Research Communication

Selenium supplementation modulates apoptotic processes in thyroid follicular cells

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Abstract

Selenium (Se) is an essential micronutrient modulating several physiopathological processes in the human body. The aim of the study is to characterize the molecular effects determined by Se-supplementation in thyroid follicular cells, using as model the well-differentiated rat thyroid follicular cell line FRTL5. Experiments have been performed to evaluate the effects of Se on cell growth, mortality and proliferation and on modulation of pro- and antiapoptotic pathways. The results indicate that Se-supplementation improves FRTL5 growth rate. Furthermore, Se reduces the proportion of cell death and modulates both proapoptotic (*p53 and Bim*) and antiapoptotic (*NF-kB* and *Bcl2)* mRNA levels. In addition, incubation with high doses of Na–Se might prevent the ER-stress apoptosis induced by tunicamycin, as assessed by membrane integrity maintenance, reduction in caspase 3/7 activities, and reduction in Casp-3 and PARP cleavage. Taken together, these results provide molecular evidences indicating the role of Se supplementation on cell death and apoptosis modulation in thyroid follicular cells. These observations may be useful to understand the effects of this micronutrient on the physiopathology of the thyroid gland. © 2017 The Authors BioFactors published by Wiley Periodicals, Inc. on behalf of International Union of Biochemistry and Molecular Biology, 00(00):000–000, 2017

Keywords: *selenium; autoimmunity; apoptosis; Hashimoto's thyroiditis*

Additional Supporting Information may be found in the online version of this article.

Volume 00, Number 00, Month/Month 2017, Pages 00–00

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Received 23 November 2016; accepted 16 January 2017

DOI 10.1002/biof.1351

Published online 00 Month 2017 in Wiley Online Library (wileyonlinelibrary.com)

1. Introduction

Selenium (Se) is an essential micronutrient playing a pivotal role in a several physiological and pathological processes including immunity [1], male fertility [2], and thyroid function [3]. Moreover, a role of Se as anticancer agent has been recently demonstrated [4,5].

Dietary selenium intake is highly variable worldwide, and a severe Se deficiency has been associated with increased mortality from cancer, poor immune function, male infertility, and cognitive decline [6] as well as Keshan disease [7] and Kashin–Beck disease [8]. In addition, the efficacy of selenium supplementation has been largely investigated in clinical trials showing that supplemental selenium reduced the incidence and mortality of at least five types of human cancers, including prostate and liver cancer [9–11].

In human body, thyroid is one of the organs with the highest selenium content. Here selenoproteins play a crucial role in the cellular defence system against H_2O_2 and reactive oxygen species (ROS) [3]. In addition, Se content directly affects deiodinases activity and therefore, indirectly affects the local activation of thyroid hormones [12,13].

Severe Se deficiency has been associated with thyroid dysfunction [14] and a low Se intake could predispose to initiation or progression of thyroid autoimmunity [15]. Moreover, in patients with Hashimoto's thyroiditis, Se supplementation reduced the thyroperoxidase antibody titer [16], and in patients with recurrent Graves' disease Se supplementation has been demonstrated to enhance the effect of antithyroid drugs [17]. However, the clinical efficacy of selenium and its effects on quality of life in patients with autoimmune thyroid disease (AITD) still remains to be proven [18].

Several papers demonstrated that, at nutritional doses, Se can be implicated in the modulation of cell cycle and apoptosis [19,20]. Aim of the present study is to investigate the direct effects of selenium on apoptosis modulation, using as model the rat thyroid follicular cells (FRTL5).

2. Experimental Procedures

2.1. Cell culture and treatment

FRTL5 thyroid cells were grown in nutrient mixture F-12 Ham (Sigma) supplemented with 10% newborn calf serum (NBCS),

