Published in final edited form as:
FEBS Lett. 2008 March 5; 582(5): 725–728.

Activation of NO Donors in Mitochondria: Peroxidase Metabolism of (2-hydroxyamino-vinyl)-triphenyl-phosphonium by Cytochrome c Releases NO and Protects Cells Against Apoptosis

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Abstract

In mitochondrial apoptosis, the formation of cytochrome c-cardiolipin complex ([CL-cyt c]) with peroxidase properties is an early event in the cascade of reactions that leads to cell death. Herein, we report the synthesis of a new prodrug, (2-hydroxyamino-vinyl)-triphenyl-phosphonium (HVTP), which compartmentalizes exclusively into mitochondria, undergoes a [CL-cyt c]-catalyzed bioactivation to nitric oxide (NO), inhibits peroxidase activity, and protects cells from apoptosis.

Introduction

Elimination of irreparably damaged or genetically pre-determined cells by apoptosis heavily relies on mitochondria. Permeabilization of mitochondrial membrane and release of pro-apoptotic factors into the cytosol designates a point-of-no-return followed by degradation of intracellular constituents and subsequent clearance of damaged cells by phagocytes. This major function of mitochondria as death-triggering machines is impossible without generation of reactive oxygen species and a dramatic change of the normal electron transporting function of cytochrome c (cyt c) to that of a peroxidase upon binding to a mitochondria-specific phospholipid, cardiolipin (CL) [1].

The central role of the [CL-cyt c] complex as a peroxidase implies that inhibition of its activity may be used for regulation of apoptotic cell death. NO readily inhibits peroxidases by forming iron-nitrosyl complexes or by interacting with the reactive intermediates at the heme-site or protein immobilized radical sites [2,3]. Moreover, NO impedes lipid peroxidation via interception of lipid radicals [3]. However, the concentrations of endogenous NO in mitochondria are not sufficient to effectively inhibit the [CL-cyt c]-dependent peroxidase pathway. Triphenylphosphonium derivatization ($(Ph)_3P^+$-) has proven efficient for the "electrophoretic" delivery of organic compounds into mitochondria [4,5]; we employed this strategy to synthesize (2-hydroxyamino-vinyl)-triphenyl-phosphonium (HVTP) as a potential substrate of [CL-cyt c]. This is based on previous studies by us and others demonstrating one-electron oxidation of oximes to iminoxyl radicals by iron complexes as the first step in their

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metabolism to NO [6,7]. Here, we report that HVTP compartmentalizes exclusively into mitochondria, undergoes a [CL-cyt c]-catalyzed bioactivation to NO, inhibits peroxidase activity, and protects cells from apoptotic death.

Methods

Preparation of HVTP

An aqueous solution (10 mL) of (2-oxo-ethyl)-triphenyl-phosphonium chloride (**I**; 1.04 g; Acros, Inc.; Geel, Belgium) and hydroxylamine hydrochloride (0.5 g) was titrated with HCl to pH 6.2 and then incubated for 14 hours at 4 °C. The final reaction solution was saturated with sodium chloride and extracted with ethyl acetate (3 × 15 mL). The unified organic layers were dried over anhydrous Na_2SO_4, filtered, mixed with n-hexane until the appearance of turbidity, and kept at −20 °C for 12 hours. The pale yellow crystals formed (0.57 g; HEPT > 98 %) were filtered and HVTP was purified by C18 HPLC.

HPLC analysis of HVTP

Analytical and semi-preparative isocratic separations of HVTP were achieved with 50% methanol containing 20 mM $LiCLO_4$ at a flow rate of 1 mL per min on a 4.6 × 250 mm Alltima (5 μC-18) and 10 × 250 mm Econosil (10 μC-18) column, respectively (Alltech Associates, Inc ; Deerfield; IL). Detection of HVTP was performed with a SPD-M10Avp photodiode array detector (Shimadzu; Kyoto, Japan) following the specific UV spectrum of the triphenylphosphonium function (λ_{max} = 224 nm; 265 nm), and by mass spectrometry (LXQ Linear Ion Trap Mass Spectrometer; Thermoelectron, San Jose, CA). HVTP was eluted as a single peak with purity of 0.99, as calculated with EZChrom 4.2 (Class VP/PDA; Shimadzu, Inc.; Columbia, MD). In separations with mobile phases with varying polarity (35 – 50 % methanol), this index remained constant, suggesting that HVTP eluted as a homogenous peak. MS, MS-MS and NMR data supporting the structure of HVTP are included in Supplementary information.

