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Inhibition of Na^+/K^+-ATPase by phenoxyl radicals of etoposide (VP-16): role of sulfhydryls oxidation

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Abstract

In the present work, we studied the effects of phenoxyl radicals, generated by tyrosinase-catalyzed oxidation of a phenolic antitumor drug, Etoposide (VP-16), on a purified dog kidney Na^+/K^+-ATPase by characterizing interactions of VP-16 phenoxyl radicals with the enzyme's SH-groups by ESR and correlating the loss of the enzymatic activity with the oxidation of its SH-groups, and oxidation of VP-16. VP-16/tyrosinase caused inhibition of Na^+/K^+-ATPase which was dependent on the incubation time and concentration of tyrosinase. The inhibition of Na^+/K^+-ATPase was accompanied by a decrease of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)-titratable SH-groups. In the presence of Na^+/K^+-ATPase, a typical ESR signal of the VP-16 phenoxyl radical could be observed only following a lag period the duration of which was proportional to the concentration of the Na^+/K^+-ATPase added. Our HPLC measurements demonstrated that Na^+/K^+-ATPase protected VP-16 against tyrosinase-catalyzed oxidation. Combined these results suggest that redox-cycling of VP-16/VP-16 phenoxyl radical by SH-groups of Na^+/K^+-ATPase occurred. Ascorbate which is known to reduce the VP-16 phenoxyl radicals, protected the enzyme against inactivation, prevented oxidation of the enzyme's SH-groups. Reduction of VP-16 phenoxyl radicals by ascorbate was directly observed by the semidehydroascorbyl radical signal in the ESR spectra. VP-16 phenoxyl radical-induced oxidation of sulfhydryls and inhibition of the Na^+/K^+-ATPase may be responsible for at least some of its clinical side effects (e.g., cardiotoxicity) which can be prevented by ascorbate.

Keywords: ATPase, Na^+/K^+-; Etoposide; Phenoxyl radical; Sulfhydryl group; Antioxidant; Ascorbate; Electron spin resonance

1. Introduction

Highly reactive hydroxyl radicals and their lipophilic membrane counterparts, lipid alkoxyl and peroxyl radicals, generated by oxidative stress may attack different amino acid residues of cytosolic and membrane proteins resulting in a relatively indiscriminative oxidative modification and/or irreversible inactivation of enzymes, receptors, channel-formers and structural proteins [1,2]. In contrast, low energy phenoxyl radicals formed as intermediates in reactive radical scavenging by phenolic antioxidants, are assumed to be relatively stable [3,4]. While phenoxyl radicals of natural phenols (e.g., vitamin E) under normal conditions are not reactive towards lipids, DNA and most amino acid residues, they readily interact with important physiological reductants, such as ascorbate and thiols [5,6]. Moreover, phenoxyl radicals formed from some phenolic compounds were shown to oxidize both small molecular weight thiols and protein sulfhydryls [7]. In particular, a widely clinically used phenolic antitumor drug, Etoposide, (VP-16) is oxidized to phenoxyl radicals in the presence of tyrosinase or peroxidase [8,9]. The radicals formed directly oxidize ascorbate and diverse thiols both in model systems and in tissue homogenates [10,11]. The redox-cycling of VP-16/VP-16 phenoxyl radical may significantly deplete endogenous antioxidants setting the stage for oxidative

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stress [11]. Previously, we have demonstrated that VP-16 phenoxyl radical-dependent oxidation of critical sulfhydryls is responsible for activation of Ca^{2+}-release channels and inhibition of Ca^{2+}-ATPase in sarcoplasmic reticulum membranes [12] which may be, at least in part, responsible for clinically well-known cardiotoxic side-effects of VP-16 [13].

The energy dependent exchange of cytoplasmic Na^+ for extracellular K^+ in mammalian cells is due to a membrane bound sodium pump, the Na^+/K^+-ATPase [14]. The enzyme, located in the plasma membrane of all animal cells, is a member of a family of ion-translocating AT-Pases that share highly homologous catalytic subunits. Na^+/K^+-ATPase consists of two membrane spanning polypeptides, an alpha subunit of 112-kDa and a beta-subunit, which is a glycoprotein of 35-kDa. The catalytic properties are associated with the alpha-subunit, which has the binding domain for ATP and the cations [15]. The enzyme contains more than 20 sulfhydryl groups which differ significantly in their reactivity and accessibility to SH-reagents [16]. Even though the exact role of the SH-groups is not known, modification of some of them causes the inhibition of the Na^+/K^+-ATPase. Na^+/K^+-ATPase, is a potential target for selective attack by phenoxyl radicals. While Na^+/K^+-ATPase has been demonstrated to be highly sensitive to oxidative stress [17,18], the role of the enzyme's functionally essential SH-groups in its oxidative damage has not been definitively established.

