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Down-regulation of the detoxifying enzyme NAD(P)H:quinone oxidoreductase 1 by vanadium in Hepa 1c1c7 cells

Anwar Anwar-Mohamed, Ayman O.S. El-Kadi *

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2N8

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ABSTRACT

Recent data suggest that vanadium (V^{5+}) compounds exert protective effects against chemical-induced carcinogenesis, mainly through modifying various xenobiotic metabolizing enzymes. In fact, we have shown that V^{5+} down-regulates the expression of Cyp1a1 at the transcriptional level through an ATP-dependent mechanism. However, incongruously, there is increasing evidence that V^{5+} is found in higher amounts in cancer cells and tissues than in normal cells or tissues. Therefore, the current study aims to address the possible effect of this metal on the regulation of expression of an enzyme that helps maintain endogenous antioxidants used to protect tissues/cells from mutagens, carcinogens, and oxidative stress damage, NAD(P)H:quinone oxidoreductase 1 (Nqo1). In an attempt to examine these effects, Hepa 1c1c7 cells and its AhR-deficient version, c12, were treated with increasing concentrations of V^{5+} in the presence of two distinct Nqo1 inducers, the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and isothiocyanate sulforaphane (SUL). Our results showed that V^{5+} inhibits the TCDD- and SUL-mediated induction of Nqo1 at mRNA, protein, and catalytic activity levels. At transcriptional level, V^{5+} was able to decrease the TCDD- and SUL-induced nuclear accumulation of Nrf2 and the subsequent binding to antioxidant responsive element (ARE) without affecting Nrf2 protein levels. Looking at post-transcriptional level; we found that V^{5+} did not affect Nqo1 mRNA transcripts turn-over rates. However, at the post-translational level V^{5+} increased Nqo1 protein half-life. In conclusion, the present study demonstrates that V^{5+} down-regulates Nqo1 at the transcriptional level, possibly through inhibiting the ATP-dependent activation of Nrf2.

Introduction

The NAD(P)H:quinone oxidoreductase 1 (NQO1) is a cytosolic flavoenzyme that catalyzes the two-electron reduction of a broad range of substrates (Korashy and El-Kadi, 2006). NQO1 plays a pivotal role in detoxifying quinones to their corresponding hydroquinone derivatives (Lind et al., 1982). Such an effect helps in maintaining endogenous antioxidants like ubiquinone and vitamin E in their reduced and active forms, thus protecting tissues from mutagens, carcinogens, and oxidative stress damage (Ross, 2004).

NQO1 gene expression can be induced through two separate regulatory elements associated with its 5′-flanking region. The first pathway includes activation of a cytosolic transcription factor, the aryl hydrocarbon receptor (AhR). The inactive form of AhR is attached to a complex of two heat shock proteins 90 (HSP90), hepatitis B virus X-associated protein (XAP2), and the chaperone protein p23 (Hankinson, 1995; Meyer et al., 1998). Upon ligand binding, the AhR–ligand complex dissociates from the cytoplasmic complex and translocates to the nucleus where it associates with the aryl hydrocarbon nuclear translocator (Arnt) (Whitelaw et al., 1994). The whole complex then acts as a transcription factor to mediate the induction of *NQO1* through activating the xenobiotic responsive element (XRE) located in its promoter region (Nebert et al., 2004). The second pathway involves activation of the antioxidant responsive element (ARE). In fact, the increased expression of *NQO1* gene expression in response to oxidative stress caused by agents such as isothiocyanate sulforaphane (SUL), *tert*-butylhydroquinone (t-BHQ) and H_2O_2 occurs primarily through this signaling pathway (Itoh et al., 1997). Perturbation in the redox status of the cell activates the nuclear factor erythroid 2-related factor-2 (Nrf2), a redox-sensitive member of the cap 'n' collar basic leucine zipper (CNC bZip) family of transcription factors (Itoh et al., 1997). Subsequently, Nrf2 dissociates from its cytoplasmic tethering polypeptide, Kelch-like ECH associating protein 1 (Keap1), and then

Abbreviations: Act-D, actinomycin D; AhR, aryl hydrocarbon receptor; ARE, antioxidant responsive element; c12, AhR-deficient Hepa 1c1c7 cells; CHX, cycloheximide; DMSO, dimethylsulfoxide; EMSA, Electrophoretic mobility shift assay; Hepa 1c1c7, murine hepatoma Hepa 1c1c7; Keap1, Kelch-like ECH associating protein 1; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor-2; PMSF, phenylmethylsulphonyl fluoride; SUL, isothiocyanate sulforaphane; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; V^{5+}, vanadium, XRE, xenobiotic responsive element.

* Corresponding author. Faculty of Pharmacy and Pharmaceutical Sciences, 3126 Dentistry/Pharmacy Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2N8. Fax: +1 780 492 1217.
E-mail address: aelkadi@pharmacy.ualberta.ca (A.O.S. El-Kadi).

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translocates into the nucleus, dimerizes with a musculoaponeurotic fibrosarcoma (MAF) protein, thereafter binds to and activate ARE (Ma et al., 2004).

The XRE- and ARE-driven regulation of *NQO1* gene was generally thought to function independently. However, the proximity of the two sequence sites suggests a possible cross-talk and functional overlap (Miao et al., 2005; Kohle and Bock, 2006). Recent reports suggest that bifunctional inducers, which activate both XRE and ARE signaling pathways, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), require direct cross-talk between the XRE- and ARE-mediated pathways for the induction of *NQO1* (Kohle and Bock, 2006). Furthermore, it has been reported that the induction of *NQO1* by ARE inducers requires the presence of AhR, suggesting a more direct cross-talk between the XRE- and ARE-mediated pathways (Ma et al., 2004; Marchand et al., 2004; Miao et al., 2005). Intriguingly, recent studies have shown that mouse Nrf2 is under the control of AhR, as evident by increased Nrf2 mRNA transcripts by AhR ligands (Miao et al., 2005). Another study have demonstrated that the expression of AhR, Cyp1a1, and Cyp2b1 is partially dependent on Nrf2, implying that Nrf2 modulates both transcription of AhR and its downstream targets (Shin et al., 2007). This was further supported by the reduction of AhR mRNA levels in Nrf2 knockout mice cells compared to wild type (Shin et al., 2007). Furthermore, the AhR mRNA levels were increased in Keap1 knockout mice cells, suggesting more direct role of Nrf2 in the regulation of AhR (Shin et al., 2007).