HPLC analysis of HVTP in isolated mitochondria and cytosol was carried out after precipitation of proteins with CH_3CN (50 %; 30 min at 0 – 4 °C; centrifugation, 5 min × 12, 000g).

EPR analysis

EPR experiments were carried out with a Jeol JES-FA100 spectrometer (Tokyo, Japan) at room temperature. Spectrometer settings were: field center, 335.237 mT; sweep width, 5 mT; modulation width, 0.08 mT; amplitude, 500; time constant, 0.03 s; and sweep time, 2 min. EPR spectra simulation was performed with a computer program created by Philip D. Morse II and Richard Reiter (ESR Simulation System 2.01, Scientific Software Services, IL).

Preparation of CL-containing liposomes

Liposomes containing dioleoyl-L-α-phosphatidylcholine (DOPC; 0.5 mM) and 1,1′2,2′-tetraoleoyl cardiolipin (DOPC-CL; 025 Mm DOPC and 0.25 mM cardiolipin) were prepared by sonication (10 × 5 sec; relaxation time, 2 min; 0 – 4 °C) with ultrasound in 25 mM sodium phosphate buffer (pH 7.0) containing 100 μM DTPA.

Cell culture

MEC +/+ and MEC −/− (courtesy of Dr. X. Wang, University of Texas, Dallas) were maintained in Dulbecco modified Eagle medium supplemented with 15% fetal bovine serum, 25 mmol/L HEPES, 50 mg/L uridine, 110 mg l/L pyruvate, 2 mmol/L glutamine, 1×

nonessential amino acids, 0.05 mmol/L 2-mercaptoethanol, 100 U/L penicillin, and 100 mg/L streptomycin.

Biomarkers of apoptosis

Cells (0.15×10^6) were seeded in 6-well plates and let attached overnight. Cells were preincubated with HVTP for 4 hr, washed with PBS (3 × 1 mL), and then treated with actinomycin D (ActD, 100 ng/ml) to induce apoptosis. At the end of incubation (18 hr), the cells were harvested by trypsinization, washed twice with PBS, and caspase-3/7 activity in cell lysates was measured with a chemiluminescence Caspase-Glo 3/7 assay kit obtained from Promega (Madison, WI). Luminescence was determined at 25 °C with a ML1000 luminescence plate reader (Dynatech Laboratories, Chantilly, VA). Caspase-3/7 activity was expressed as luminescence arbitrary units per milligram of protein produced within 1 h incubation.

PS externalization was determined with an annexin V-FITC apoptosis detection kit (BioVision, Mountain View, CA). Collected cells were resuspended in the binding buffer and incubated with the annexin V-FITC conjugate and propidium iodide for 5 min in the dark. Cell fluorescence was analyzed with a FACScan flow cytometer (Beckton-Dickinson) with simultaneous monitoring of green fluorescence for annexin V-FITC (530 nm, 30 nm band-pass filter, FL-1) and red fluorescence associated with propidium iodide (long-pass emission filter that transmits >650 nm light, FL-3). For each sample, 10,000 events were recorded and analyzed.

Isolation of mitochondria

Cells (1×10^6) were seeded in 100 mm cell culture dishes and let attached overnight. Cells were incubated with HVTP (50 μM; 0.5 – 4 hr) then collected by trypsinization and washed twice with PBS. Mitochondria fraction was isolated by differential centrifugation. Briefly, cells were suspended in mitochondria isolation buffer (MIB, 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.4) and lysed by Dounce homogenization. Unbroken cells, nuclei and debris were removed by 10 min centrifugation at 700×g at 4ºC. A mitochondria rich fraction was obtained by 10 min centrifugation at 5,000×g and washed twice with MIB.

Assessment of NO production

Cells (0.15×10^6) were seeded in 6-well plate and let attached overnight. HVTP (100 μM) was added 4 hr prior to induction of apoptosis by actinomycin D (ActD, 100 ng/ml). At the end of incubation with ActD (18 hr), the cells were incubated with DAF-2-DA (10 μM for 30 min at 37°C), harvested by trypsinization, washed twice with PBS, and fixed with 4% paraformaldehyde for 10 min. DAF-2 signal was determined with FACScan flow cytometer (Beckton-Dickinson, San Jose, CA) with monitoring green fluorescence (530 nm band-pass filter, FL-1 channel). For each analysis, 10,000 events were recorded.