In the present work, we studied the effects of phenoxyl radicals, generated by tyrosinase-catalyzed oxidation of VP-16, on a purified dog kidney Na^+/K^+-ATPase by characterizing interactions of VP-16 phenoxyl radicals with the enzyme's SH-groups by ESR and correlating the loss of the enzymatic activity with the oxidation of its SH-groups, and oxidation of VP-16.

2. Materials and methods

2.1. Materials

Methanol (HPLC) grade was purchased from Aldrich (Milwaukee, WI). Deferoxamine mesylate (DFO) was purchased from Ciba (NJ). Protein assay kit was obtained from Bio-Rad (Hercules, CA). VP-16 was a generous gift from Bristol-Myers Squibb (Syracuse, NY). Tyrosinase from mushroom and other chemicals were obtained from Sigma (St. Louis, MO).

Na^+/K^+-ATPase from outer medulla of canine kidney was prepared as previously described [18]. Hydrolytic activity of Na^+/K^+-ATPase was determined as γ-P^{32}-release from radiolabelled ATP in the medium containing NaCl 100 mM, KCl 25 mM, $MgCl_2$ 3 mM, ATP 2 mM, MOPS 30 mM (pH 7.4, 37°C) [18,19]. Specific ATPase activity was about 1500 μmol P_i/mg per h (37°C) and was inhibited by 1 mM ouabain by no less than 95%.

2.2. Assay of SH-groups in Na^+/K^+-ATPase

Concentration of sulfhydryls in Na^+/K^+-ATPase was determined using 5,5′-dithiobis-(2-nitrobenzoic acid), (DTNB), Ellman reagent [20]. Absorbance spectra were recorded on a Hitachi-557 spectrophotometer under constant temperature control. The DTNB blank was subtracted from the reference beam and absorbance was measured at 412 nm. For correction of the scattering input in the absorbance measured, optical density at 550 nm was subtracted from that at 412 nm. In order to identify free SH-groups of the enzyme, aliquots containing 50–100 μg of protein were preincubated 30 min at 37°C in 10 mM potassium-phosphate buffer (pH 8.5) containing 1 mM EDTA and 8 M urea. In order to prevent the interference of VP-16 radicals with the reagents in the assay medium, samples were diluted 10-fold and tyrosinase activity was inhibited by denaturing reagent (8 M urea).

2.3. Protein concentration measurement

Protein concentration in Na^+/K^+-ATPase was determined by the Lowry method with bovine serum albumin as a standard [19] and by protein assay kit (Bio-Rad).

2.4. Tyrosinase + VP-16 induced oxidation of Na^+/K^+-ATPase

The ATPase protein was incubated 5–60 min at 37°C in the presence of different concentrations of mushroom tyrosinase and VP-16. Reaction was started by the addition of VP-16 (25–200 μM) to incubation medium containing 80 mM potassium-phosphate buffer (pH 7.4), 50 μM DFO, to chelate metal ions, tyrosinase (1–3.8 U/ μl) and 0.3 mg/ml Na^+/K^+-ATPase. Aliquots were taken to assay ATPase activity and content of SH-groups Na^+/K^+-ATPase.

2.5. Tyrosinase-catalyzed oxidation of VP-16 in the presence and in the absence of Na^+/K^+-ATPase

For measurements of VP-16 phenoxyl radical formation, VP-16 (200 μM) and tyrosinase (3.8 U/μl) were incubated in 50 mM potassium-phosphate buffer (pH 7.5 at 25°C), containing DFO 50 μM in the presence or absence of Na^+/K^+-ATPase as described elsewhere [9]. The phosphate buffer was pretreated with Chelex-100 to remove possible metal ion contaminants.