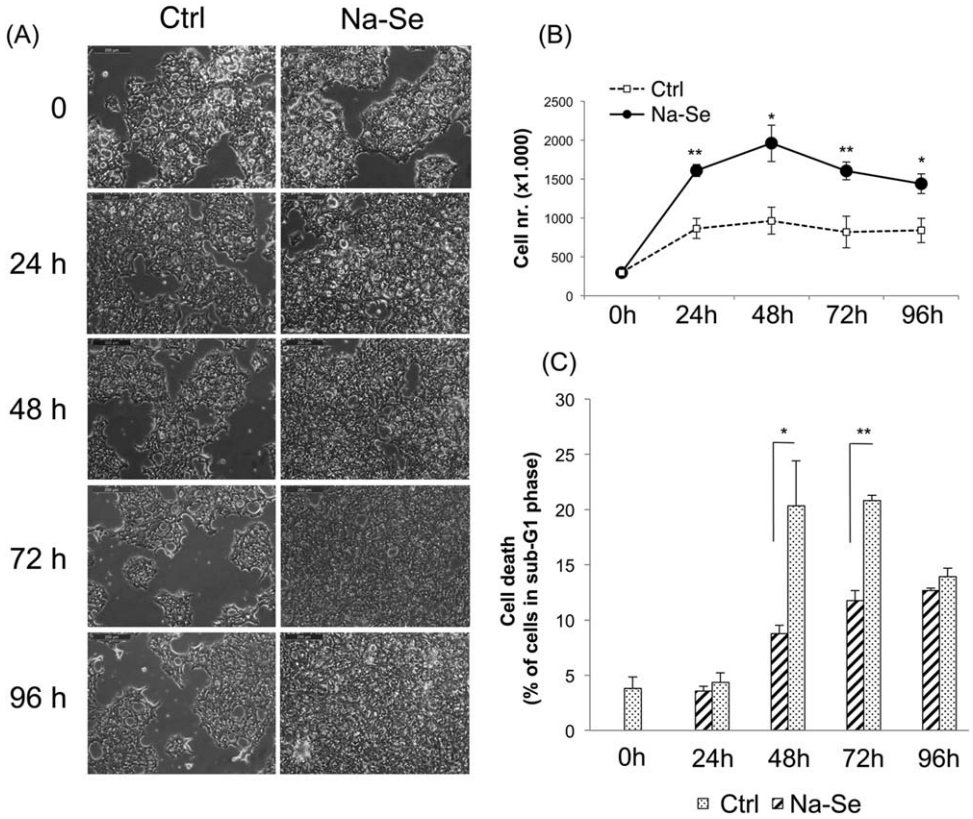

FIG 1

*Selenium effects on cell growth. A. Effect of Na–Se supplementation on FRTL5 cells morphology. Cells were maintained in medium containing only 0.5% serum or in the same medium supplemented with 100 nM Na–Se from 0 to 96 h. B. Cell count of both treated (Na–Se) and untreated cells (Ctrl). Na–Se increases the total number of cells. In graphs means ± SEM of four independent experiments are shown (*P < 0.05; **P < 0.01; Student's t test). C. Percentage of cells in sub-G1 phase measured by FACS analysis. Se supplementation significantly reduces sub-G1 FRTL5 cells at 48 and 72 h. In graphs means ± SEM of three independent experiments are shown (*P < 0.05; **P < 0.01; Student's t test).*

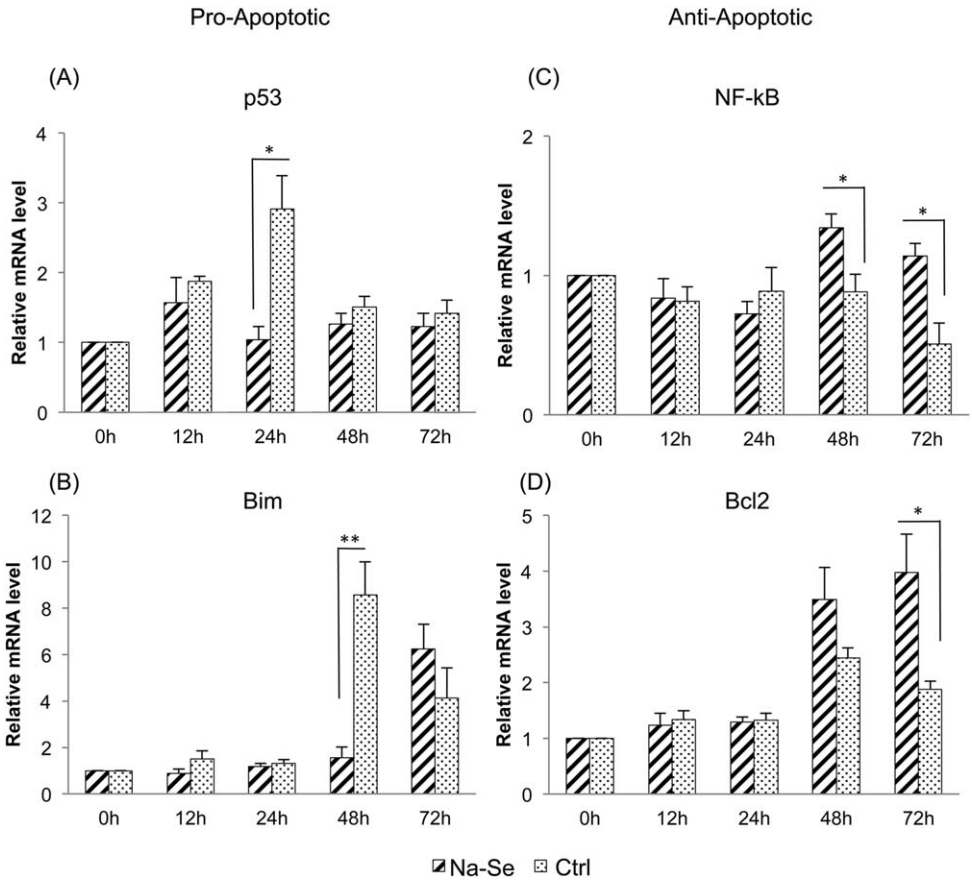

FIG 2

*Real-time PCR. A–D. FRTL5 cells were treated from 12 to 72 h with (Na–Se) or without (Ctrl) 100 nM Na–Se. mRNA levels of proapoptotic (*p53 *and* Bim*) (A,B) and antiapoptotic (*NF-kB *and* Bcl2*) (C,D) genes were measured by real-time PCR. Expression levels are normalized against the housekeeping gene beta actin. Histograms are the means of four (*p53, Bim, *and* Bcl2*) or five (*NF-kB*) independent experiments ± SEM (*P < 0.05 **P < 0.01; Student's* t *test).*