Conglomerates of studies have examined the toxic effects of individual AhR ligands, yet there have been very few studies on the combined toxic effects of AhR ligands and other environmental co-contaminants. Among these, environmental co-contaminants of most concern are heavy metals, typified by vanadium (V^{5+}). Humans and other species may be exposed to V^{5+} through the atmosphere, food, and water. Foods contain V^{5+} in the concentration of 10–60 μg (Evangelou, 2002). Samples from the drinking water supplies in the U.S.A. showed that the concentration of V^{5+} can reach up to 10 μg V^{5+}/L (Craun et al., 1981). Furthermore, weight training athletes are reported to use up to 18.6 mg V^{5+} per day (Barceloux, 1999).

Recently, we have demonstrated that V^{5+} down-regulates the bioactivating enzyme Cyp1a1 which is solely under the control of XRE, through a transcriptional mechanism (Anwar-Mohamed and El-Kadi, 2008). In addition, the translocation of the transformed AhR was inhibited by V^{5+} probably through an ATP-dependent mechanism. Therefore, the current study aims to address the possible effect of this metal on the *Nqo1* gene expression in Hepa 1c1c7 cells.

Materials and methods

Materials. Ammonium metavanadate (NH_4VO_3), cycloheximide (CHX), 2,6-dichlorophenolindophenol, fluoroscamine, anti-goat IgG peroxidase secondary antibody, protease inhibitor cocktail, dicoumarol, and isothiocyanate sulforaphane were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent was purchased from Invitrogen (Grand Island, NY). High-Capacity cDNA Reverse Transcription Kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Actinomycin-D (Act-D) was purchased from Calbiochem (San Diego, CA). Chemiluminescence Western blotting detection reagents were from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). NAD(P)H:quinone oxidoreductase 1 (Nqo1) rabbit polyclonal primary antibody was generously provided by Dr. David Ross (University of Colorado, Denver, CO). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) primary antibody, anti-goat and anti-rabbit IgG peroxidase secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Nuclear factor erythroid 2-related factor-2 (Nrf2) primary antibody, and anti-mouse IgG peroxidase secondary antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). [γ^{32}P]ATP was supplied by the DNA Core Services Laboratory, University of Alberta. All other chemicals were purchased from Fisher Scientific (Toronto, ON).

Cell culture. Hepa 1c1c7 cell lines, ATCC number CRL-2026, or mutant AhR-deficient Hepa 1c1c7 (c12) cell lines, ATCC number CRL-2710 (Manassas, VA), were maintained in Dulbecco's modified Eagle's medium (DMEM), without phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 50 μg/ml amikacin, 100 IU/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37 °C in a 5% CO_2 humidified incubator.

Chemical treatments. Cells were treated in serum free medium with various concentrations of V^{5+} (25–250 μM) in the presence of 1 nM TCDD or 5 μM SUL. TCDD and SUL were dissolved in dimethylsulfoxide (DMSO) and maintained in DMSO at −20 °C until use. V^{5+} was prepared freshly in double de-ionized water. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

RNA extraction and quantitative real-time PCR. After incubation with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen Co.), and quantified by measuring the absorbance at 260 nm. For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 1.0 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. Real-time PCR reactions were performed on an ABI 7500 real time PCR system (Applied Biosystems), using SYBR® Green PCR Master Mix (Applied Biosystems). The amplification reactions were performed as follows: 10 min at 95 °C, and 40 cycles of 94 °C for 15 s and 60 °C for 1 min. The primers used in the current study were chosen from previously published studies (Nioi et al., 2003; Williamson et al., 2005; Dong et al., 2008; Ma et al., 2007) and are listed in Table 1. The primers were purchased from Integrated DNA technologies (IDT, Coralville, IA). The fold change in the level of Nqo1 (target gene) between treated and untreated cells, corrected by the level of β-actin, was determined using the following equation: fold change $= 2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

Protein extraction and Western blot analysis. After incubation with the test compounds for the indicated time points, cells were washed thoroughly twice with PBS containing EDTA, thereafter collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μl/ml of protease inhibitor cocktail. The cell homogenates were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 ×*g* for 10 min at 4 °C. Proteins from Hepa 1c1c7 cells (25 μg), or c12 (100 μg) were resolved by denaturing electrophoresis, as described previously (Elbekai and El-Kadi, 2004). Briefly, the cell homogenates were

Table 1
Primers sequences used for real-time PCR reactions

Gene	Forward primer	Reverse primer
AhR	*CGGCTTCTTGCAAAACACAGT*	*GTAAATGCTCTCGTCCTTCTTCATC*
β-actin	*TATTGGCAACGAGCGGTTCC*	*GGCATAGAGGTCTTTACGGATGTC*
Nqo1	*GCAGGATTTGCCTACACAATATGC*	*AGTGGTGATAGAAAGCAAGGTCTTC*
Nrf2	*CGAGATATACGCAGGAGAGGTAAGA*	*GCTCGACAATGTTCTCCAGCTT*

dissolved in 1× sample buffer, boiled for 5 min, separated by 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked for 24 h at 4 °C in blocking buffer containing 5% skim milk powder, 2% bovine serum albumin and 0.05% (v/v) Tween-20 in Tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris–base). After blocking, the blots were incubated with a primary polyclonal goat anti-mouse Nqo1 antibody for 2 h at room temperature, or primary polyclonal goat anti-mouse Gapdh antibody for 24 h at 4 °C in TBS containing 0.05% (v/v) Tween-20 and 0.02% sodium azide. Incubation with a peroxidase-conjugated goat anti-rabbit IgG for Nqo1, and rabbit anti-goat IgG secondary antibody for Gapdh was carried out in blocking buffer for 2 h at room temperature. The bands were visualized with the enhanced chemiluminescence method according to manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ). The intensity of Nqo1 protein bands were quantified, relative to the signals obtained for Gapdh protein, using ImageJ software.

Determination of Nqo1 enzymatic activity. After incubation with the test compounds for the indicated time points, cells were washed thoroughly twice with PBS containing EDTA, thereafter collected in 500 µl homogenate buffer containing 25 mM Tris–HCl pH 7.4, 250 mM sucrose, and 5 µM FAD. The cells were then homogenized for 1 min and then sonicated for 5 s on ice, thereafter the cell homogenates were centrifuged at 12,000 ×*g* for 15 min, and the supernatant was transferred to new microcentrifuge tubes which were kept in −80 freezer till further use. The Nqo1 activity was determined by the continuous spectrophotometric assay to quantitate the reduction of its substrate, 2,6-dichlorophenolindophenol (DCPIP) as described previously (Korashy and El-Kadi, 2006; Preusch et al., 1991). Briefly, 20 µg of cell homogenate protein was incubated with 1 ml of the assay buffer [40 µM DCPIP, 0.2 mM NADPH, 25 mM Tris–HCl, pH 7.8, 0.1% (v/v) Tween 20, and 0.7 mg/ml bovine serum albumin, 0 or 30 µM dicoumarol]. The rate of DCPIP reduction was monitored over 90 s at 600 nm with an extinction coefficient (ε) of 2.1 mM^{-1} cm^{-1}. The Nqo1 activity was calculated as the decrease in absorbance per min per mg of total protein of the sample which quantitates the dicoumarol-inhibitable reduction of DCPIP.