2.6. HPLC assay of VP-16

Aliquots of the reaction mixture (25μl) were taken at given time intervals and transferred into 0.3 ml of 50% aqueous CH_3OH. The dispersion thus formed was filtered through a C-18 cartridge (1 ml Sep-Pak cartridge, Waters Division of Millipore, Millipore, Milford, MA) and the

filtrate was used for HPLC assays. HPLC analysis of VP-16 was performed on a Shimadzu LC-10A system equipped with an LC-600 pump and an M10A photodiode array detector. Separation of the VP-16 oxidation mixture on a C18 reverse phase column (Ultrasphere ODS, 5 mm particle size, 4.6 × 250 mm, Beckman) was carried out with a mobile phase of 50% aqueous CH_3OH adjusted to pH 3.1 with CH_3COOH at a flow rate of 1.3 ml/min. Under these conditions, the retention time for VP-16 was 4.6 min.

2.7. ESR-spectroscopy of VP-16 phenoxyl radicals

ESR measurements were performed on a JEOL JES-RE1X spectrometer at 25°C in gas permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm wall thickness; obtained from Alpha Wire, Elizabeth, NJ). The tube approx. 10 cm length was filled with 60 μl of a mixed sample, folded twice and placed into an open 3.0 mm internal diameter ESR quartz tube in such a way that all of the sample was within the effective microwave irradiation area. Spectra of VP-16 phenoxyl radicals were recorded at 335.5 mT - center field, 20 mW - microwave power, 0.05 mT - field modulation, 0.03 s - time constant.

2.8. Measurement of the lag period in the oxidation reaction of the VP-16 by tyrosinase

Na^+/K^+-ATPase was added to the incubation mixture containing VP-16, and DFO (time = 0) and the final volume was adjusted to 60 μl with potassium-phosphate buffer (pH 7.5 at 25°C). To start the reaction tyrosinase was added. The concentrations of Na^+/K^+-ATPase in incubation medium were from 25.0 to 100.0 μg/ml. The lag period was determined as the time between adding tyrosinase and the appearance of the VP-16 phenoxyl radical ESR spectrum at a signal to noise ratio larger than 2.

3. Results

3.1. VP-16 Phenoxyl radicals in the presence and absence of Na^+/K^+-ATPase

A characteristic signal of the VP-16 phenoxyl radical appeared in the ESR spectra immediately after the addition of tyrosinase (Fig. 1). The magnitude of the ESR signal increased over 50–60 min of incubation and reached a plateau thereafter (Fig. 2A). In the presence of Na^+/K^+-ATPase, the ESR signal of the VP-16 phenoxyl radical could be observed only following a lag period the duration of which was proportional to the concentration of the Na^+/K^+-ATPase added (Fig. 2 A and B).

3.2. Effect of Na^+/K^+-ATPase on the VP-16 oxidation

We used an HPLC assay to measure the effect of Na^+/K^+-ATPase on the rate of tyrosinase-catalyzed oxidation of VP-16. Na^+/K^+-ATPase protected VP-16 against oxidation (Fig. 3). While in the control (without Na^+/K^+-ATPase) the observed initial rate of the VP-16 oxidation was 36.4 μM/10 min, the rate decreased to less than 1.6 μM/10 min in the presence of 300 μg Na^+/K^+-ATPase/ml. This 'lag period' in the VP-16 oxidation caused by Na^+/K^+-ATPase was followed by an increased rate of oxidation, comparable to that in the control. These results in combination with our ESR data provide evidence for the recycling of VP-16 via reduction of its phenoxyl radicals by Na^+/K^+-ATPase.

3.3. Inhibition of Na^+/K^+-ATPase

Na^+/K^+-ATPase was inhibited up to 80% during 60 min of incubation with VP-16 and tyrosinase (Fig. 4). The residual 20% of activity could not be inhibited by increases in concentrations of VP-16 and/or tyrosinase. The time course of inhibition was linear during the first 10 min

