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Research Paper

# Sodium selenite supplementation does not fully restore oxidative stress-induced deiodinase dysfunction: Implications for the nonthyroidal illness syndrome

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ABSTRACT

Nonthyroidal illness syndrome (NTIS) is marked by low T3 and high reverse T3 levels. The physiopathology is poorly understood but involves oxidative stress-induced disruption of the iodothyronine deiodinases, which activate or inactivate thyroid hormones. Selenium, an essential trace element, exerts antioxidant function mainly through the thioredoxin reductase (TRx) and glutathione peroxidase (GPx) redox-regulating systems. We evaluated the effect of sodium selenite on IL6-induced disruption on deiodinase function. Cell lines expressing endogenous deiodinases type 1(D1), 2(D2) or 3(D3) (HepG2, MSTO, and MCF-7 cells, respectively) were used in an intact cell model that mimics the deiodination process under physiological conditions of substrate and cofactor, in the presence or not of IL6, with or without selenite. Deiodinase activity was quantified by the amount of iodine-125 in the medium (D1 and D2) or by ion-exchange chromatography (D3). Oxidative stress was evaluated by measuring reactive species (RS), carbonyl content as well as enzymatic and non-enzymatic antioxidant defenses. *Results*: IL6 induced ROS and carbonyl content in all 3 cell lines (all $P<0.001$). Increased ROS was paralleled by D1 and D2-decreased T3-production ($P<0.01$) and increased D3-catalyzed T3-inactivation ($P<0.001$). Selenite decreases the IL6-induced ROS and carbonyl content, while enhances Gpx and Trx activities. Nevertheless, it failed on restoring D1 or D2 function and only attenuates D3 activation ($P<0.05$). In conclusion, although sodium selenite reduces IL6-induced redox imbalance it does not fully repair deiodinase function. These results shed light on NTIS physiopathology and might explain why low T3 levels are unaffected by selenium supplementation in sick patients.

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## 1. Introduction

The conversion of peripheral thyroxine (T4) to 3,5,3′-triiodothyronine (T3) accounts for 80% of all T3 produced in euthyroid individuals. This critical step in thyroid hormone metabolism is catalyzed by two enzymes, the type 1 (D1) and type 2 (D2) iodothyronine deiodinases, via outer-ring deiodination of the prohormone T4. Type 3 iodothyronine deiodinase (D3) catalyzes the inner (5)-ring deiodination of T4 and T3, thus inactivating thyroid hormone activity [1]. The deiodination reactions are catalyzed by an as yet undefined cofactor, probably a thiol, which acts as a reducing agent, regenerating the active enzyme. All deiodinases contain the amino acid selenocysteine in their active site, an essential residue for efficient catalysis [2–4].

The nonthyroidal illness syndrome (NTIS) refers to changes in peripheral thyroid hormone levels, characterized by low T3 and high serum reverse T3 levels. This set of alterations is observed in $>70\%$ of critically ill patients in almost every form of illness and is correlated with increased mortality [5,6]. Changes in deiodinase activities have been postulated to play an important role in the altered circulating levels of thyroid hormones in NTIS [7]. Cytokines are elevated as a generalized response to illness and available data suggest that interleukin (IL)-1β, tumor necrosis factor-α (TNFα) and, particularly IL6, play a central role in in NTIS [7]. The increased levels of IL6 lead to increases in superoxide radical ($O_2^{\bullet -}$) production through the enzyme complex of the NAD(P)H oxidase system, a major pathway of increased reactive oxidative species (ROS) generation [8]. The resulting oxidative environment impairs D1 and D2 function while inducing the expression of D3 [9]. The decreases in D1 and D2 activities occur despite increases in D1 and D2 protein levels, indicating that IL6-induced oxidative stress decreases the catalytic activity of D1 and D2, possibly by interfering with the effects of the yet unknown endogenous cofactor(s). Indeed, N-acetylcysteine (NAC), an antioxidant that restores intracellular reduced gluthatione (GSH) levels, prevents the

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IL6-induced effects on intracellular redox state [9,10].

Selenium, an essential trace element, has biological functions vitally important to human health, exerting critical intracellular antioxidant function mainly as selenoproteins [11–13]. Sodium selenite, the most commonly used form of selenium supplementation, acts through the enzymatic gluthatione peroxidase (GPx) and thioredoxin (TRx) antioxidant defense system. The GPx isoforms (GPx1–GPx6), implicated in maintaining cellular redox homeostasis, reduce hydrogen and lipid peroxides, using GSH as cofactor. TRx enzyme isoforms (TRx1–TRx3) play a role on the cellular thiol-dependent redox mechanisms as a disulfide NAD(P)H-dependent reducer [12–14]. Several studies have investigated the effects of selenium supplementation in acutely ill patients; however, the results of these studies are conflicting. While selenium supplementation, most commonly with sodium selenite, did not improve the clinical outcome or decrease mortality in some studies [15,16], others reported an inverse correlation between plasma selenium levels and sepsis-related organ failure assessment [17]. Notably, decreases in selenium plasma levels have been inversely correlated with survival rate in patients with severe sepsis or septic shock [18,19].

The aim of the present study was to evaluate the antioxidative effect of sodium selenite on the deiodination process under IL6-induced oxidative stress in an intact cell system model that mimics the NTIS.

## 2. Methods

### 2.1. Reagents

Reagents were obtained from Invitrogen (Life Technologies Inc., NY, USA), Calbiochem-Novabiochem, or Sigma-Aldrich (St. Louis, MO, USA). Outer ring-labeled [$^{125}$I]T3 and [$^{125}$I]T4 (specific activity 4400 Ci/mmol) were obtained from PerkinElmer (Boston, MA, USA). Purification of [$^{125}$I]T4 or [$^{125}$I]T3 was performed on sephadex LH-20 columns just before it was used to reduce $^{125}I^-$ to $<1\%$.

### 2.2. Cell culture and condition studies

Human mesothelioma (MSTO-211H) and human embryonic kidney epithelial (HEK-293) cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Hepatocarcinoma (HepG2) and human breast carcinoma (MCF-7) cell lines, were obtained from Banco de Células do Rio de Janeiro (RJ, Brazil). MSTO-211 and MCF-7 cells were cultured in RPMI-1640 (Roswell Park Memorial Institute) medium supplemented with 10% fetal bovine serum, while HepG2 and HEK-293 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air, and the culture medium was changed three times per week. The passage number of all cell lines was less then 10–15 at the time of experiments.

In all the experiments to evaluate the effect of IL6 and sodium selenite on intracellular oxidative parameters, enzyme activity and mRNA cells were cultured with IL6, in an attempt to reproduce the pathophysiological conditions as observed in NTIS patients [20], with or without 100 nM sodium selenite (Na(2)SeO(3)) per 24 h [21]. In all experiments 20 mM NAC was used as control, since it is able to completely restore deiodinase function in the presence of augmented reactive oxygen species [9].