penicillin (100 IU/mL) streptomycin (100 μg/mL), 1 mM nonessential amino acids and a hormone mixture containing: bovine TSH (1 mU/mL), insulin (10 μg/mL), hydrocortisone (3.6 ng/mL), human apo-transferrin (5 μg/mL), glycyl-L-histidyl-L-lysine acetate (20 ng/mL) and somatostatin (10 ng/mL) (Sigma-Aldrich). Cells were maintained in a humidified incubator containing 5% CO_2 at 37°C and the experiments were performed at 60% confluency in normal medium.

To induce Se depletion, FRTL5 cells were grown in a starvation medium containing only 0.5% serum. This determined a reduction of Se concentration in the medium to about 0.85 ng/mL. Next, to evaluate the specific effect of Se on deprived cells, sodium selenite (Na–Se) (Sigma Aldrich) was added to a final concentration of 100 nM (~18 ng/mL) or 500 nM (~86 ng/mL). These values corresponded to low/normal Se concentration in human serum [21–23]. Both concentrations did not modify the proliferation of FRTL5 cells, as demonstrated by EC50 determination, using from 100 to 4000 nM Na–Se (data not shown). To verify that Se supplementation was successful, relative *GPx1* mRNA was measured by Real-Time qPCR and GPx1 activity was indirectly evaluated with the GPx Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's protocol (data not shown).

To induce apoptosis, cells were co-treated for 24 h with 2 μg/mL of the cell death inductor Tunicamicyn (TN, Sigma-Aldrich).

2.2. FACS analysis

FRTL5 were grown in 60 mm dishes with or without the addition of 100 nM Na–Se. Attached and detached cells were collected and fixed in ice-cold 70% ethanol in PBS. After an overnight incubation at −20°C, cells were washed twice with PBS, resuspended in 0.015M propidium iodide (PI, Sigma-Aldrich) in PBS for 20 min and analysed for the emission in FL1 and FL3 channels, as previously described [24].

2.3. Rna isolation and quantitative RT-PCR

FRTL5 cells were seeded in 60 mm dishes. Two hours before treatment, cell medium was changed to 0.5% NBCS and then 100 nM Na–Se was added up to 72 h.

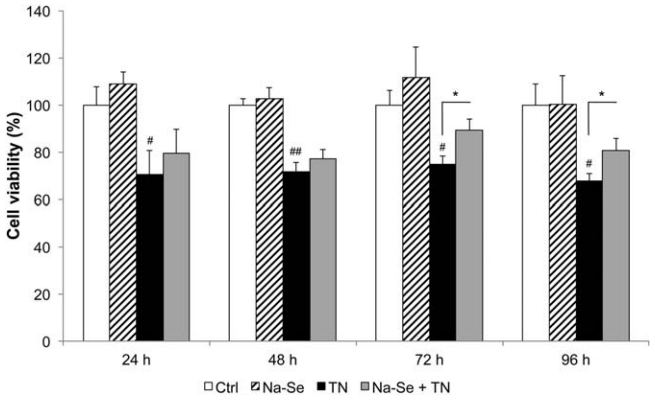

FIG 3

Cell viability assay. FRTL-5 cells were treated for 24, 48, 72, and 96 h with 500 nM Na–Se and with 2 μg/mL tunicamycin (TN) for the last 24 h. The histogram shows the percentage of cell viability compared to untreated starved cells (Ctrl, white column). Na–Se treatment (dashed columns) did not change the percentage of viable cells. Treatment with TN (black columns) reduces cell viability when compared to the starved cells (#P < 0.05, ##P < 0.01; Student's t *test). Pretreatments with Na–Se (gray columns) for 72 and 96 h significantly contrasted TN effect. Histograms are the means ± SEM of three independent experiments (*P < 0.05; Student's* t *test).*

Total RNA was extracted using TRIzol reagent in accordance with the manufacturer's instructions (Life Technologies) and its purity was assessed by the A_{260}/A_{230} absorbance ratio.

Relative mRNA expression of *p53*, *Bim, NF-kB, Bcl2, Casp8ap2,* and *Bax* was determined using the $2^{-\Delta\Delta Ct}$ method in comparison to T_0. Primers' sequence will be provided upon request. RNA amount was normalized using rat beta actin gene as internal reference.