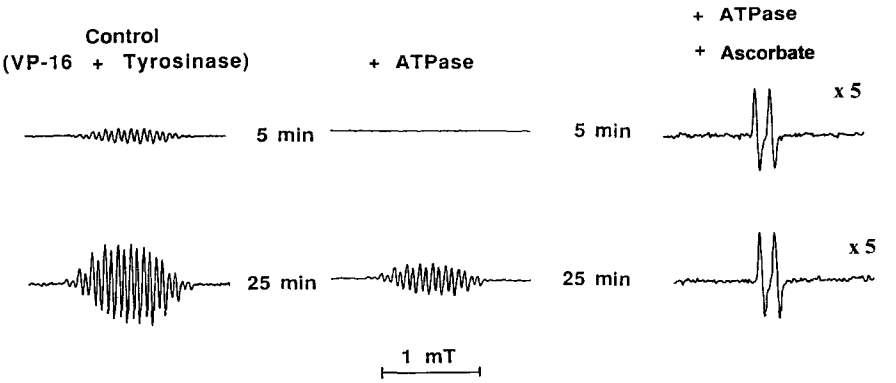

Fig. 1. ESR spectra of VP-16 phenoxyl radicals, and ascorbyl radicals generated by tyrosinase in the presence and absence of Na^+/K^+-ATPase (upper traces, on the 5th min; lower traces, on the 25th min). Samples contained: VP-16 (200 μM), tyrosinase (3.8 U/μl), deferoxamine (50 μM), KCl (80 mM) in 50 mM potassium-phosphate buffer (pH 7.5 at 25°C), Na^+/K^+-ATPase (50 μ/ml), ascorbate 2 mM. ESR conditions: center field 335.5 mT, sweep width 5 mT, field modulation 0.05 mT, gain 100 (gain 500 in the measurement of ascorbate radical spectra).

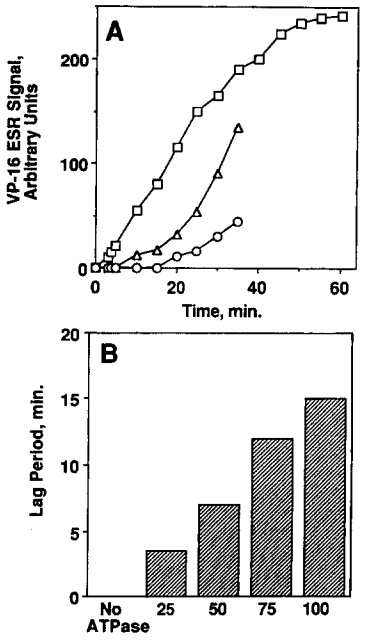

Fig. 2. (A) Time-course of ESR signals of VP-16 phenoxyl radicals generated by tyrosinase-catalyzed oxidation of VP-16 in the absence and presence of different amounts of Na^+/K^+-ATPase: □, control (no Na^+/K^+-ATPase), △, 50 μg/ml and ○, 100 μg/ml Na^+/K^+-ATPase. (B) Dependence of the lag period in tyrosinase-catalyzed oxidation of VP-16 on the concentration of Na^+/K^+-ATPase. ESR signal measurements. Experimental conditions were as described in the legend to Fig. 1.

and slowed down progressively at longer times of incubation (Fig. 4). The inhibitory effect was proportional to the amount of tyrosinase added (Table 1). Neither VP-16 alone, nor tyrosinase alone yielded any significant inhibition of the enzymatic activity (data not shown). Ascorbate protected Na^+/K^+-ATPase against oxidative inactivation by VP-16 phenoxyl radicals (see below 3.5). The enzyme was equally sensitive to VP-16/tyrosinase-induced oxidation in the presence of Na^+ or K^+ ions suggesting that conformational changes caused by ion-binding had no

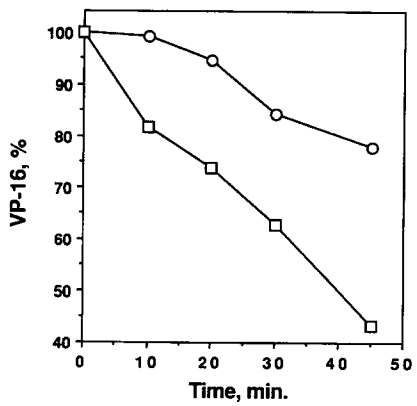

Fig. 3. Tyrosinase-catalyzed oxidation of VP-16 in the presence (○) and absence (□) of Na^+/K^+-ATPase. Incubation conditions: Na^+/K^+-ATPase (0.3 mg/ml), VP-16 (200 μM); other conditions were as given in the legend to Fig. 1.