### 2.3. Deiodinase activity for D1 and D2 in intact cells

Intact cell assay for D1 and D2 was performed as previously described [22]. Cells were cultured for 24 h in 1 mL serum-free 0.1% BSA plus 740 nM of total T4 (resulting in an FT4 concentration of 2.7%; [23]), including approximately 100,000 cpm/mL [$^{125}$I]T4 in the presence or not of IL6, sodium selenite or NAC. Experiments were performed in duplicate or triplicate for each condition and repeated at least three times. At the completion of the experiment, 300 μL medium were removed, added to 200 μL horse serum and protein precipitated by 100 μL 50% trichloroacetic acid (TCA). After vortexing, tubes were centrifuged at 12,000 *g* for 2 min. The $^{125}I^-$ generated was expressed as the fraction of the total T4 counts minus the nonspecific deiodination in untransfected HEK-293 cells, which does not express any deiodinase activity ($<5\%$ of total [$^{125}$I]T4 counts), and corrected for the 50% reduction in the specific activity relative to T4. Net T3 production was calculated by multiplying the fractional conversion by the T4 concentration in the media (740 pM) and expressed as total T3 production/mg protein per 24 h. Results were expressed as iodide production. Using high-performance liquid chromatography, the authors previously demonstrated that net iodide release in this particular system – although not in skeletal muscle – is specific and equivalent to T3 production [22,24].

### 2.4. Deiodinase activity for D3 in intact cells

For the studies with D3, cells in 6-well plates were cultured for 24 h in 1 mL serum-free 0.5% BSA (resulting in an FT3 fraction of 3.5%; [25]) in DMEM plus 195 pM T3 (FT3, ~7 pM), including approximately 200,000 cpm/mL [$^{125}$I]T3 in the presence or not of IL6, sodium selenite or NAC. Sephadex LH-20 chromatography was used to measure the D3 activity in intact cells [9]. Briefly, at completion of the experiment, 300 μL medium was collected, and the reaction was stopped with 200 μL horse serum and 100 μL 50% TCA, followed by centrifugation at 12,000 *g* for 2 min to precipitate the non-metabolized [$^{125}$I]T3. The supernatant was used for the determination of [$^{125}$I]T2 and [$^{125}$I]T1 produced. The LH-20 column (bed volume 2 mL) was equilibrated with 1:1 0.1 M HCl, an equal volume of 0.1 M HCl was added to 500 μL samples and the mixture was then applied. Stepwise elution was performed by successive application of $2\times1$ mL 0.1 M HCl (for $^{125}I^-$ release), $6\times1$ mL 20% ethanol ([$^{125}$I] for T1 release), and $4\times1$ mL 50% ethanol in 0.1 M NaOH (1:1 v/v [$^{125}$I] for T2 release). The 1 mL fractions were collected and counted for radioactivity. Results are presented as the mean values derived from at least two independent experiments. Nonspecific deiodination was $<1.5\%$. Net D3 activity was calculated by multiplying the fractional conversion by the T3 concentration in the media and expressed as T3 inactivation (fmol/mg protein per 24 h). The reaction was saturated by excess unlabeled T3.

### 2.5. Real-time PCR

Using the same incubation conditions above, after 24 h incubation total RNA was extracted from MSTO-211, HepG2 and MCF-7 cells with an RNeasy kit (Qiagen, Hilden, Germany) and used to synthesize complementary DNA (cDNA) (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen). The generated cDNAs were used in a real-time PCR with a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in ABI Prism 7500 Sequence Detection System (Applied Biosystems). Standard curves representing five-point serial dilutions of cDNA of the experimental and control groups were analyzed and used as calibrators of the relative quantification of product generated in the exponential phase of the amplification curve. The $r^2$ was $>0.99$, and the amplification efficiency varied between 80% and 100%. Samples were measured by relative quantification (change in expression in the experimental group vs. control; i.e., untreated vs. treated cells). The data generated by the ABI Prism 7500 system

SDS software (Applied Biosystems) were then transferred to a spreadsheet (Excel, Microsoft Corporation, USA), and the experimental values were corrected to that of the cyclophilin A standard [22,26]. The following oligonucleotides were used: hD1 (5′-AAGAGGCTCTGGGTGCTCTTGG-3′ and 5′-GGTTCTGGTGATTTCTGATGTC-3′), hD2, 5′-ACTTCCTGCTGGTCTACATTGATG-3′ and 5′-CTTCCTGGTTCTGGTGCTTCTTC-3′; hD3, 5′-TCCAGAGCCAGCACATCCT-3′ and 5′-ACGTCGCGCTGGTACTTAGTG-3′; cyclophilin A (internal control), 5′-GTCAACCCCACCGTGTTCTTC-3′ and 5′-ACTTGCCACCAGTGCCATTATG-3′. The internal control cyclophilin A was unaffected by the presence of IL6.

## 3. Detection of reactive species

Intracellular ROS production was performed by staining cells with the chloromethyl derivative of 5-(and 6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate (CM-$H_2$-DCFDA) (Invitrogen, Life Technologies Inc., NY, USA), which can permeate the cell membrane. This technique is an assay of generalized oxidative stress rather than of any particular reactive species, and is not a direct assay of hydrogen peroxide, lipid peroxides, superoxide or nitric oxide [27]. A stock solution of the dye was reconstituted in 100% DMSO to a concentration of 1 mM immediately before use. After removing growth medium and washing cells with pre-warmed phosphate-buffered saline (PBS), 70% confluent MSTO, HepG2 or MCF-7 cells on six-well plates were incubated at 37 °C for 30 min with pre-warmed PBS containing CM-$H_2$-DCFDA at a final concentration of 5 μM. Cells were scraped, centrifuged at 1500 rpm for 3 min, ressuspended and counted. 12,000 cells were then incubated at 37 °C with 1000 ng/L IL6 in the presence or absence of 100 mM Na(2)SeO(3) or 20 mM NAC. The same number of cells was used as negative control. Fluorescence was measured at wavelengths of 490 nm (excitation) and 535 nm (emission). Samples were analyzed in triplicate. Data were expressed as fold increase in fluorescence compared with buffer-treated cells (control).