2.4. MTT assay

To evaluate cell viability, 2.0×10^4/well FRTL5 cells were seeded in 96-well plates. The cells were pretreated with 100, 250, and 500 nM Na–Se from 24 to 96 h and TN (2 μg/mL) was added for the last 24 h.

MTT solution (5 mg/mL) was added, incubated 1 h at 37°C and then replaced with solvent (isopropyl alcohol, 10%HCl) in a constant stir for 5 min at room temperature (RT). Absorbance was measured at 570 nm using a Victor X4 plate reader (Perkin Elmer, Italy). The percentage of cell survival in treated cells was expressed as absorbance normalized to control cells.

2.5. Cell Tox green cytotoxicity assay

Cell Tox Green Cytotoxicity Assay (Promega), a system evaluating the membrane integrity, has been used to measure cell death. This system uses a proprietary asymmetric cyanine dye that is excluded from viable cells but stains the DNA of dead cells.

After the pretreatment with 500 nM Na–Se from 24 to 96 h and the addition of 2 μg/mL TN for the last 24 h, CellTox solution (20 μL of CellTox Green Dye in 10 mL of Assay Buffer) was added and incubated for 15 min at RT, shielded from light. Fluorescence was measured at 450–500 nm_{Ex}/520–530 nm_{Em} using a Victor X4 plate reader (Perkin Elmer, Italy). Vehicle-treated cells were used as control.

2.6. Western blotting

After the Na–Se (500 nM) and TN treatment, detached and attached cells were lysed in detergent buffer (1% NP-40, 10% glycerol, 137 mM NaCl, 20 mM Tris pH7.6, 20 mM NaF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin, 200 μM Na_3VO_4, 1 mM PMSF) on ice for 30′. The homogenate was centrifuged for 20 min at 1200 g and 4°C and supernatant was collected. Total protein extracts (30 μg) were separated in a 15 or 6% SDS-PAGE for Casp-3 and PARP detection respectively, and electroblotted to a nitrocellulose membrane. Filters were probed with antibodies against Casp-3 (#9665, Cell Signaling), PARP (#9542, Cell Signaling), and beta actin (A4700, Santa Cruz), revealed and quantified with the ImageQuant Las 4000 system (GE Healthcare).

2.7. Caspase 3/7 assay

Caspase 3 and 7 activities in FRTL5 cells were measured using a Caspase-Glo assay kit (Promega) according to manufacturer instructions. Briefly, the proluminescent substrate containing the DEVD (sequences are in a single-letter amino acid code) is cleaved by caspase 3 and 7. After caspase cleavage, a substrate for luciferase (aminoluciferin) is released producing a luminescent signal.

FRTL5 cells were growth in 96-well plate and treated with Na–Se (500 nM) and TN as previously described. Cells were equilibrated to RT. Next, 100 μL of Caspase-glo 3/7 reagent were added and incubated for 2 hours at RT, shielded from light. Finally, the activity of caspases 3 and 7, measured as luminescence, was read using a luminometer (Victor X4, Perkin Elmer, Italy).

2.8. Statistical analyses

Results were means of 3–5 independent experiments performed in triplicates. Statistical analysis has been performed using the Student's *t*-test with the SPSS statistical software.

3. Results

3.1. Selenium effect on cell growth

The addition of 100 nM Na–Se to the culture medium of starved FRTL5 determined a significant increase in cell survival at each of the studied time point (Figs. 1A and 1B).

FACS analysis showed that, after 48 and 72 h treatment, the percentage of FRTL5 cells in sub-G1 phase was significantly lower in Se-supplemented compared to control cells (Fig. 1C). This indicates that Se supplementation increased cell survival by reducing the percentage of dying cells.

3.2. Selenium effect on apoptosis

To assess whether the inhibition of cell death is determined by a reduction in apoptosis, mRNA expression of pro- and antiapoptotic genes has been measured upon Se treatment (Fig. 2).