Western Blots

Cyt c (1.5 μM) was incubated for 15 min at 25 °C in 20 mM HEPES (pH7.4) containing CL (12.5 μM)/DOPC (125 μM) liposomes. Thereafter, samples were transferred to a water bath (37° C) and 4 aliquots of 25 μM H_2O_2 were added with a time interval of 15 min. After termination of the reactions with catalase (final activity, 500 units/mL), equal amounts of protein (350 ng) were resolved by SDS PAGE (12.5%) and transferred on to nitrocellulose membranes. The membranes were blocked in 5% (w/v) dried milk powder in TBS (Tris-buffered saline) at 4 °C, incubated with anti-cyt c (1:2000 dilution) overnight, washed several times, and then incubated with HRP-conjugated goat anti-mouse antiserum for 60 min at 25 ° C. The protein bands were visualized by using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Results and discussion

Interaction of [CL-cyt c] with HVTP was monitored in the presence of H_2O_2 and 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetra-methyl-1-himidazol-1-yloxy-3-oxide (carboxy-PTIO), a specific molecular probe for NO that exhibits a five-line EPR spectrum with hyperfine splitting constants (in Gauss) of $a_N^1 = a_N^2 = 8.1$ (Figure 1A1). Carboxy-PTIO readily reacts with NO to form imino nitroxide (IMND) that has a distinct seven-line EPR spectrum ($a_N^1 = 4.5$ G; $a_N^2 = 9.8$ G)[6]. Cyt c, H_2O_2, HVTP and dioleoyl-L-α-phosphatidylcholine (DOPC)-liposomes lacking CL did not change the spectrum of carboxy-PTIO to any significant extent (Fig. 1*A*1-3; A4, computer simulation of the spectrum of carboxy-PTIO). In the presence of CL, however, the magnitude of the EPR spectrum of carboxy-PTIO decreased with concomitant appearance of a seven-line EPR spectrum that was assigned to IMND (Fig. 1*A*5–8; B9, computer simulation of the spectrum of carboxy-PTIO). The initial rate of IMND production strongly depended on the content of CL (Figure 1*C*). As an end-reaction product of the oxidation of HVTP by [CL-cyt c], NO was independently identified chemiluminometrically using Sievers Nitric Oxide Analyzer (Supplementary information). HVTP inhibited typical for peroxidases H_2O_2-dependent oligomerization of cyt c ($[cyt. c]_n$; Fig. 1*D*).

It has been established that cyt c-Fe^{III} interacts with ROOH to form cyt c-Fe^{IV}=O and RO [8], and that Fe^{IV}=O complexes oxidize aminoxyls to aminoxyl radicals with concomitant generation of NO [6,7,9]. Hence, the [CL-cyt c]-catalyzed release of NO from HVTP may be tentatively explained by the reaction sequence **1** → **8**, where the release of $Fe^{(IV)}$=O following the breakdown of **4** to alkoxyl radical closes the catalytic cycle (Scheme 1).

Reverse phase HPLC measurements established that in mouse embryonic cells, HVTP compartmentalized predominantly into mitochondria (Fig. 2). In these experiments, the identity of HVTP was confirmed via comparison of the retention times and the UV spectra of the compounds eluted with the retention time and the spectrum of authentic HVTP (data not shown). Recently, Asin-Cayuela et al. conducted detailed analysis of the uptake of triphenylphosphonium compounds by mitochondria [5] and concluded that all mitochondrial compartments and membranes are enriched with $(Ph)_3P^+$-R; peak-concentrations of triphenylphosphonium compounds could be expected in the matrix of non-damaged mitochondria. They further reported that depolarized mitochondria rapidly release non-polar triphenylphosphonium compounds back into the cytosol. Given that mitochondria depolarize during isolation, the data presented in Fig. 2 may underestimate the total mitochondrial content of HVTP in intact cells.