### 3.1. Determination of thioredoxin reductase and peroxide reductase activity

Thioredoxin reductase activity (TRx) was determined using the Thioredoxin Reductase Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacture's instructions. Briefly, after 24 h incubation in the presence or not of IL6, sodium selenite or n-acetylcysteine cells were lysed with a buffer containing 150 mmol/L NaCl, 50 mmol/L Tris–HCl, 1% Triton X-100, 1% sodium dodecyl sulfate, 1% deoxycholate, 1 mmol/L NaF and 1 mmol/L EDTA, and protease inhibitors. After centrifugation at 14,000 × *g* for 10 min at 4 °C, protein concentrations of supernatants were determined using the Bradford method, and protein was then incubated in 100 mmol/L of potassium phosphate with 10 mmol/L EDTA and 0.24 mmol/L NADPH with and without a TRx reductase inhibitor of all isoforms. The reaction was started by adding 5,5′-dithiobis(2-nitrobenzoic) acid and monitored spectrophotometrically at 412 nm. Enzymatic activity was quantified using the following equation: Unit/mL = A412 nm/min (thioredoxin reductase) × dil × vol/enzvol (dil = sample dilution factor; vol = volume of reaction in mL; and enzvol = volume of enzyme in mL). Results were expressed as U/mg protein.

Glutathione peroxide activity (GPx) was determined using the Glutathione Peroxidase Cellular Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, after 24 h incubation in the presence or not of IL6, sodium selenite or NAC cells crude homogenate were determined by monitoring the NADPH disappearance at 340 nm. The specific activity was calculated as U/mg protein.

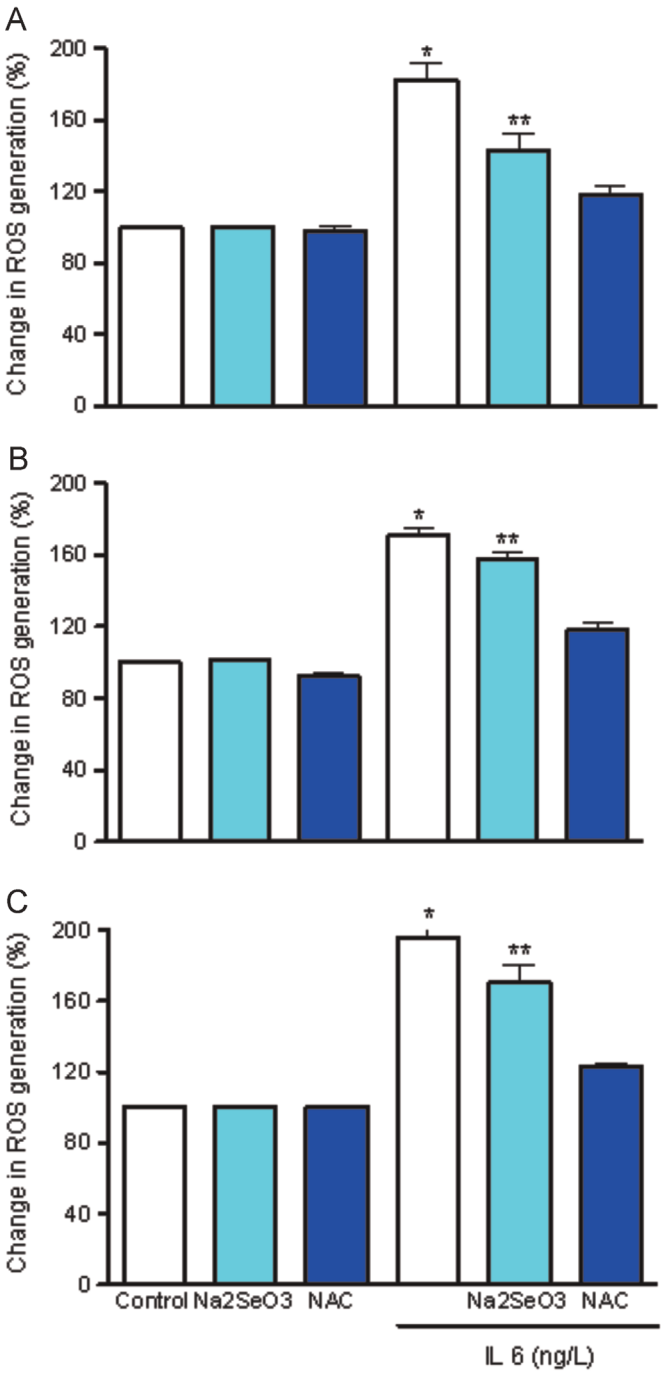


**Fig. 1.** Reactive oxygen species (ROS) generation in cells endogenous expressing type 2 (MSTO; A), type 1 (HepG2; B) or type 3 (MCF-7; C) deiodinases. *$P < 0.001$ refers to the difference between control and IL6 treated (1000 ng/L) while **$P < 0.001$ refers to the difference between sodium selenite supplementation in controls as compared with IL6 treated cells. Data are mean ± SD of at least three independent experiments. NAC, N-acetylcysteine.

### 3.2. Nonenzymatic antioxidant defenses

Reduced glutathione (GSH) levels were measured after the same incubation conditions above, according to a standard method [28]. Briefly, cells in culture dishes were washed with phosphate-buffered saline (PBS) and harvested in the presence of 300 μL of 20 mM sodium phosphate and 140 mM KCl buffer pH 7.4. Proteins were precipitated by adding sodium metaphosphoric