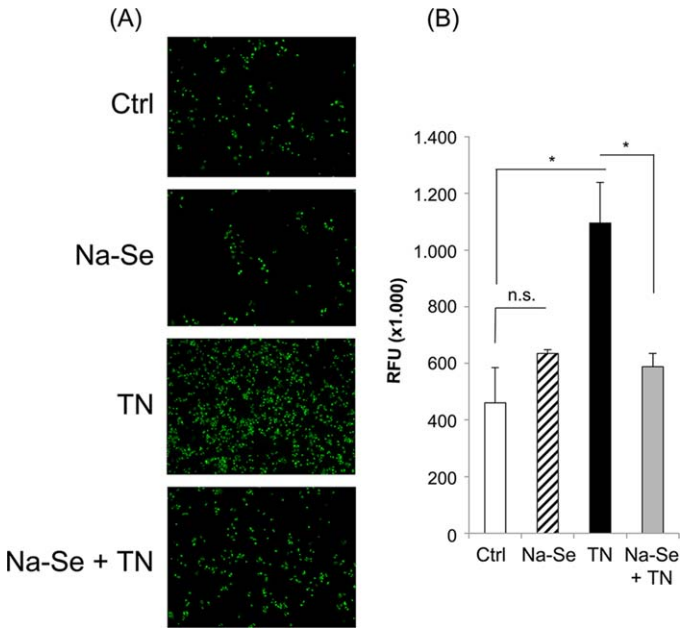

FIG 4

*Cell Tox green cytotoxicity assay. A. FRTL-5 cells were treated for 96 h with 500 nM Na–Se and/or with 2 μg/mL tunicamycin (TN) for the last 24 h. After the addition of the reagents, fluorescence stains death cells, lacking membrane integrity. B. As demonstrated by fluorescence quantification, TN treatment significantly increased cell mortality compared to untreated control (*P < 0.05; Student's t test), while, after 96 h of Na–Se pretreatment, fluorescence was reduced when compared to TN alone (*P < 0.05; Student's t test). Histograms represent fluorescent signals expressed as means ± SEM of four independent experiments.*

The addition of 100 nM Na–Se to starved culture medium reduced the transcription of the proapoptotic genes *p53* at 24 h (Fig. 2A) and *Bim* at 48 h (Fig. 2B) and increased the expression of the antiapoptotic *NF-kB* (Fig. 2C), and *Bcl2* (Fig. 2D) at 48 and 72, and at 72 h, respectively.

The protective effect of Na–Se on apoptosis was also evaluated after induction of endoplasmatic reticulum (ER) stress with the addition of 2 μg/mL tunicamycin (TN). Supplementation with Na–Se (100, 250, and 500 nM) produced no changes in cell viability (Supporting Information Figs. 1 and 3, dashed bars), while TN determined a reduction in cell viability at each time point (Supporting Information Figs. 1 and 3, black bars). No significant prevention of TN-induced apoptosis was observed at lower Na–Se doses (100 and 250 nM, Supporting Information Fig. 1, gray bars), while pretreatment with 500 nM Na–Se protected FRTL5 cells against TN-induced apoptosis at 72 and at 96 h (Fig. 3, gray bar). These data indicate that a long incubation with high doses of selenium might prevent the cell death determined by a pharmacologically-induced ER stress.

TN-induced mortality was also investigated using the CellTox Green Cytotoxicity Assay (Fig. 4). TN treatment alone significantly increased cell mortality compared to control ($P < 0.05$), while, after 96 h of 500 nM Na–Se pretreatment, fluorescence signal was reduced, suggesting a higher membrane integrity (TN vs. Na–Se+ TN, $P < 0.05$).

3.3. Role of selenium in FAS pathway

FAS pathway has been investigated by evaluation of *Casp8ap2* and *Bax* expression, Caspase-3 and PARP cleavage and Caspase-3/7 activity (Fig. 5). FRTL5 cells have been pretreated (24, 48, 72, and 96 h) with 500 nM Na–Se, with or without the addition of TN (2 μg/mL) for the last 24 h.

The results indicated that 96 h of Na–Se pretreatment reduced TN-stimulated transcription levels of the proapoptotic genes *Casp8ap2* and *Bax* (Figs. 5A and 5B). Se-pretreatment also reduced the cleaved forms of both Casp-3 (Fig. 5C) and PARP (Fig. 5E), and this effect was significant at 96 h for both Casp-3 and PARP (Figs. 5D and 5F, respectively) when compared with TN alone. Finally, 96 h with 500 nM Na–Se pretreatment reverted the increased caspase 3/7 activity induced by TN (Fig. 5G; $P < 0.05$ Na–Se+ TN vs. TN).

These data indicate that supplementation with 500 nM Na–Se produces a significant inhibition of the FAS cascade that has been activated by the addition of TN.

4. Discussion

Apoptosis is a physiologic and programmed cell death mechanism involved in the regulation of development, differentiation, and homeostasis [25]. This process is the result of a finely controlled gene modulation, where both survival and disruptive genes interact in a complex network leading to cells death. Alterations in the regulation of apoptosis have been associated with the pathogenesis of many disorders linked to Se deficiency. It has been demonstrated that master regulators of apoptosis such as p53, Bax, and Bcl2 are all involved in Se deficiency-induced myocardial cell death [26]. In rooster testis, dietary Se supplementation can affect spermatogenesis process and regulate the apoptosis of germ cells [27], while Se deficiency exerts significant harmful effects [28]. Moreover, very recently it has been demonstrated that selenium deficiency can induce vascular smooth muscle cells apoptosis, with the involvement of several selenoproteins [29].