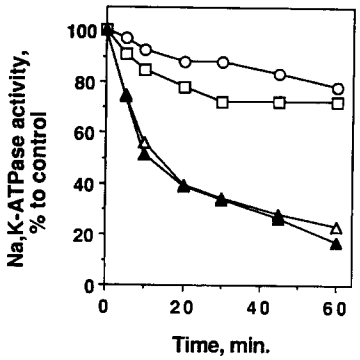

Fig. 4. Time-course of Na^+/K^+-ATPase activity during its incubation with tyrosinase and VP-16. Effect of ascorbate and ATP: ○, control (no VP-16), △, complete system; □ plus ascorbate (2 mM); ▲, plus ATP (3 mM, no ascorbate). Na^+/K^+-ATPase 0.3 mg/ml; other conditions were as given in the legend of Fig. 1.

effect on the inactivation of the Na^+/K^+-ATPase in our system (data not shown). Saturation of Na^+/K^+-ATPase with ATP has been shown to prevent the reaction of the enzyme with certain SH-modifying reagents [21,22]. At saturating concentrations (3 mM), ATP did not protect the enzyme against inactivation by VP-16/tyrosinase (Fig. 4).

3.4. Oxidation of SH-groups of Na^+/K^+-ATPase

Our enzyme preparation contained 14 SH-groups/molecule titratable by DTNB. The enzyme SH-groups decreased almost linearly over time in the course of incubation with VP-16/tyrosinase (Fig. 5). Incubation of Na^+/K^+-ATPase with VP-16 and tyrosinase for 60 min at 37°C resulted in oxidation of about nine SH-groups. Thus, the inhibition of the enzyme was accompanied by a pronounced decrease of DTNB-titratable SH-groups (Fig. 5).

3.5. Effects of ascorbate on the interaction of VP-16 phenoxyl radicals with Na^+/K^+-ATPase

Since ascorbate has been shown to reduce VP-16 phenoxyl radicals back to phenols [6] we studied whether it could prevent the inactivation of Na^+/K^+-ATPase by VP-16/tyrosinase-induced oxidation. In the presence of

Table 1
Dependence of Na^+/K^+-ATPase activity on tyrosinase concentration

Tyrosinase concentration (U/μl)	Na^+/K^+-ATPase activity (% of the control)	
	5 min incubation	60 min incubation
0	97	79
1.0	90	71
2.0	85	53
3.8	77	23

Incubation conditions: 0.3 mg/ml Na^+/K^+-ATPase, 80 mM potassium-phosphate buffer (pH 7.4 at 37°C), 50 μM DFO and 200 μM VP-16.

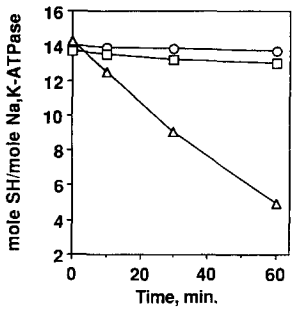

Fig. 5. Time-course of Na^+/K^+-ATPase thiols content during tyrosinase-catalyzed oxidation of VP-16. Effect of ascorbate: ○, control (no VP-16), △, complete system, □, in the presence of ascorbate (2 mM). Experimental conditions: Na^+/K^+-ATPase 0.3 mg/ml; other conditions as given in the legend to Fig. 1.

ascorbate, a typical signal of semidehydroascorbyl radical was continuously observable in the ESR spectra for 60 min of incubation both in the presence and in the absence of Na^+/K^+-ATPase (Fig. 1). No ESR signals of VP-16 phenoxyl radicals could be detected up to 60 min of incubation. Thus, one-electron reduction of VP-16 phenoxyl radicals by ascorbate resulting in its one-electron oxidation intermediate, semidehydroascorbyl radical took place. The enzyme activity was effectively protected against VP-16/tyrosinase-induced inhibition by the addition of excess ascorbate (2 mM) (Fig. 4). The enzyme retained 85% activity at 10 min of incubation in the presence of ascorbate (which is close to the 94% activity in control). Without ascorbate, the activity decreased to 55%. In addition, the reduction in the number of SH-groups/molecule of Na^+/K^+-ATPase was also prevented in the presence of 2 mM ascorbic acid (Fig. 5).