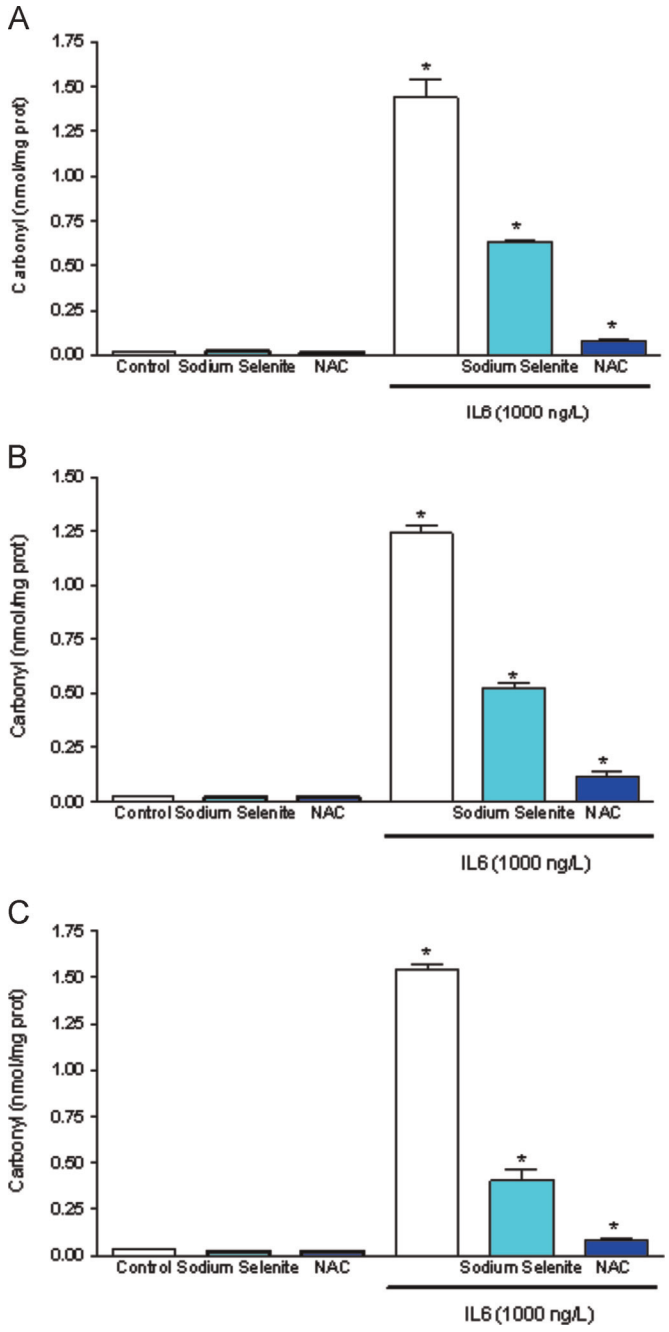


**Fig. 2.** Effect of sodium selenite or N-acetylcysteine (NAC) on carbonyl content in IL6 (1000 ng/L) treated cells endogenous expressing type 2 (MSTO; A), type 1 (HepG2; B) or type 3 (MCF-7; C) deiodinases. Data are mean ± SD of at least three independent experiments. $^{*}P < 0.001$.

acid to a final concentration of 1:1. Samples were centrifuged for 10 min at 7000 *g*. Fifteen microliters of cell preparation were incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min in the presence of 20 volumes (1:20, v/v) of 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. The calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol/mg protein.

Total glutatione (tGS) and oxidized gluthatione (GSSG) concentrations were determined by following the enzymatic recycling method described by Teare et al. (1993) with some modifications. Briefly, cells were homogenized in 4 (w/v) volumes of a solution of sulfosalicylic acid (11%) and Triton X-100 (0.11%) (1:1 ratio). A brief incubation of 5 min at 4 °C under continuous shaking was followed, the samples were then centrifuged at 10,000 *g* for 10 min (4 °C), and the supernatant was collected for analyses of glutathione levels. For GSSG measurement, 10 µL of thes supernatant were added to 110 µL of a GSH masking buffer (100 mM phosphate buffer, 1 mM EDTA, 1.1% 2-vinylpyridine), pH 7.4, and incubated for 1 h at room temperature. The samples prepared for tGS and GSSG were subjected to enzymatic analysis in a recycling buffer system containing 300 µM NADPH, 225 µM DTNB, 1.6 U/mL GR and 1.0 mM EDTA in 100 mM phosphate buffer (pH 7.4). The linear increase in absorbance at 405 nm over time was monitored using a microplate reader (Spectramax M5, Molecular Devices, California, US). A standard curve was built with known amounts of GSH (100uM) and GSSG (3.47, 6.95, and 13.89 µM).

### 3.3. Determination of protein carbonyl content

Duplicate aliquots of 0.3 mg of protein homogenate of each cell type were incubated with 500 µL of 10 mM 2,4-dinitrophenylhydrazine or 1.0 mL of 2 M HCl (blank tube). After 30 min, 250 µL of 50% trichloroacetic acid was added. The samples were centrifuged at 8000 *g* for 30 min to obtain protein pellets, which were immediately washed with ethanol–ethyl acetate 1:1 (v/v). The final protein pellets were resuspended in 500 µL of 8 M urea buffer and incubated at 50 °C for 90 min. The difference between the 2,4-dinitrophenylhydrazine-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 370 nm. Carbonyl content was calculated using the millimolar absorption coefficient of hydrazone (e370 nm = 21,000,000 $M^{-1}$ $cm^{-1}$), and the results were expressed in nmol carbonyl/mg protein.

### 3.4. Statistical analysis

Unless otherwise specified, results are presented as mean ± SD. Data were analyzed using two-tailed Student's *t* tests or two-way ANOVA followed by post-hoc Duncan multiple-range tests when *F* was significant. Prism 5.0 (GraphPad Software Inc., USA) was used for statistical analysis; $P < 0.05$ was considered to be statistically significant.

## 4. Results

### 4.1. Sodium selenite partially reverses the oxidative stress generated by IL6

First, we assessed whether sodium selenite had an effect on the IL6-induced oxidative stress by measuring the amount of intracellular ROS generated. The treatment with NAC (20 mM) was used as a positive control. Using as reference IL6 levels as observed in critically ill patients, cells were exposed to 1000 ng/L IL6 [20] with or without sodium selenite [21]. The experiments were performed using MSTO-211, HepG2 or MCF-7 cell lines, which endogenously express D2 [29], D1 [30], or D3 [30] enzymes, respectively. All cell lines were pre-incubated for 30 min with CM-$H_2$-DCFDA and then treated with 1000 ng/L IL6 in the presence or absence of sodium selenite or NAC.

IL6 induced ROS in all 3 cell lines ($P < 0.001$; Fig. 1A–C). Pretreatment with sodium selenite slightly attenuated IL6-induced reactive species detection in MSTO-211, HepG2 and MCF-7 cells (approximately 25%, 12% and 15%, respectively, all $P < 0.05$; Fig. 1A–C, respectively) while NAC completely abolished it ($P < 0.001$; Fig. 1A–C).

Next, we evaluated the amount of carbonyl formation, one of

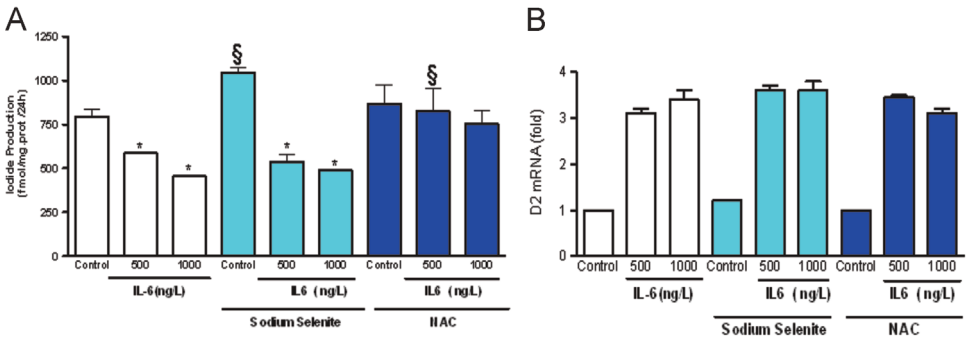


**Fig. 3.** Effect of sodium selenite on type 2 deiodinase activity and expression. Sodium selenite does not reverse the inhibitory effect of interleukin IL6 on deiodinase type 2 (D2)-catalyzed T4-to-T3 conversion in endogenous D2 expressing MSTO-211 cells, as observed in the presence of N-acetylcysteine (NAC) (A). The IL6-induced increases on *DIO2* mRNA levels in MSTO-211 cells is not reverted by sodium selenite or NAC (B). $^{*}P<0.001$, difference between controls and IL6 treated cells; $^{\S}P<0.001$, difference between controls in the presence or not of sodium selenite. Data are mean ± SD of at least three independent experiments. The net iodide release in this system is specific and equivalent to T3 production.