Preparation of nuclear extracts. Nuclear extracts from Hepa 1c1c7 cells were prepared according to a previously described procedure (Andrews and Faller, 1991) with slight modifications. Briefly, Hepa 1c1c7 cells grown on 100-mm petri dishes were treated for 4 h with vehicle, 1 nM TCDD, or 5 µM SUL in the presence and absence of 50 µM V^{5+}. Thereafter, cells were washed twice with cold PBS, pelleted, and suspended in cold buffer A [10 mM Hepes–KOH, 1.5 mm $MgCl_2$, 10 mM KC1, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulphonyl fluoride (PMSF)] pH 7.9, at 4 °C. After 10 min on ice, the cells were centrifuged at 12,000 ×*g* and the pellets were suspended again in high salt concentration cold buffer C (20 mM Hepes–KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF) to extract nuclear proteins. The cells were then incubated on ice with vigorous agitation every 5 min for 30 min followed by centrifugation for 15 min at 14,000 ×*g* at 4 °C. The nuclear extracts (supernatant) were stored at −80 °C till further use.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts protein concentrations were determined using the method of Lowry (Lowry et al., 1951). To visualize the ability of V^{5+} to alter the DNA binding of the Nrf2 to ARE, a complementary pair of synthetic oligonucleotides containing the sequence 5′-GAT CTG GCT CTT CTC ACG CAA CTC CG-3′ and 5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′, corresponding to the mouse Nqo1 ARE binding site, were synthesized and radiolabeled with [γ^{32}P]ATP as previously described (Nioi et al., 2003). Binding reactions using aliquots of 7.5 µg nuclear extracts and excess radiolabeled oligonucleotides were allowed to proceed for 20 min at 20 °C in a buffer containing 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 25 mM HEPES, 3 µg poly(dI–dC), and 0.4 mM KCl. To determine the specificity of binding to the oligonucleotide, a 100-fold M excess of unlabeled ARE probe was added to the binding reaction prior to addition of the γ^{32}P-labeled probe. Protein–DNA complexes were separated under non-denaturing conditions on a 4% polyacrylamide gel using 0.5×TBE (90 mM of Tris borate, 90 mM of boric acid, 4 mM of EDTA) as a running buffer. The gels were dried and the protein–DNA complexes were visualized by autoradiography after 6 h exposure.

Nqo1 mRNA stability. The half-life of Nqo1 mRNA was analyzed by an Act-D-chase assay. Cells were pre-treated with 1 nM TCDD for 12 h. Cells were then washed and incubated with 5 µg/ml Act-D, to inhibit further RNA synthesis, immediately before treatment with V^{5+} (50 µM). Total RNA was extracted at 0, 6, 12, and 24 h after incubation with the metal. Real-time PCR reactions were performed using SYBR® Green PCR Master Mix (Applied Biosystems). The fold change in the level of Nqo1 (target gene) between treated and untreated cells, corrected by the level of β-actin, was determined using the following equation: fold change $= 2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$. The mRNA half-life values were determined from semilog plots of fold-change versus time.

Nqo1 protein stability. The half-life of Nqo1 protein was analyzed by the CHX-chase assay. Cells were pre-treated with 1 nM TCDD for 24 h. Cells were then washed and incubated with 10 µg/ml CHX, to inhibit further protein synthesis, immediately before treatment with V^{5+} (50 µM). Cell homogenates were extracted at 0, 12, 24, 36 and 48 h after incubation with the metal. Thereafter, Nqo1 protein was determined by Western blot analysis. The intensity of Nqo1 protein bands was quantified, relative to the signals obtained for Gapdh protein, using the ImageJ software. The protein half-life values were determined from semilog plots of integrated densities versus time.

Statistical analysis. The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows (Systat Software, Inc, CA). A one-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test was carried out to assess statistical significance. The differences were considered significant when $P<0.05$.

Results

Concentration-dependent effect of V^{5+} on TCDD-mediated induction of Nqo1 mRNA

To examine the ability of V^{5+} to modulate *Nqo1* gene expression, Hepa 1c1c7 cells were treated with various concentrations of V^{5+} in the presence of 1 nM TCDD (Fig. 1A). Thereafter, Nqo1 mRNA was assessed using real-time PCR. The concentrations of V^{5+} used hereafter were chosen after determining the ability of wide range of concentrations to modulate the *Nqo1* gene expression without significantly affecting cell viability (Anwar-Mohamed and El-Kadi, 2008). Initially, TCDD alone caused 470% increase in Nqo1 mRNA levels that was inhibited in a dose-dependent manner by V^{5+}, starting at the lowest concentration tested which is 25 µM (45%), and reaching the maximum inhibition at the concentration of 250 µM (80%) (Fig. 1A).

Concentration-dependent effect of V^{5+} on TCDD-mediated induction of Nqo1 protein and catalytic activity

To examine whether the observed inhibitory effect of V^{5+} on the Nqo1 mRNA is reflected at the protein and catalytic activity levels,