4. Discussion

Exposure of isolated membrane fractions to different kinds of reactive oxygen species ($\cdot OH$, peroxyl and alkoxyl radicals, FeO^{2+}) demonstrated an extremely high sensitivity of Na^+/K^+-ATPase to oxidation [17]. In vivo treatments associated with the production of oxidative stress (e.g., ischemia/reperfusion of the heart, hyperbaric oxygenation, have been also shown to decrease the activity of the enzyme [23,24]. Mechanisms of oxidative modification of the Na^+/K^+-ATPase by reactive oxygen species such as contribution of annulus lipid oxidation versus protein oxidation and specific role of sulfhydryl oxidation remain to be elucidated [17–19].

4.1. VP-16 phenoxyl radicals inhibit Na^+/K^+-ATPase

We have shown previously that phenoxyl radicals generated from a phenolic antitumor drug, VP-16, react with GSH as well as with SH-groups of proteins [11]. While the VP-16 radical is reduced in the reaction, the sulfhydryl oxidation leads to inhibition of activity of several enzymes, including Ca^{+2} ATPase and the Ca^{2+}-release channel [12,25]. Redox-cycling of VP-16/VP-16 phenoxyl radical by thiols was suggested to result in cytotoxic effects most likely via thiol pumping [11], i.e., a futile depletion of low molecular weight thiols and oxidation of protein sulfhydryls [26].

The reaction of the low energy phenoxyl radicals with proteins is selective for the cysteine sulfhydryls [27]. Therefore we used the tyrosinase/VP-16 as a model system to elucidate the effects of phenoxyl radicals on Na^+/K^+-ATPase. The inhibition of the enzyme was clearly associated with the tyrosinase-catalyzed activation of VP-16. Neither VP-16 nor tyrosinase alone significantly inhibited the enzyme. In combination, VP-16 and tyrosinase inhibited Na^+/K^+-ATPase in a concentration dependent manner. Thus one can suggest that the inhibition was due either to the oxidation of the enzyme SH-groups by the phenoxyl radicals or to the binding of the VP-16 oxidation products to the enzyme.

Our results are consistent with the suggestion that phenoxyl radical-mediated oxidation of the enzyme sulfhydryls rather than effects of VP-16 oxidation products (e.g., *o*-quinone of VP-16) were involved in inactivation of the Na^+/K^+-ATPase. This conclusion is supported by: (i) disappearance of the VP-16 phenoxyl radical ESR signal was observed upon addition of Na^+/K^+-ATPase, (ii) decrease of the enzyme SH-groups in the course of its incubation with tyrosinase/VP-16, (iii) decrease in the rate of tyrosinase-catalyzed VP-16 oxidation in the presence of Na^+/K^+-ATPase, and (iv) strong protective effect of ascorbate (which reduced VP-16 phenoxyl radicals by a one electron process accompanied by the formation of ESR detectable semidehydroascorbyl radicals) in preventing both the loss of enzymatic activity and oxidation of SH-groups. Combined our results give evidence that VP-16 phenoxyl radical-induced oxidation of the Na^+/K^+-ATPase SH-groups was most likely responsible for the enzyme inhibition.

4.2. VP-16 phenoxyl radical-induced oxidation of SH-groups of Na^+/K^+-ATPase

Na^+/K^+-ATPase consists of two subunits, α and β, which are present in equimolar amounts. The Na^+/K^+-ATPase dimeric protomer (a functional unit) has a molecular mass of about 145–150 kDa [18,27,28]. Based on the pronounced difference in the reactivity toward *N*-ethylmaleimide and effects on the enzymatic activity, Esmann et al. classified SH-groups of Na^+/K^+-ATPase into three categories [29]. In a purified pig kidney Na^+/K^+-ATPase, 10–20 sulfhydryl groups have been reported to be available for different SH-reagents [15]. At least one of the SH-groups is located in the ATP binding site [21,22] suggesting that its oxidation can cause inhibition of the

enzymatic activity. Kirley et al. identified a DTNB-titratable SH-group which was found to be essential for the binding of ouabain, a specific inhibitor of Na^+/K^+-ATPase [30]. Studies of rabbit kidney Na^+/K^+-ATPase using alkylating reagents demonstrated the difference in the sensitivity of the enzyme in different conformation: E_1-conformer (induced by binding of sodium) was protected against the attack by radiolabelled *N*-ethylmaleimide by ATP, while E_2-conformer (resulting from binding of potassium) was protected by ouabain [31,32]. Modification of some SH-groups of the enzyme did not affect the activity, suggesting their structural role [29]. In line with this, six sulfhydryl groups were identified in beta-subunit of the enzyme: two sulfhydryl groups were in reduced form and four were involved in disulfide bond formation [33].