HVTP was designed to impede the peroxidase activity of [CL-cyt c]. These complexes do not exist in normal mitochondria; instead, they are formed in mitochondria of cells undergoing early apoptosis. The formation of the complexes becomes possible due to collapse of CL asymmetry in mitochondrial membranes and its *trans*-migration from the inner to the outer mitochondrial membrane. Whereas typical distribution of mitochondrial components changes markedly during apoptosis, we hypothesized that HTVP, even if localized predominantly in the mitochondrial matrix, gets access to [CL-cyt c] during apoptosis. To verify this hypothesis, we have assessed the antiapoptotic potential of HVTP as a function of the cellular content of cyt c. To this end, we induced actinomycin D (ActD)-dependent apoptosis in control and cyt c-deficient mouse embryonic cells (MEC+/+ and MEC−/−, respectively). In apoptotic HVTP-loaded MEC+/+, which exhibit high [CL-cyt c] peroxidase activity [1], DAF-2 was converted to a benzotriazole, suggesting that HVTP was metabolized to NO (Fig. 3*A*). In contrast, no production of NO was observed in MEC−/− (Fig. 3*B*). Although DAF-2 is converted to a fluorescent benzotriazole by nitric oxide-derived nitrosating species, rather than by NO [10], it has been successfully used to quantitate NO in intact cells [11]. In parallel, HVTP impeded

the activation of caspases 3/7 and the externalization of phosphatidylserine (PS), which are two characteristic biomarkers of apoptosis (Fig. 4*A*,*B*).

In conclusion, this work describes a new type of pro-drug that is selectively targeted to and specifically activated in mitochondria to NO. Since [CL-cyt c]-catalyzed oxidation of phospholipids (LPO) occurs very early in mitochondrial apoptosis, inhibitors of this process may be used as anti-apoptotic agents. Positively charged compounds containing an aminoxyl function may prove particularly useful because of their preferential compartmentalization into mitochondria and bioactivation to NO in close proximity to the sites of [CL-cyt c], procaspases, and induction of lipid peroxidation. Studies to further assess the pharmacology of HVTP and its structural analogues are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work is supported by NIH NIAID U19 AI068021 and the Human Frontier Science Program.

References

1. Kagan VE, et al. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nat Chem Biol 2005;1:223–32. [PubMed: 16408039]
2. Vlasova II, Tyurin VA, Kapralov AA, Kurnikov IV, Osipov AN, Potapovich MV, Stoyanovsky DA, Kagan VE. Nitric oxide inhibits peroxidase activity of cytochrome c.cardiolipin complex and blocks cardiolipin oxidation. J Biol Chem 2006;281:14554–62. [PubMed: 16543234]
3. Kanner J, Harel S, Granit R. Nitric oxide, an inhibitor of lipid oxidation by lipoxygenase, cyclooxygenase and hemoglobin. Lipids 1992;27:46–9. [PubMed: 1608303]
4. Murphy MP, Smith RA. Drug delivery to mitochondria: the key to mitochondrial medicine. Adv Drug Deliv Rev 2000;41:235–50. [PubMed: 10699318]
5. Asin-Cayuela J, Manas AR, James AM, Smith RA, Murphy MP. Fine-tuning the hydrophobicity of a mitochondria-targeted antioxidant. FEBS Lett 2004;571:9–16. [PubMed: 15280009]
6. Caro AA, Cederbaum AI, Stoyanovsky DA. Oxidation of the ketoxime acetoxime to nitric oxide by oxygen radical-generating systems. Nitric Oxide 2001;5:413–24. [PubMed: 11485379]
7. Wang CCY, Ho DM, Groves JT. Models of nitric oxide synthase: iron(III) porphyrin-catalyzed oxidation of fluorenone oxime to nitric oxide and fluorenone. J Am Chem Soc 1999;121:12094–12103.
8. Barr DP, Mason RP. Mechanism of radical production from the reaction of cytochrome c with organic hydroperoxides. An ESR spin trapping investigation. J Biol Chem 1995;270:12709–16. [PubMed: 7759524]
9. Jousserandot A, Boucher JL, Henry Y, Niklaus B, Clement B, Mansuy D. Microsomal cytochrome P450 dependent oxidation of N-hydroxyguanidines, amidoximes, and ketoximes: mechanism of the oxidative cleavage of their C=N(OH) bond with formation of nitrogen oxides. Biochemistry 1998;37:17179–91. [PubMed: 9860831]
10. Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, Nagano T. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. Anal Chem 1998;70:2446–53. [PubMed: 9666719]
11. Ku CJ, Karunarathne W, Kenyon S, Root P, Spence D. Fluorescence determination of nitric oxide production in stimulated and activated platelets. Anal Chem 2007;79:2421–6. [PubMed: 17288406]

