marker); *lanes 5, 6* and *7*: samples -Se/-VE. *B*, Field inversion gel electrophoresis was run at 180 V in 1% agarose gels. *Lanes 1* and *3*: sample +Se/+VE; *lanes 2* and *4*: sample -Se/-VE; *lane 5*: 100-bp ladder; *lane 6*: lambda ladder pulsed field gel marker (48.5 to 1020 kb). The sizes of the markers are indicated at the right of each figure

are undetectable by conventional electrophoresis. We have adapted a form of field inversion gel electrophoresis to optimize the separation of DNA fragments eventually produced in muscle cells under stressful conditions, since a prerequisite for the analysis of high molecular weight DNA is the elimination of mechanical or enzymatic damage to the DNA during the processing of cells. Even using modified procedures, we did not observe the hallmark profile of DNA fragmentation in the samples of muscle tissue. According to some investigators, DNA fragmentation is not a requirement in programmed cell death, but rather, can be a late and nonessential event (18). In addition, nucleases involved in DNA degradation have been demonstrated mostly by indirect evidence.

It has been previously mentioned that little is known about the initiating events and factors involved in apoptotic cell death, in particular in skeletal muscle. While apoptosis plays an important role in the physiological turnover of cells and tissues, necrosis is usually confined to pathologic states. However, there is increasing evidence now that necrosis and apoptosis may share certain common factors and pathways, which include death domain receptors, mitochondrial permeability transition pores or mitochondrion-nuclear translocation of the apoptosis initiating factor (19). Also, apoptosis of multinucleated muscle fibers in which successive fiber segments are controlled by individual nuclei may be different from that seen in mononucleated cells. It has been shown that not all nuclei of a muscle fiber might display DNA fragmentation at the same time (20). Apoptosis of individual myonuclei and the subsequent degradation of the associated sarcoplasmic segment probably contribute to the process of muscle fiber atrophy, which may occur after denervation.

These reports support the observation that the oxidative stress responsible for exudative diathesis symptoms in chick muscle can also lead to caspase activation and ultimately to apoptosis, although most molecular events involved in the process still remain unclear.

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