The time course of the enzyme inhibition by tyrosinase-induced VP-16 phenoxyl radicals was linear during the first 10 min and slowed down at longer incubations. This was most likely caused by the different roles of the respective SH-groups in enzyme inactivation. The loss of 43% of activity during the first 10 min was accompanied by the oxidation of one to two SH-groups/mol of enzyme which are likely to be essential for the enzyme activity. Oxidation of 3–4 additional SH-groups/mol of enzyme lead to the total inhibition of 70% of activity. Apparently, the additional sulfhydryls are less important for enzyme function. After 60 min incubation, about nine SH-groups were oxidized yielding 80–85% inhibition of the enzyme. The residual enzymatic activity was not sensitive to further enhanced generation of phenoxyl radicals provided by increased concentrations of tyrosinase and/or VP-16. This low level of activity may be characteristic of the enzyme with nine of its fourteen SH-groups modified by interactions with the VP-16 phenoxyl radicals which may result in different oxidation products (e.g., disulfides, mixed sulfides, and sulfoxygenated derivatives). Further studies are necessary to elucidate the role(s) of these products in catalytic activity. Alternatively, the enzyme preparation used may be heterogeneous hence not all the enzyme molecules are equally susceptible to the attack by the VP-16 phenoxyl radicals.

Saturation with ATP did not protect the enzyme against inactivation by tyrosinase/VP-16: the time course of the Na^+/K^+-ATPase inactivation was identical in the presence and absence of ATP. Thus, the SH-groups within the ATP-binding domain are an unlikely target of phenoxyl radicals.

In order to compare the sensitivity of the two Na^+/K^+-ATPase-conformers to phenoxyl radicals, we have substituted potassium for sodium in the incubation medium. This substitution had no effect on the loss of the enzyme activity in the presence of tyrosinase/VP-16. While E_1- and E_2-conformers have been shown to exert different sensitivity to SH-reagents [31,34], VP-16 phenoxyl radicals turned to be equally effective in inhibiting the enzyme in these different conformations.

4.3. Toxicological and pharmacological implications

VP-16 is frequently used in the US as a first-line drug for treating small lung cancer, germ cell tumors, lymphomas, and more recently Kaposi's sarcoma associated with AIDS. The drug is also used to treat a variety of leukemias including acute nonlymphocytic leukemia [35]. Teniposide (VM-26), chemically a close relative of VP-16 is also a very potent antineoplastic drug. Both VP-16 and VM-26 are successfully used not only as single agents but also in combination chemotherapy as well as in concurrent chemoradiotherapy [36]. While myelosuppression is the major toxic effect of these two drugs, cardio-, pulmonary and neurotoxicity are considered significant in limiting efficiency and intensification of cancer therapy [36–39].

In contrast to anthracycline anticancer drugs, toxic side-effects of VP-16 cannot be easily derived from their ability to intercalate into mitochondrial respiratory chain and to generate oxygen radicals. Instead, high reactivity and selectivity of VP-16 phenoxyl radicals toward protein SH-groups may be the major contributor to its toxic effects. The importance of this reaction is accentuated by our results which demonstrated that a single injection of VP-16 (10 mg/kg body weight) considerably decreased the concentration of protein thiols and GSH in some organs of the rat. The decrease in total titratable protein thiols occurred at the same time as the decrease in GSH concentration (Kagan et al., unpublished data). It is therefore important to identify the proteins whose sulfhydryls are sensitive to oxidation by VP-16 phenoxyl radicals and which determine toxic side-effects of chemotherapy. We suggest that VP-16 phenoxyl radical-induced oxidation of sulfhydryls and subsequent inhibition of the Na^+/K^+-ATPase may be responsible for at least some of its clinical side effects. Therefore, effective protection of Na^+/K^+-ATPase by ascorbic acid described here could suggest future strategies to decrease or prevent side effects of VP-16 and related phenolic antitumor drugs.

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