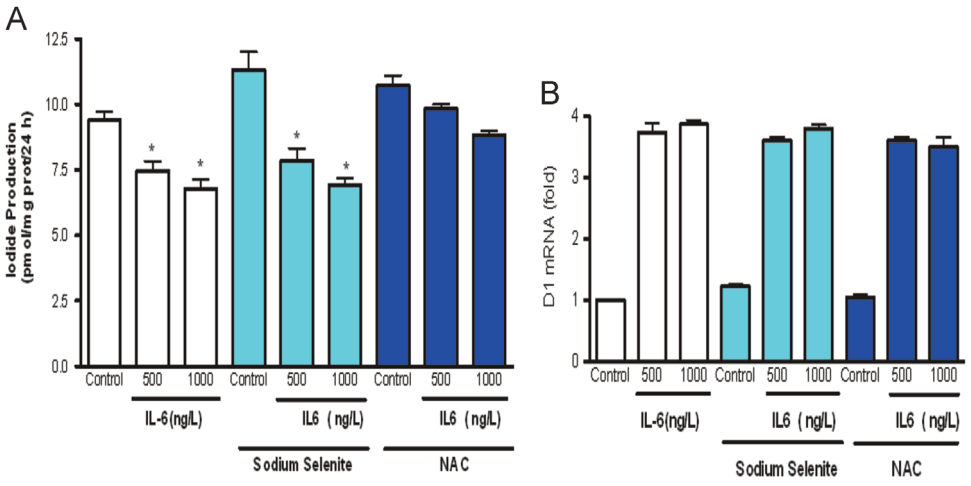


**Fig. 4.** Effect of sodium selenite on type 1 deiodinase activity and expression. Sodium selenite does not reverse the inhibitory effect of IL6 on deiodinase type 1 (D1)-catalyzed T4-to-T3 conversion in HepG2 cells, as observed with NAC (A). The IL6-induced increases on *DIO1* mRNA levels in HepG2 cells is not reverted by sodium selenite or NAC (B). $^{*}P<0.001$, difference between control and IL6 treated cells; $^{\S}P<0.001$, difference between controls in the presence or not of sodium selenite. Data are mean ± SD of at least three independent experiments. The net iodide release in this system is specific and equivalent to T3 production.

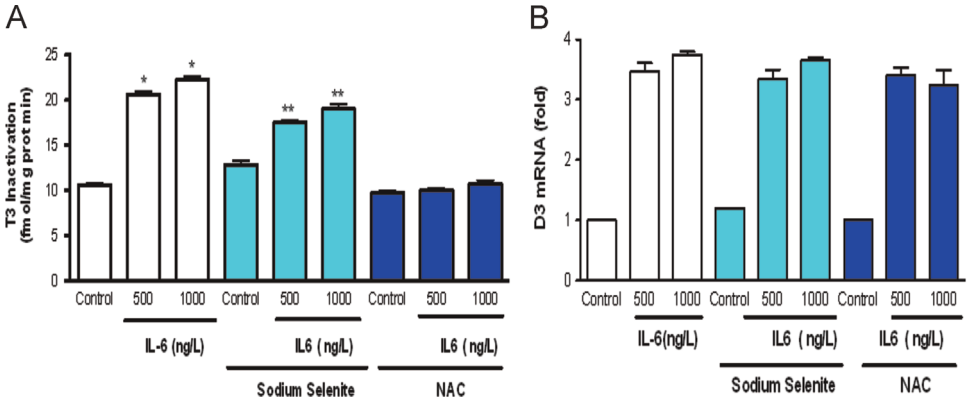


**Fig. 5.** Effect of sodium selenite on type 3 deiodinase activity and expression. Sodium selenite partially reverses the effect of IL6 on deiodinase type 3 (D3) catalyzed T3 inactivation while NAC completely abolishes it (A). There was no effect on the IL6 induced DIO3 messenger RNA (mRNA) (B). $^{*}P<0.001$, difference between control and IL6 treated cells; $^{**}P<0.001$, difference between presence or not of sodium selenite; $^{\S}P=0.01$, difference between controls in the presence or absence of sodium elenite. Data are mean ± SD of at least three independent experiments.

the most extensively used markers of oxidative damage to proteins [13]. In MSTO-211 cells, IL6 induced carbonyl formation (0.03 vs. 1.44 nmol carbonyl/mg protein; $P<0.001$; Fig. 2A). Pretreatment with sodium selenite partially corrected the IL6-induced carbonyl formation, while NAC completely reversed it (1.44 vs. 0.63 vs. 0.08 nmol carbonyl/mg protein, respectively; $P<0.001$; Fig. 2A). A similar effect of IL6 on carbonyl formation was observed in HepG2 (0.023 vs. 1.24 nmol carbonyl/mg protein, respectively; $P<0.001$; Fig. 2B) and MCF-7 cells (0.03 vs. 1.54 nmol carbonyl/mg protein; $P<0.001$; Fig. 2C).