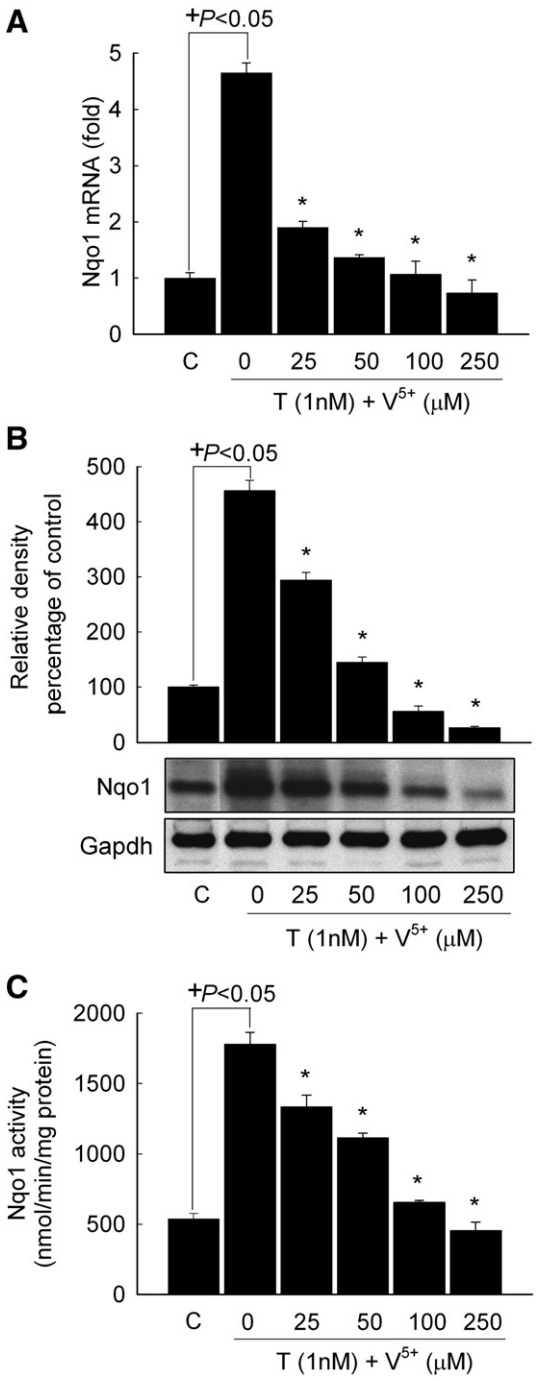

Fig. 1. Concentration-dependent effect of V^{5+} on TCDD-mediated induction of Nqo1 at mRNA, protein, and catalytic activity in Hepa 1c1c7 cells. Hepa 1c1c7 cells were treated with increasing concentrations of V^{5+} in the presence of 1 nM TCDD for 6 h for mRNA or 24 h for protein and catalytic activity. A, First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa1c1c7 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P<0.05$, compared to control (C) (concentration = 0 μM); (*) $P<0.05$, compared to respective TCDD (T) treatment. B, Protein (25 μg) was separated on a 10% SDS-PAGE. Nqo1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to Gapdh signals, which was used as loading control. One of three representative experiments is shown. C, Nqo1 enzyme activity was determined spectrophotometrically using DCPIP as substrate. Values are presented as mean ± SE ($n=6$). (+) $P<0.05$, compared to control (C); (*) $P<0.05$, compared to respective TCDD (T) treatment.

Hepa 1c1c7 cells were treated for 24 h with increasing concentrations of V^{5+} in the presence of 1 nM TCDD. Figs. 1B and C show that TCDD alone caused 450% and 350% increase in Nqo1 protein and catalytic activity, respectively. On the other hand, V^{5+} significantly reduced the TCDD-mediated induction of Nqo1 at protein and activity levels in a dose-dependent manner (Figs. 1B and C). This inhibition pattern was consistent with that observed at the mRNA levels, in which the initial significant inhibition took place with the lowest concentration tested, 25 μM V^{5+}. V^{5+} at the concentration of 25 μM inhibited the TCDD-mediated induction of Nqo1 protein and catalytic activity levels by 35% and 20%, respectively. On the other hand, the maximal inhibition took place with the highest concentration tested, 250 μM V^{5+}, in which the TCDD-mediated induction of Nqo1 protein and catalytic activity levels were inhibited by 90% and 70% in comparison to the TCDD-induced Nqo1 protein and catalytic activity levels (Figs. 1B and C).

Effect of co-exposure to V^{5+} and SUL on Nqo1 mRNA, protein, and catalytic activity in Hepa 1c1c7 cells

It is well established that TCDD-mediated induction of Nqo1 occurs through the AhR and Nrf2 signaling pathways. The fact that V^{5+} inhibited TCDD-mediated induction of *Nqo1* gene expression raised the question whether V^{5+} will behave similarly in the presence of SUL which induces *Nqo1* gene expression through the Nrf2 pathway only (Itoh et al., 1997; Venugopal and Jaiswal, 1996). For this purpose, Hepa 1c1c7 cells were treated with 5 μM SUL in the presence and absence of 50 μM V^{5+}. If V^{5+} exerts its effect solely through the AhR pathway; we expect to see no effect of V^{5+} on SUL-mediated induction of Nqo1.

Fig. 2A shows that SUL alone significantly increased Nqo1 mRNA levels by 400% in comparison to control. Interestingly, V^{5+} was able to inhibit this induction by 50% compared to SUL alone. Furthermore, this inhibition was further translated to the protein and catalytic activity levels, in which SUL alone showed significant induction of Nqo1 protein and catalytic activity levels by 300% (Figs. 2B and C). On the other hand the co-exposure to V^{5+} and SUL significantly decreased the SUL-induced Nqo1 protein and catalytic activity levels by more than 50%, in comparison to SUL alone (Figs. 2B and C).

Effect of co-exposure to V^{5+} and SUL on Nqo1 mRNA, protein, and catalytic activity in AhR-deficient, Hepa 1c1c7 (c12) cells

We took a genetic approach to examine the role of V^{5+} in decreasing *Nqo1* gene expression through the Nrf2 signaling pathway. For this purpose we used the AhR-deficient, Hepa 1c1c7 (c12) cells.

Our results show that SUL (5 μM) significantly induced Nqo1 mRNA, protein and catalytic activity levels by 600%, 450%- and 370%, respectively (Figs. 2A–C). In contrast, V^{5+} alone was able to cause a significant induction of Nqo1 mRNA levels, yet this induction was not adequate to produce a significant induction in the protein or catalytic activity levels. Interestingly, V^{5+} was able to inhibit SUL-mediated induction of Nqo1 at the mRNA, protein and catalytic activity levels by 40%, 50%, and 55%, respectively compared to SUL alone (Figs. 2A–C).

Effect of V^{5+}on the mRNA levels of AhR, and Nrf2

In the current study we showed that V^{5+} decreased TCDD- and SUL-mediated induction of Nqo1 mRNA at the transcriptional level. Nqo1 is regulated by both AhR and Nrf2. Therefore, it was of interest to examine the effect of V^{5+} on the transcription of AhR and Nrf2. Our results demonstrated that V^{5+} did not affect the gene expression of AhR and Nrf2 (Fig. 3). Thus, V^{5+} mediated inhibition of Nqo1 gene expression is not occurring through the inhibition of AhR or Nrf2 transcription.