The results described in this work indicate that Se supplementation exerts a specific effect on apoptosis prevention in thyroid follicular cells. In particular, Se supplementation improves FRTL5 cells growth and proliferation, and reduces cell death. The resulting changes are coherently coordinated by the downregulation of proapoptotic genes *p53* and *Bim*, and by the upregulation of antiapoptotic genes *NF-kB* and *Bcl2*. During inflammation, NF-kB transcription factor antagonize tumour necrosis factor-α-induced apoptosis. NF-kB reduces ROS accumulation [30] and modulates the activity of JNK pathway. We observed an upregulation of *NF-kB* after Se supplementation, confirming the role of this trace element in the protection from apoptosis in FRTL5 cells. This is in contrast with

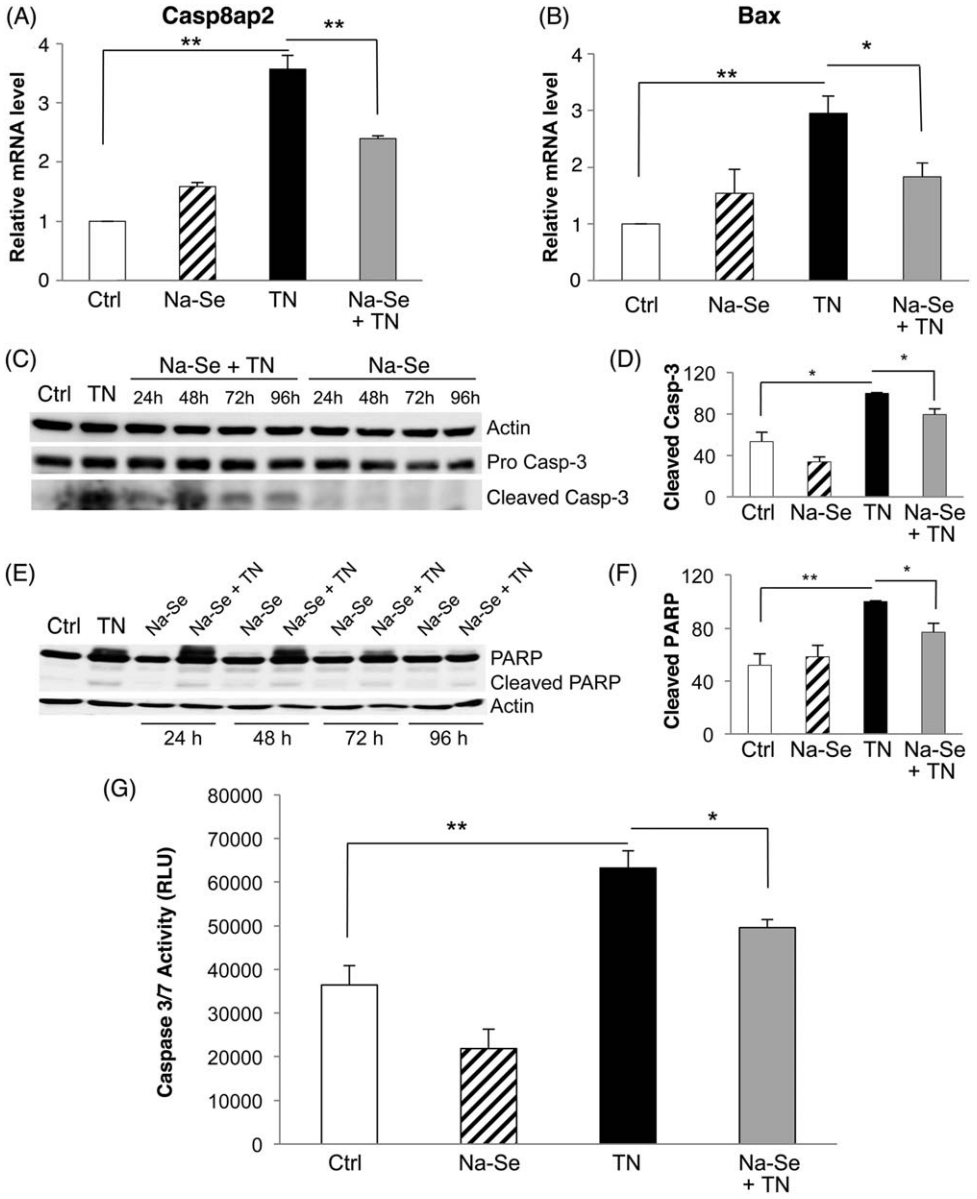

FIG 5

*Role of Selenium in FAS pathway. FRTL5 cells have been pretreated (24, 48, 72, and 96 h) with 500 nM Na–Se, with or without the addition of TN (2 μg/mL) for the last 24 h. A,B. Relative mRNA level of proapoptotic genes (*Casp8ap2 *[A] and* Bax *[B]) were investigated. TN significantly increased* Casp8ap2 *and* Bax *transcription. After 96 h of Na–Se pretreatment (gray columns), TN-stimulated transcription levels were reduced in comparison to TN alone (black columns). Histograms represent means ± SEM of three independent experiments. C,D. Casp-3 cleavage was evaluated by western blots (C). The graph (D) shows the results observed at 96 h. Quantification of Casp-3 cleaved band is expressed as percentage relative to the cleaved band after treatment with TN alone. Histograms represent means ± SEM of three independent experiments. E,F. PARP cleavage was evaluated by western blots (E). The graph (F) shows the results observed at 96 h. Quantification of PARP cleaved band is expressed as percentage relative to the cleaved band after treatment with TN alone. Histograms represent means ± SEM of five independent experiments. G. Caspase 3/7 activities were quantified in FRTL5 cells. TN significantly increases caspase 3/7 activities when compared to untreated cells (Ctrl). Pretreatment for 96 h with 500 nM Na–Se significantly reduced TN-stimulated luminescence signal. Histograms represent means ± SEM of four independent experiments. Statistical comparisons have been performed with the Student's* t *test (*$P < 0.05$, **$P < 0.01$)