### 4.2. Sodium selenite does not prevent the inhibitory effect of IL6 on

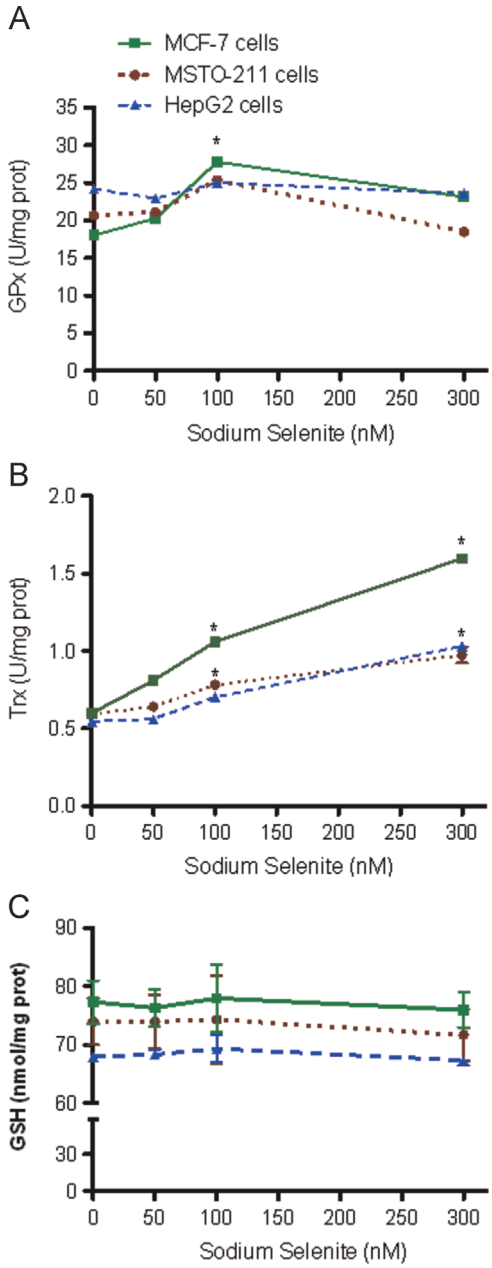


**Fig. 6.** Effect of increasing levels of sodium selenite (0–300 nM) on glutathione peroxidase activity (A), thioredoxin reductase (TRx) activity (B) and GSH (C) levels in MSTO-211 (•), HepG2 (▲) or MCF-7 (■) cells. Data are mean ± SD of at least three independent experiments.*$P < 0.01$.

*D2 and D1-catalyzed T3 production*

The conversion of T4-to-T3 by D2 decreases in the presence of increased levels of IL6 (Fig. 3A). The addition of sodium selenite to the culture media slightly increased T4-to-T3 conversion in control cells but failed to prevent the IL6 induced dose-dependent D2 inhibition, as observed with NAC addition ($P < 0.001$; Fig. 3A). Pretreatment with sodium selenite or NAC did not prevent the IL6-induced increase in the levels of *DIO2* mRNA (Fig. 3B).

Similar experiments were performed using cells expressing endogenous D1 (HepG2 cells, 30). The addition of sodium selenite to the culture media slightly increase the conversion of T4-to-T3, but failed to prevent IL6 induced D1 inhibition ($P < 0.0001$; Fig. 4A), which was restored in the presence of NAC. As observed with *DIO2*, sodium selenite or NAC did not reverse the IL6 -induced increase in *DIO1* mRNA levels (Fig. 4B).

### 4.3. Sodium selenite partially reverses the effect of IL6 on D3 activity

Next, we evaluated the effect of sodium selenite on D3 expression using MCF-7 cells [30]. The addition of IL6 induced D3-catalyzed T3 inactivation in a dose dependent fashion ($P < 0.001$). In contrast to the results obtained for D2 and D1, the sodium selenite supplementation attenuated the IL6-induced increase in D3 activity ($P < 0.05$; Fig. 5A). On the other hand, D3 activity return to the baseline levels with NAC addition. Either sodium selenite or NAC addition altered the IL6-induced up-regulation of *DIO3* mRNA (Fig. 5B).

### 4.4. Sodium selenite augments TRx and GPx levels in a dose dependent fashion while it does not change the intracellular GSH concentrations

To further explore the mechanisms involved on the rescue of IL6 mediated oxidative changes on deiodinase function, we sought to evaluate the effect of sodium selenite on the intracellular levels of GPx and TRx at baseline conditions or under IL6 induced oxidative stress. Total glutathione (tGS), GSH and oxidized gluthathione (GSSG) were also evaluated under the same conditions. Results were compared with those obtained with NAC treatment.

Sodium selenite supplementation increased GPx and TRx activities in a dose dependent fashion in all cell lines ($P < 0.01$; Fig. 6A and B), but it did not alter the intracellular GSH levels (all $P > 0.05$; Fig. 6C).

Next we determined the extension of GPx and TRx consumption in the presence of IL6-induced oxidative stress and whether sodium selenite supplementation could rescue IL6 mediated effects. IL6 induced oxidative stress decreased both GPx and TRx activities in all cell lines whereas sodium selenite counteracted the IL6 effects (all $P < 0.05$; Fig. 7A–F). NAC had no effect on GPx or TRx levels (all $P > 0.05$; Fig. 7A–F).

We also evaluated the effect of sodium selenite on the non-enzymatic antioxidant compounds measuring tGS, GSH, and GSSG levels. Sodium selenite had no effect on IL6 induced effects on tGS, GSH or GSSG levels whereas NAC reverts all of them (Fig. 8A–I). Accordingly, IL6-induced oxidative stress results in decreases in GSH:GSSG ratio in all cell lines (MSTO, 96 vs. 22; MCF-7, 100 vs. 29; and HepG2, 92 vs. 26; control vs. IL6 treated, respectively, all $P < 0.01$). The decreases in GSH:GSSG ratio observed in IL6 treated cells was not reversed by sodium selenite (MSTO, 96 vs. 25; MCF-7, 95 vs. 30; HepG2, 90 vs. 28; control vs. IL6 treated, respectively, all $P < 0.01$). However, this ratio was completely restore with the addition of NAC to the medium (MSTO, 96 vs. 123; MCF-7, 114 vs. 114; HepG2 cells, 104–103; control vs. IL6 treated, respectively, all $P > 0.05$).

## 5. Discussion

Perturbations in the intracellular redox status, as observed in critically ill patients, can disrupt the function of deiodinases. Here, we have demonstrated that sodium selenite partially corrects the augmented ROS levels and protein damage generated by pathologically elevated concentrations of IL6. However, it does not prevent the inhibitory effect of IL6 on D2- or D1-catalyzed T3 production while only attenuates the induced D3-catalyzed thyroid hormone inactivation. This might be explained by the lack of effect of sodium selenite on restoring the intracellular GSH levels, despite its role as protecting antioxidant enzymes involved in the intracellular redox balance.