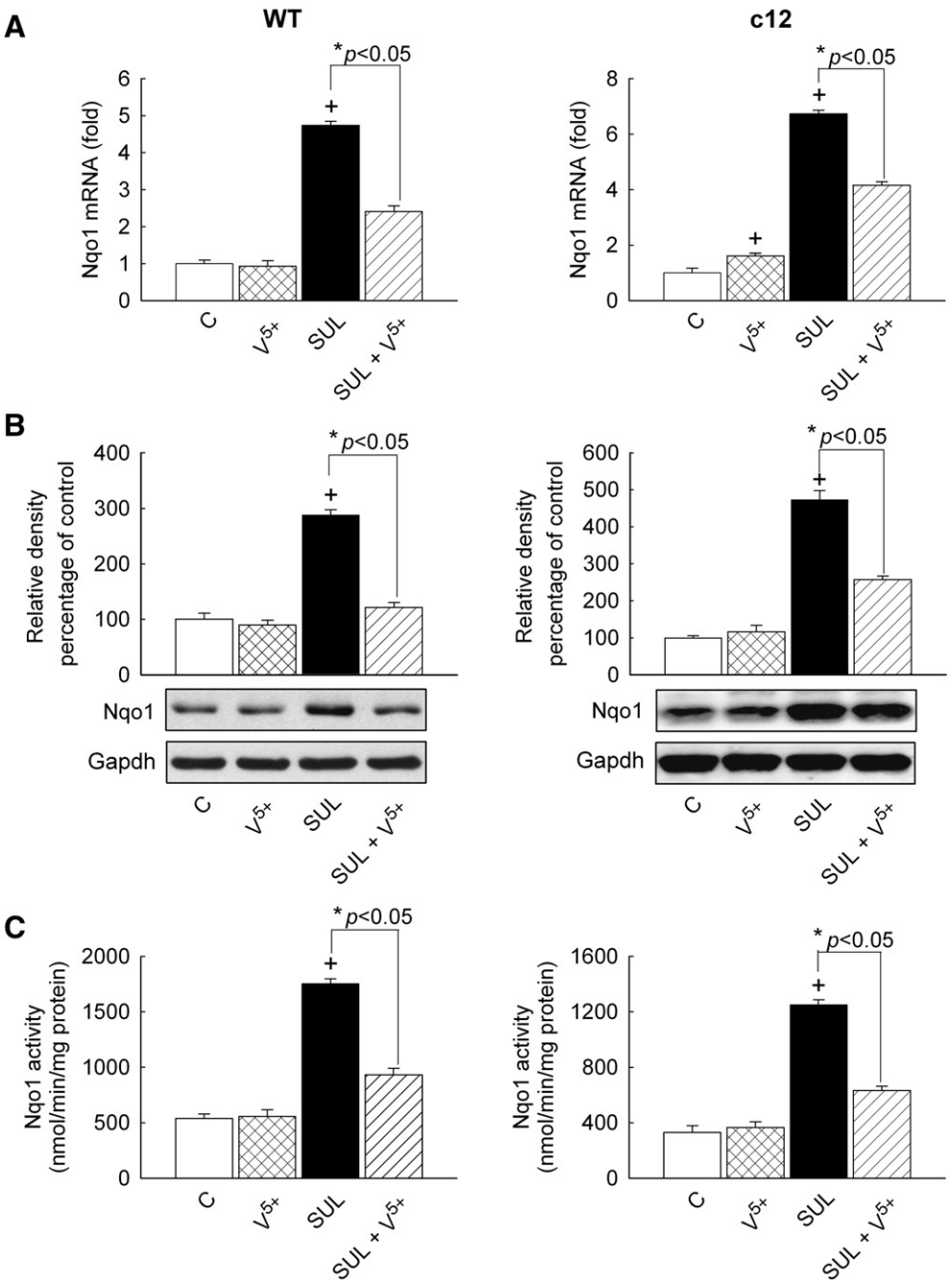

Fig. 2. Effect of co-exposure to V^{5+} and SUL on Nqo1 at mRNA, protein, and catalytic activity in Hepa 1c1c7 cells, and AhR-deficient Hepa 1c1c7 cells (c12). Hepa 1c1c7 or c12 cells were treated with 50 μM V^{5+} in the absence and presence of 5 μM SUL for 6 h for mRNA or 24 h for protein and catalytic activity. A, First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa 1c1c7 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. (+) $P<0.05$, compared to control (C) (concentration = 0 μM); (*) $P<0.05$, compared to respective sulforaphane (SUL) treatment. B, Proteins from Hepa 1c1c7 cells (25 μg), or c12 cells (100 μg) were separated on a 10% SDS-PAGE. Nqo1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to Gapdh signals, which was used as loading control. One of three representative experiments is shown. C, Nqo1 enzyme activity was determined spectrophotometrically using DCPIP as substrate, and dicoumarol as specific Nqo1 inhibitor. Values are presented as mean ± SE ($n = 6$). (+) $P<0.05$, compared to control (C); (*) $P<0.05$, compared to respective sulforaphane (SUL) treatment.

Transcriptional inhibition of Nqo1 gene induction by V^{5+}

Our previous data have shown that V^{5+} completely abolished the TCDD-induced nuclear formation of AhR/Arnt/XRE complex (Anwar-Mohamed and El-Kadi, 2008). Therefore, it was of great importance to examine whether or not V^{5+} exerts similar effect with respect to the Nrf2/ARE binding complex. In an effort to determine whether V^{5+} interferes with the nuclear binding of Nrf2 to the ARE, we examined the potential effect of V^{5+} on SUL- and TCDD-induced translocation of Nrf2 to the nucleus and the subsequent binding to ARE by EMSA. For this purpose, Hepa 1c1c7 cells were treated with vehicle, V^{5+}, SUL, SUL plus V^{5+}, TCDD and TCDD plus V^{5+} for 4 h, followed by extraction of nuclear extracts. Fig. 4A shows that SUL and TCDD significantly increased the Nrf2/ARE binding, which reflects an increase in the nuclear accumulation of Nrf2 protein. Despite increasing Nrf2 nuclear accumulation, there was a noticeable difference in the magnitude of Nrf2/ARE complex formation between TCDD and SUL as noted by their representative bands, the latter being more potent. Interestingly, V^{5+} was able to inhibit the TCDD- and SUL-mediated induction of nuclear accumulation of Nrf2 and its subsequent binding to the ARE

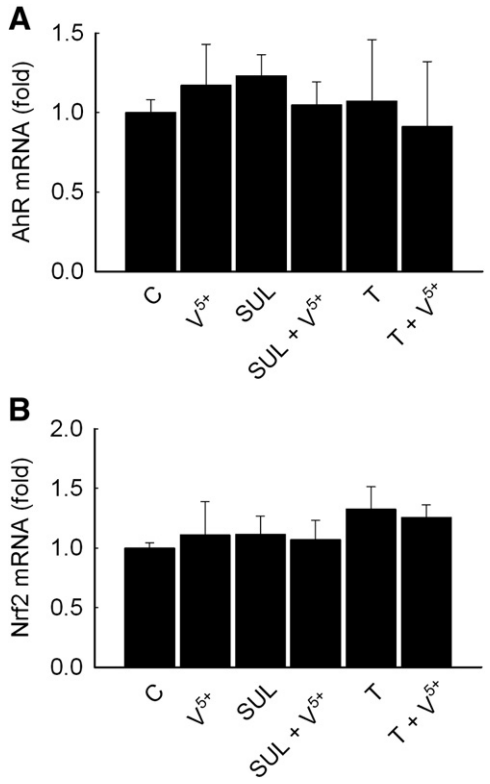

Fig. 3. Effect of V^{5+} on the gene expression AhR (A), and Nrf2 (B). Hepa 1c1c7 cells were treated for 6 h with vehicle, V^{5+} (50 μM), SUL (5 μM), SUL (5 μM) plus V^{5+} (50 μM), TCDD (1 nM), or TCDD (1 nM) + V^{5+} (50 μM). First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa 1c1c7 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments.