what has been reported in previous studies suggesting that Se reduces NF-kB nuclear translocation and prevents an excess of immune response [31]. This discrepancy can be the consequence of different mechanisms by which Se acts in different tissues, but we cannot exclude that the phenotype is modulated by complex interactions with environmental factors, not reproducible in the *in vitro* model. p53 is an additional transcription factor that can be influenced by Se supplementation.

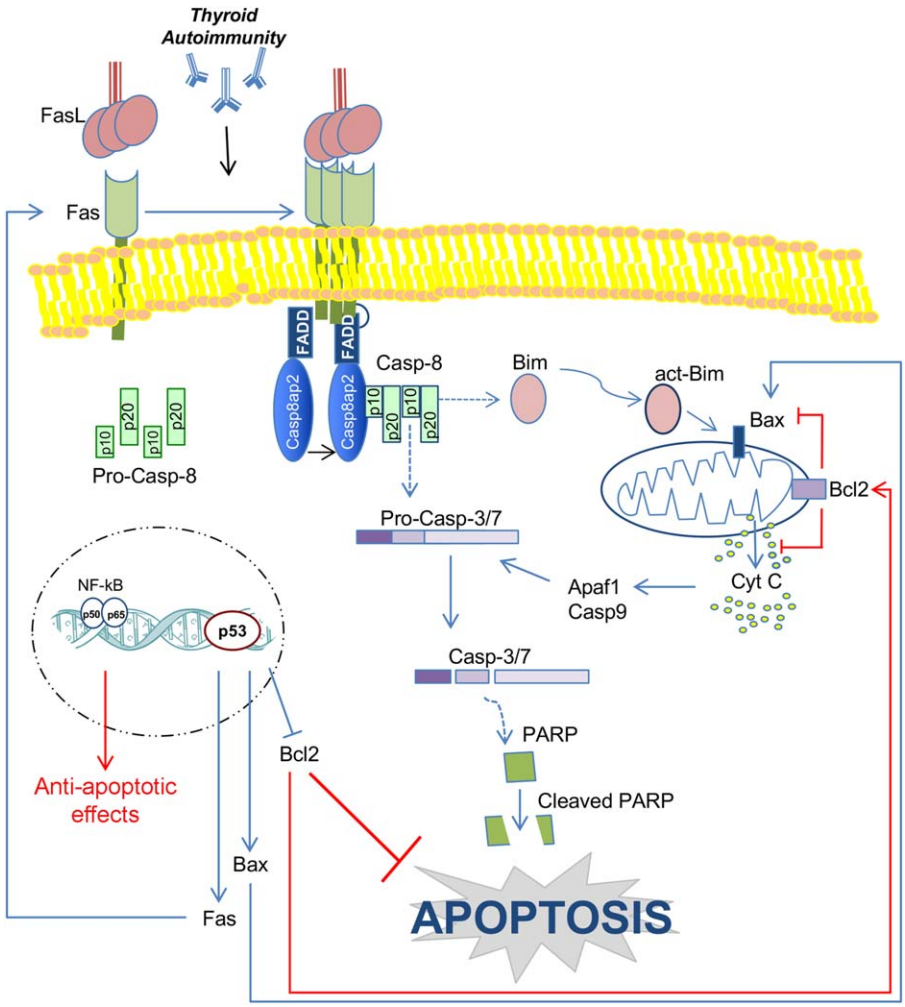

FIG 6

Schematic representation of the Fas/Fas-ligand pathway inducing apoptosis in thyroid follicular cells. Thyroid autoimmunity induces massive apoptosis by up-regulation of FAS/FAS-ligand and down regulation of Bcl2 protein in thyroid follicular cells. Activated caspase-8 cleaves pro-caspases 3/7 directly and indirectly via Bid and Bim. This indirect way determines conformational changes in Bax, which are responsible for cytochrome-c release. This causes apoptosome formation and casp-9 activation, responsible for procaspase-3/7 cleavage. Caspase-3/7 lead the activation of other executor proteases and, finally, apoptosis. Our results demonstrated that Na–Se reduces the expression of the proapoptotic Casp8ap2 resulting in decreased activation of caspase-8 and consequentially of Bim, Bax, and caspase-3/7. Finally, this reduces PARP cleavage with consequent inhibition of apoptosis. Moreover, the Se induces down-regulation of p53 resulting in higher expression of Bcl2. This, sequestering part of the activated Bax, inhibits apoptotic processes. In figure, lines are in blue if the pathway promotes apoptosis and in red if the proteins are associated with apoptosis inhibition.