Selenium supplementation has been advised in critically ill

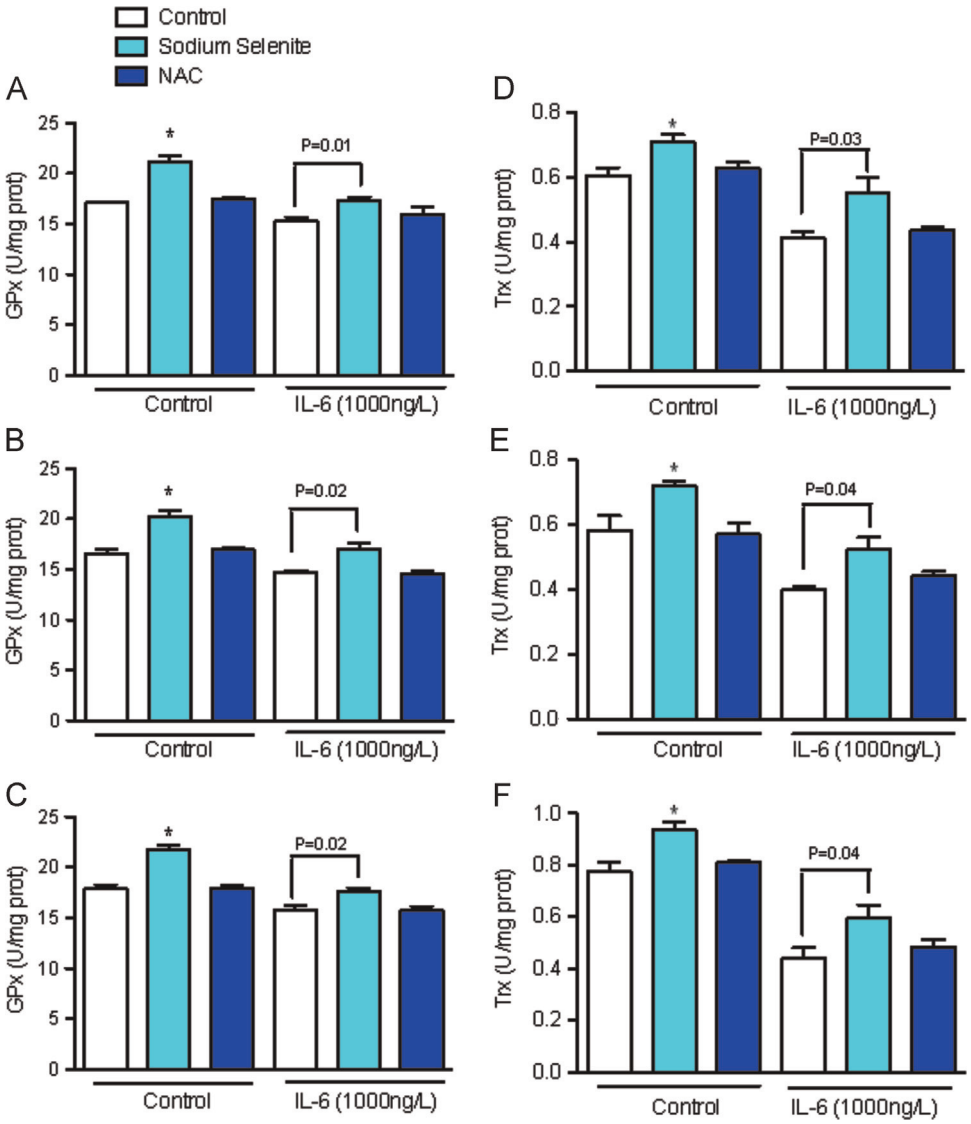


**Fig. 7.** Effect of sodium selenite or N-acetylcysteine (NAC) on glutathione peroxidase (GPx) and thioredoxin reductase (TRx) activity in controls and IL6 (1000 ng/L) treated cells. The column on the left shows the effect of sodium selenite or NAC on intracellular GSH levels in controls and IL6 (1000 ng/L) treated MSTO-211 (A), HepG2 (B), or MCF-7 (C) cells. The column on the right shows the effect of sodium selenite or NAC on intracellular TRx levels in MSTO-211 (D), HepG2 (E), or MCF-7 (F) cells. Data are mean ± SD of at least three independent experiments.

patients since low plasma selenium levels have been associated with oxidative stress, which might lead to the accumulation of severe damage in proteins, nucleic acids, lipids and other macromolecules [8,12,19,31]. Sodium selenite is the most common form of supplementation of selenium in sick patients. Indeed, in agreement with clinical studies that show improvement on the oxidative parameters of critically ill patients [15], here we show in cell culture model, that the addition of sodium selenite to the media counteracted 30–50% of the IL6-induced ROS generation and decreases the formation of carbonyl groups by 50–60% in all cell lines. Nevertheless, the addition of NAC, an antioxidant that restores intracellular cysteine and GSH levels, was more effective in diminishing the IL6 generated ROS and carbonyl formation (Figs. 1 and 2).

Changes in the intracellular redox state are known to disrupt deiodinase function [9,10]. The deiodinases are oxireductases that catalyze iodine removal from the outer or inner ring of thyroid hormones [32]. The D1 enzyme catalyzed reaction follows ping-pong kinetics with two substrates, an iodothyronine and a still unidentified thiol-containing cofactor whereas the D2 and D3 proteins exhibit sequential reaction kinetics, meaning that the substrate and the thiol-containing cofactor interact with the enzyme simultaneously [33,34]. The putative thiol cofactor(s), critical for the deiodinase function, may be depleted in situations of oxidative stress, thus impairing the catalytic reactions [12]. Accordingly, NAC prevents the IL6 or $H_2O_2$-induced effects on deiodinase activities as well as changes on thyroid hormone levels as observed in NTIS [9,10,31]. Based on the antioxidant properties of selenium compounds, one could expect that supplementation with sodium selenite could also restore deiodinase activity. Nevertheless, our results show that sodium selenite supplementation did not restore D2 or D1 function (Figs. 3 and 4). In contrast with the intact cell activity inhibition, IL6-induces an augment of all deiodinase transcripts, not reversed by sodium selenite or NAC. Although at first glance contradictory, this apparent discrepancy further indicates impairment of the enzyme function, probably due to the depletion of the endogenous cofactor, since the IL6 induced increases in deiodinase mRNAs would predict a parallel augment in deiodinase activities, as shown in DTT catalyzed sonicate assays [9]. The IL6 effect probably involves the p38 MAPK and ERK pathways, since it is abolished by specific inhibitors [9,35,36], while the MAPK cascades activates cAMP-