(lanes 4 and 6). The specificity of the Nrf2/ARE band was confirmed by competition assay using 100-fold M excess of unlabeled ARE (lane 7). Collectively, our data indicate that V^{5+} inhibited the translocation of Nrf2 and/or its subsequent binding to ARE.

Effect of V^{5+} on Nrf2 nuclear accumulation

In order to examine whether the observed effect of V^{5+} on the Nrf2/ARE binding is actually due to decreasing the nuclear accumulation of Nrf2, we examined the effect of V^{5+} on the constitutive, SUL-induced, and TCDD-induced nuclear accumulation of Nrf2 using Western blot analysis. Our results showed that V^{5+} alone did not affect the nuclear accumulation of Nrf2. On the other hand, SUL and TCDD increased the nuclear accumulation of Nrf2 (Fig. 4B). Interestingly, we found that V^{5+} was able to inhibit the SUL- and TCDD-induced nuclear accumulation of Nrf2, suggesting that V^{5+} inhibits the nuclear accumulation through either decreasing the Nrf2 protein level or inhibiting its nuclear translocation.

Time-dependent effect of V^{5+} on total Nrf2 protein level

In an attempt to investigate whether V^{5+} inhibited Nrf2 accumulation via increasing its degradation we measured the Nrf2 level in the total cell lysate of Hepa 1c1c7 cells treated with V^{5+} for different time points. Our results demonstrated that V^{5+} did not affect the short-lived Nrf2 protein at all time points tested (Fig. 4C).

Post-transcriptional modification of Nqo1 mRNA by V^{5+}

The level of mRNA expression is not only a function of the transcription rate, but is also dependent on the elimination rate, through processing or degradation. Therefore, we examined the effect of V^{5+} on the stability of TCDD-induced Nqo1 mRNA transcripts, using Act-D chase experiment. If V^{5+} alters Nqo1 mRNA stability, a decrease in half-life would be expected to take place. Fig. 5 shows that TCDD-induced Nqo1 mRNA decayed with a half-life of 20.38 ± 0.68 h. Furthermore, V^{5+} did not alter the half-life of TCDD-induced Nqo1 mRNA significantly as it reached 21.65 ± 0.72 h, indicating that the

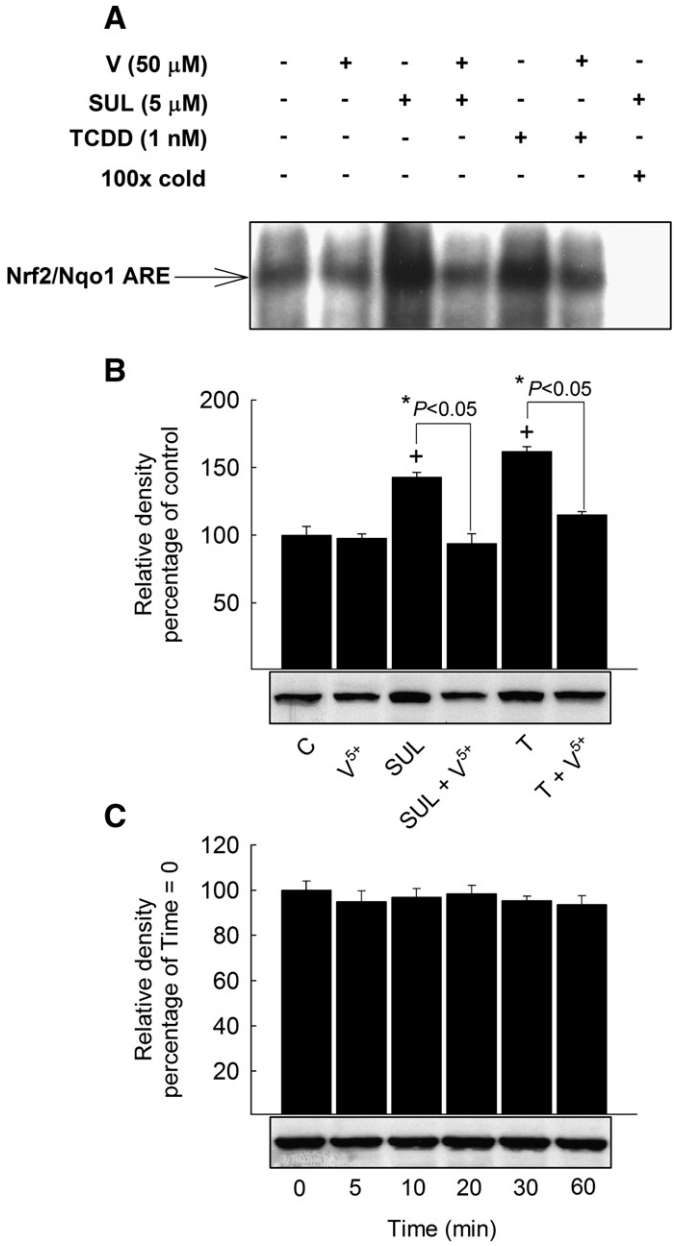

Fig. 4. Effect of V^{5+} on: Nrf2/ARE binding (A), nuclear accumulation of Nrf2 protein (B), and total Nrf2 protein level (C). Hepa 1c1c7 cells were treated for 4 h with vehicle, V^{5+} (50 μM), SUL (5 μM), SUL (5 μM) plus V^{5+} (50 μM), TCDD (1 nM), or TCDD (1 nM) + V^{5+} (50 μM). A, The nuclear proteins (7.5 μg) were mixed with [γ^{32}P]-labeled ARE, and the formation of Nrf2/ARE complexes was analyzed by EMSA. The specificity of binding was determined by incubating the protein treated with SUL with 100-fold molar excess of cold ARE. The arrow indicates the specific shift representing the Nrf2/ARE complex. This pattern of Nrf2 alteration was observed in three separate experiments, and only one is shown. B, Nuclear proteins (50 μg) were separated on a 10% SDS-PAGE. Nrf2 protein was detected using the enhanced chemiluminescence method. One of three representative experiments is shown. C, Total cell lysate protein (25 μg) was separated on a 10% SDS-PAGE. Nrf2 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to Gapdh signals (not shown), which was used as loading control. One of three representative experiments is shown. (+) $P<0.05$, compared to control (C); (*) $P<0.05$, compared to respective TCDD (T) or sulforaphane (SUL) treatment.