The activation of p53 positively regulates a large number of targets, including the proapoptotic protein Bax [32], and inhibits the transcription of other factors, including the antiapoptotic gene *Bcl2* [33]. In our system, Se supplemented cells express low levels of *p53* and, as expected, high levels of *Bcl2* compared to untreated cells. Moreover, *Bax* expression after TN-induced apoptosis, is reduced in Se supplemented cells.

Apoptosis is an active process mediated by programmed signalling pathways, whose activation can be caused by several extra- or intracellular stimuli leading to cell death [34]. Several pharmacological agents, such as TN, can also induce these effects. TN determines the cell death by disrupting endoplasmic reticulum (ER) integrity [35]. The present study demonstrates that Se supplementation prevents TN-induced apoptosis and cell death in FRTL5 cells. Indeed, Se exposition produces a decrease in proapoptotic and an increase in antiapoptotic genes' mRNA levels. Furthermore, Se preincubation contributes to maintain membrane integrity reducing both Casp-3 and TN-induced caspase 3/7 activities and consequent reduction of PARP inactivation.

The results reported here indicate that at least 72 h and 500 nM Na–Se pretreatment are necessary to produce a protective effect against TN-induced apoptosis. This might suggest that high doses of selenium for a long period are required to protect thyroid follicular cells from damage-induced mortality, and these data may support the recent observations by

Esposito and co-workers, indicating that there is no effect on thyroid function, when a short-term supplementation with L-selenomethionine is given to euthyroid patients with Hashimoto's thyroiditis [36].

In the last years, several papers suggested that apoptosis can be associated with the development of autoimmune thyroid diseases (AITD) [37–41]. Indeed in AITD, an abnormal Bcl2 expression [42] and a dysregulation in the FAS/FAS-ligand pathway [43–45] have been reported. Moreover, follicles of patients with Hashimoto's thyroiditis show increased FAS/FAS-ligand proteins expression and high apoptotic rate, compared to normal thyroid [42]. The FAS/FAS-ligand pathway (Fig. 6) can follow different ways accordingly to the cell type. In type I cells (including thymocytes and resting T-lymphocytes) the activation of effector caspases (−3 and −7) is directly determined by Caspase-8 (Casp-8). In type II cells, the FAS-mediated apoptosis signalling requires a further cell cascade amplification of Casp-8 via BH3-only proteins (Bid and Bim) and subsequent recruitment of Bax and Bak [46]. In this scenario, FRTL5 cells seem to belong to type II cells. Our results indicate that Se treatment reduces FAS-mediated apoptosis with a modulation of *Casp8ap2* and *Bax* expression and consequent inhibition of caspase 3/7 activities.

In the last years, beneficial effects of Se-supplementation have been reported in patients with Hashimoto thyroiditis [16] and Graves' disease [17]. Data are not clear, and the real efficacy of Se-supplementation in AITD is still an unresolved issue [18]. Large-scale studies are necessary to understand the effects of selenium on the clinical course of AITD and on the quality of life in these patients [47,48]. However, it is possible hypothesize that modulation of apoptosis, determined by Se, can contribute to the reduction of antithyroid antibodies and to the improvement of the thyroid ultrasound picture in patients with AITD.

We are aware that the study presents some limitations. First, the experiments have been performed with Na–Se and not with different forms of selenium. Sodium selenite has been already used in previous studies [26,27,49], and it is known to produce its effects quickly in terms of glutathione consumption to become available for selenoprotein biosynthesis. Moreover, in the experiments described here, the doses of Na–Se that have been used were comparable to low (100 nM) or normal (500 nM) concentrations in human serum [21–23]. The Se-supplementation determined a final Se concentration in culture medium corresponding to about 18 ng/mL (100 nM) or 86 ng/mL (500 nM). The results suggest that if Se concentrations are similar to the normal Se levels in plasma (100–120 ng/mL) [23] a protective effect against apoptosis is already present. Therefore, the translation of the results observed in this work to the clinical practice, may suggest that, to protect against thyroid apoptosis, a nutritional integration with selenium is necessary only when selenium deficiency is present. Finally, it will be interesting to evaluate the effects of selenium supplementation on apoptosis in animal models, to confirm *in vivo* the results reported here.

Despite these limitations, this work provides molecular evidences indicating the role of Se supplementation on cell death and apoptosis modulation in thyroid follicular cells, and these observations may be useful to understand the effects of this micronutrient on the physiopathology of the thyroid gland.

Acknowledgements

This work has been partially supported by a grant from the Italian Ministry for University and Research (PRIN 2012 to PEM). Carmela Passaro is currently supported by an American-Italian Cancer Foundation Post-Doctoral Research Fellowship.

Conflict of Interest

All the authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. The authors declare no support from any commercial organization for the submitted work.

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