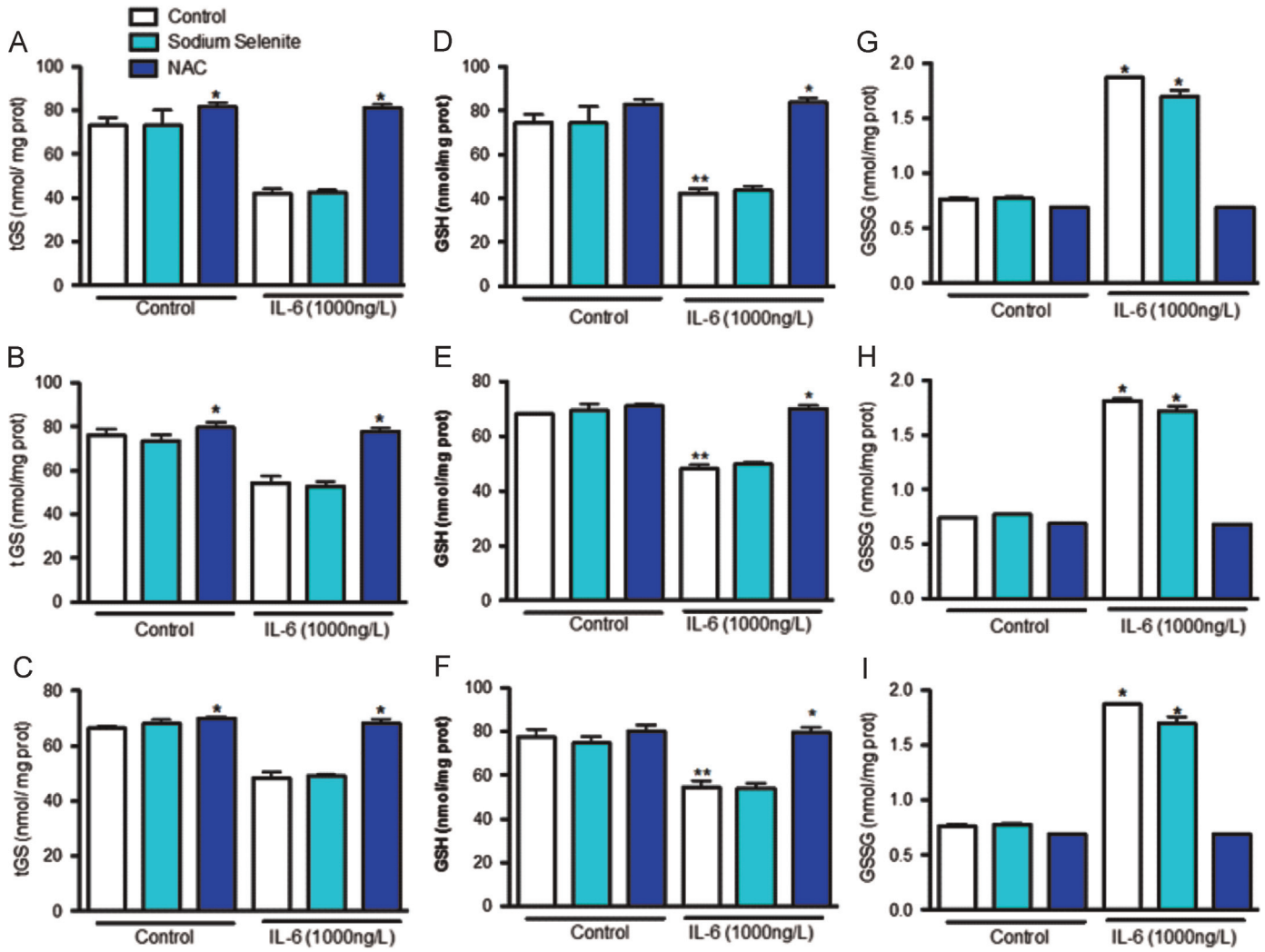


**Fig. 8.** Effect of sodium selenite or N-acetylcysteine (NAC) on total gluthatione (tGS), and GSH and GSSG in controls and IL6 (1000 ng/L) treated cells. The column on the left shows the effect of sodium selenite or NAC on intracellular tGS levels in controls and IL6 (1000 ng/L) treated MSTO-211 (A), HepG2 (B), or MCF-7 (C) cells. The column on the middle shows the effect of sodium selenite or NAC on intracellular GSH levels in MSTO-211 (D), HepG2 (E), or MCF-7 (F) cells. The column on the right shows the effect of sodium selenite or NAC on intracellular GSSG levels in MSTO-211 (D), HepG2 (E), or MCF-7 (F) cells. Data are mean ± SD of at least three independent experiments.

response element-binding protein (CREB), a known inducer of Dio1, Dio2 and Dio3 gene transcription [35,37]. Interestingly, CREB has been recently involved in the oxidative-stress response [38].

Other mechanisms besides depletion of a putative thiol co-factor(s) could be involved on ROS-induced inhibition of deiodinase activity. Upon oxidation, the cysteine residues within proteins can be modified to different products, including disulfide bonds and GSH-mixed disulfides [39]. However, the observed derangement of the deiodinase function is probably not due to protein damage itself. As shown here, notwithstanding the improvement on the protein related oxidative parameters by sodium selenite supplementation, it fails to prevent the inhibition on IL6 induced D2 or D1 activities. Although we cannot rule out other oxidative stress induced protein modifications that may lead to changes in deiodinase function, the fact that thiol DTT cofactor addition (in vitro assays) and NAC treatment fully restore enzyme function argue against an oxidative damage, post-translational modification or protein degradation [9].

Remarkably, despite the structural similarities among the deiodinases, their response to cellular oxidative imbalance appears to be rather different. Contrasting with the results obtained with D2 and D1, we observed a partial effect of sodium selenite on IL6-induced D3 activation (Fig. 5). Although we do not know how to explain these observations, we speculate that differences in cellular location may explain the different responses of D3 to diverse oxidative conditions. A still controversial extracellular location of the catalytic portion of the D3 enzyme [40–42] might have a role in favoring access to a less oxidative environment due to the partial correction of redox imbalance secondary to the sodium selenite supplementation. This hypothesis was previously investigated by evaluating the effects of IL6 induced decreases on intracellular GSH levels on transiently expressed D1, D2, or D3 [9]. Interestingly, IL6 decreases D3 activity in a dose dependent fashion. Of note, D3 activity, but not D2 or D1 function, was fully restored by the addition of GSH to the culture media, which might indicate that extracellular GSH are accessible only for the D3 molecule, overcoming the effects of an intracellular cofactor deficiency [9].

The observed difference in the antioxidant effects of sodium selenite and NAC on the oxidative stress induced changes on deiodinase function might be explained by their different mechanisms of action. While NAC is the major cysteine donator, not only to GSH but also to several thiol enzymes, sodium selenite acts through the GPx and TRx system and does not directly restore intracellular cysteine/thiol levels [8,12,13,39], which might be critical to proteins dependent on thiols [8]. Indeed, here we further

demonstrated that, contrasting with NAC, sodium selenite fails to prevent the IL6 induced decreases in intracellular tGS and GSH levels (Fig. 7A–F). Taken together, these results support the hypothesis that the inhibition of D2 and D1 functions in an oxidized environment is mainly due to the depleted amount of endogenous thiols consumed by ROS.

In conclusion, we demonstrate that sodium selenite partially corrects the oxidative stress generated by high concentrations of IL6 but, contrasting with NAC, it does not prevent the IL6-induced deiodinase dysfunction, which seems to depend on the restoration of intracellular cysteine levels. These observations add to our current understanding on NTIS pathogenesis and may explain the lack of correlation between selenium supplementation and thyroid hormone levels in critically ill patients [43].

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