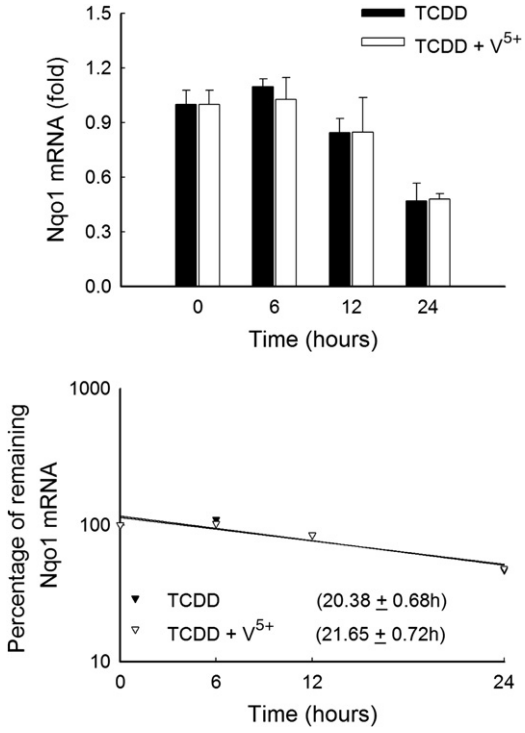

Fig. 5. Effect of V^{5+} on Nqo1 mRNA half-life. Hepa 1c1c7 cells were grown to 90% confluence in six-well cell culture plates, and then treated with 1 nM TCDD for 12 h. The cells were then washed and incubated in a fresh media containing 50 μM V^{5+} plus 5 μg/ml Act-D, a RNA synthesis inhibitor. First-strand cDNA was synthesized from total RNA (1 μg) extracted from Hepa1c1c7 cells. cDNA fragments were amplified and quantitated using ABI 7500 real-time PCR system as described under Materials and methods. Duplicate reactions were performed for each experiment, and the values presented are the means of three independent experiments. mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2>0.85$) to a semilog plot of mRNA amount, expressed as a percent of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SE, $n=3$). *$p<0.05$ compared with TCDD.

decrease of Nqo1 mRNA transcripts in response to V^{5+} was not due to any post-transcriptional decrease in the mRNA stability. These results confirm the transcriptional inhibition of *Nqo1* gene expression by V^{5+}.

Post-translational modification of Nqo1 protein by V^{5+}

The fact that V^{5+} inhibited TCDD-mediated induction of Nqo1 protein raised the question whether V^{5+} could modify the Nqo1 protein stability. Therefore, the effect of V^{5+} on the TCDD-induced Nqo1 protein half-life was determined using CHX-chase experiments. Fig. 6 shows that Nqo1 protein induced by TCDD degraded with a half-life of 30.44 + 1.82 h. Interestingly, V^{5+} increased the Nqo1 protein half life to 37.17 ± 2.14 h.

Discussion

Metallic vanadium does not occur in nature, however, it does occur in over 65 known naturally occurring mineral salts, and is the major metal in fossil fuels (Tsiani and Fantus, 1997). During Gulf War II (Desert storm) oil slicks in some Gulf States polluted the water and coastlines of these countries killing thousands of sea birds (Small, 1991). Moreover, during this war more than 700 oil wells were burnt in Kuwait, forming a very dense black cloud that covered some Gulf States (Browning, 1991). The burning wells emitted oxides of carbon, nitrogen, sulfur, and hydrocarbon particulates (Browning, 1991). Interestingly, heavy metals such as V^{5+} which is present in Kuwait crude oil in the order of 10–30 mg/kg, was also observed (Browning, 1991; Bakan, 1991). A study conducted soon after this war in Bahrain reported that the concentrations of polycyclic aromatic hydrocarbons (PAH) and V^{5+} were 5.3 and 26 ng/m^3, respectively (Madany and Raveendran, 1992).

Mounting evidence now support the role of NQO1 in protecting against toxic and neoplastic effects of pro-oxidant chemicals. It is believed that NQO1 achieves that through three different mechanisms. The first being its direct catalytic action and this occurs if the chemical insult happens to be a quinone. The second mechanism would be its indirect antioxidant effect. Thirdly, it stabilizes p53 protein which serves primarily as a transcriptional factor, and plays important role in preserving genomic integrity, or the elimination of damaged or tumorigenic cells (Talalay and Dinkova-Kostova, 2004).

Recent data suggest that V^{5+} compounds exert protective effects against chemical-induced carcinogenesis, mainly through modifying various xenobiotic metabolizing enzymes (Evangelou, 2002). Yet, it has been seen that high V^{5+} concentrations are found in tissue samples of actual tumors as compared to those in normal tissues (Evangelou, 2002). These observations would be consistent with our study in which V^{5+} was shown to cause decreases in Nqo1 activity that, in turn, might reduce its normal protective effect against mutagens/carcinogens. Thus, V^{5+} might actually act to promote carcinogenic outcomes in cells rather than protect them against certain forms of chemical-induced carcinogenesis. Interestingly, data from our laboratory and others showed that heavy metals other than V^{5+} are capable of modifying Nqo1 through different stages of its regulatory pathway.

In the current study we hypothesize that V^{5+} down-regulates the inducible *Nqo1* gene expression through inhibiting both the AhR and Nrf2 signaling pathways. Hence the main objective of the current study was to determine the potential effect of co-exposure to V^{5+} and

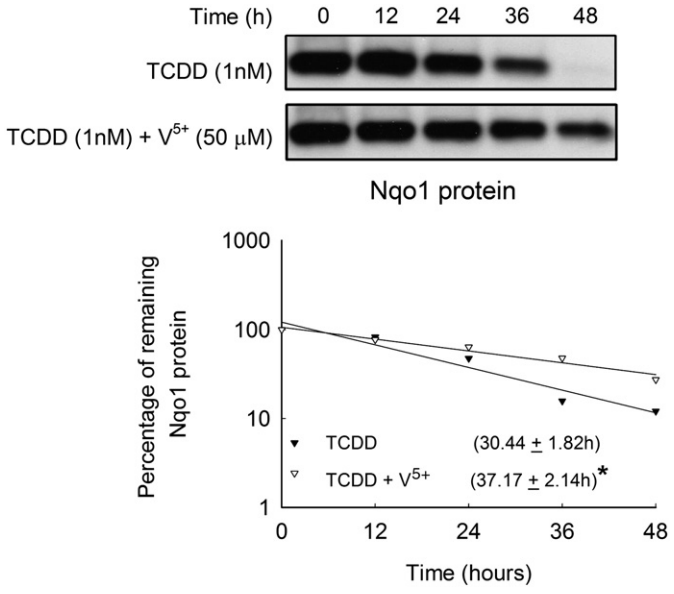

Fig. 6. Effect of V^{5+} on the Nqo1 protein half-life. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates, and then treated with 1 nM TCDD for 24 h. Thereafter, the cells were washed and incubated in fresh media containing 50 μM V^{5+} plus 10 μg/ml CHX, a protein translation inhibitor. Nqo1 protein was extracted at the designated time points after the addition of CHX. Protein (25 μg) was separated on a 10% SDS-PAGE. Nqo1 protein was detected using the enhanced chemiluminescence method. The intensity of Nqo1 protein bands were normalized to Gapdh signals, which was used as loading control (data not shown). All protein decay curves were analyzed individually. The half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2>0.85$) to a semilog plot of protein amount, expressed as a percent of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SE, $n=3$). *$p<0.05$ compared with TCDD.

TCDD or SUL, as bifunctional and monofunctional inducers, respectively, on *Nqo1* gene expression. Furthermore, we investigated the molecular mechanisms by which V^{5+} modulates the expression of *Nqo1* using wild type and AhR-deficient cells.

Initially we tested the ability of V^{5+} to modulate the expression of Nqo1 in Hepa 1c1c7 cells. Our results clearly demonstrated that V^{5+} significantly inhibited the TCDD- and SUL-mediated induction of Nqo1 at mRNA, protein, and activity levels in both wild type and AhR-deficient cells. These results suggest that V^{5+} inhibits Nqo1 expression through the AhR/XRE and the Nrf2/ARE signaling pathways.

Being able to inhibit inducible *Nqo1* gene expression in addition to its similar previous reported effect on *Cyp1a1* (Anwar-Mohamed and El-Kadi, 2008), V^{5+} was suspected to exert its effect through inhibiting the transcription of AhR or Nrf2. To address this question we examined the effect of V^{5+} on the transcription of both AhR and Nrf2, the two transcriptional factors that regulate the *Nqo1* gene expression. Our results demonstrated that V^{5+} did not affect the mRNA levels of both AhR and Nrf2, further confirming that the inhibitory effect of V^{5+} on *Nqo1* gene expression was not due to any decrease in the expression of either AhR or Nrf2.

The transcriptional regulation of *Nqo1* gene expression by V^{5+} was supported by a series of evidence, the first being its ability to inhibit TCDD- and SUL-mediated induction of Nqo1 mRNA in both wild type and AhR-deficient cells. The second evidence was the ability of V^{5+} to inhibit the TCDD- and SUL-induced Nrf2/ARE binding as evident by EMSA. The third evidence was the ability of V^{5+} to inhibit the TCDD- and SUL-induced nuclear accumulation of Nrf2. The fourth evidence was the inability of V^{5+} to significantly alter the Nqo1 mRNA half-life.

We have previously shown that V^{5+} inhibits the AhR translocation through an ATP-dependent mechanism. Since one of the two Nqo1 regulatory pathways involves activation and subsequent translocation of the AhR, it is thus expected that V^{5+} through inhibiting the ATP-dependent translocation of AhR would inhibit the *Nqo1* gene expression. In addition, it has been previously reported that the Nrf2 activity is ATPase-dependent (Zhang et al., 2006). Bearing in mind that V^{5+} is a potent ATPase inhibitor, it is not surprising to observe a decrease in Nrf2 nuclear accumulation. Therefore we suggest that the inhibitory effect of V^{5+} on *Nqo1* gene expression is occurring primarily through an ATP-dependent mechanism.

Previous reports have shown that Keap1 negatively regulates the transcription of ARE-reporter and endogenous detoxication genes by Nrf2 (McMahon et al., 2003). Keap1 is a cytoplasmic protein that interacts with the cytoskeleton, and is thought to control the subcellular distribution of Nrf2 (McMahon et al., 2003). This interaction between Keap1 and Nrf2 increases the rate of Nrf2 degradation by the proteasome. Current studies indicate that under oxidative stress conditions, cysteine residues 273 and 288 in Keap1 are modified, and thus abolishing the Keap1-Nrf2 interaction (McMahon et al., 2003). Breaking this interaction benefits Nrf2 on both sides, the first is increasing its sub-cellular levels, and the second is its translocation to the nucleus (Nioi et al., 2003). Despite the fact that V^{5+} inhibited the nuclear accumulation of Nrf2 protein, there was still a possibility that V^{5+} might have participated in increasing the degradation of Nrf2 protein through its well known 26S proteasomal pathway. Therefore, we examined the time-dependent effect of V^{5+} on the total Nrf2 protein levels. Our results demonstrated that V^{5+} did not affect the total Nrf2 protein levels, further confirming that V^{5+} inhibited the Nrf2 shuttling to the nucleus without affecting its protein levels.

We have previously shown that heavy metals do not affect the Nqo1 mRNA and protein turn-over rates (Korashy and El-Kadi, 2006). Yet it was of great importance to determine the effect of V^{5+} on the Nqo1 post-transcriptional and post-translational levels. The cellular mRNA level at any time point is a function of the rate of its production, through transcriptional mechanism, and the rate of its degradation. Our results showed that Nqo1 transcripts are long-lived with an estimated half-live of approximately 21 h. These results are in agreement with previous studies that reported a half-life of more than 17 h for Nqo1 mRNA in Hepa 1c1c7 cells (Ma et al., 2004). Furthermore, our results showed that Nqo1 is a long-lived protein with an estimated half-life of 30.44 ± 1.82 h, which is in agreement with previously published studies (Siegel et al., 2001). Interestingly, the stability of Nqo1 protein was increased upon treatment with V^{5+} and reached around 37 h, inferring presence of post-translational modification by V^{5+}.

The discrepancy between the effect of V^{5+} on Nqo1 activity levels and its effect on Nqo1 protein half-life could be explained by the strong binding of V^{5+} to sulfhydryl compounds such as glutathione and cysteine (Macara et al., 1980; Legrum, 1986). Pool of data suggest that the effect of V^{5+} on enzymatic activity may be most likely due to a covalent interaction with the enzyme cysteine residues, and thus decreasing its catalytic activity (Crans and Simone, 1991).

In conclusion, the present study demonstrates that V^{5+} down-regulates Nqo1 primarily through a transcriptional mechanism. Furthermore, the translocation of the Nrf2 was inhibited by V^{5+} possibly through an ATP-dependent mechanism. Nqo1 contains multiple copies of XRE and ARE, and the activation of which will serve to strengthen the cellular defense systems. Importantly, Nqo1 is an important detoxifying enzyme that utilizes a one step two electron reduction to eliminate potential cancer candidates. To our knowledge this is the first study to report the effect V^{5+} on the detoxifying enzyme Nqo1, and may provide further evidence for metal-induced carcinogenesis.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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