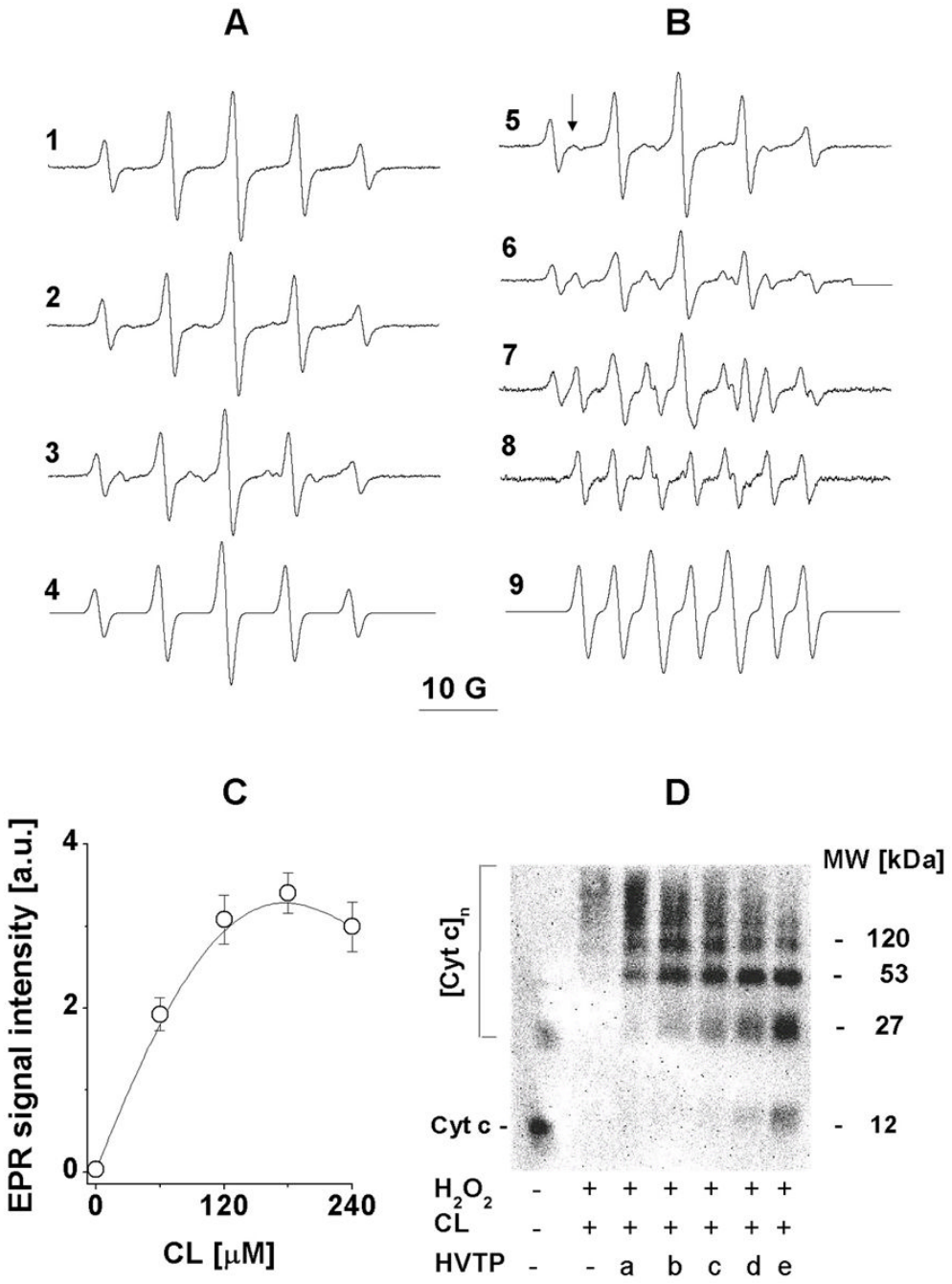


Figure 1.

HVTP undergoes [CL-cyt c]-catalyzed oxidation to NO and inhibits peroxidase activity. Reactions were carried out at 25 °C in 25 mM sodium-phosphate buffer (pH 7.0) containing DTPA (100 μM). Cyt *c*, carboxy-PTIO, HVTP, and H_2O_2 were used at concentrations of 5, 10, 50, and 200 μM, respectively. *A*, *B*- HVTP, cyt c and H_2O_2; *1-3*- DOPC(500 μM)-liposomes; *5-7*-CL(125 μM)-containing DOPC (375 μM) liposomes. EPR spectra were recorded 1 min (traces 1 and 5), 10 min (traces 2 and 6), and 30 min (traces 3 and 7) after addition of H_2O_2. Spectrum 8 was obtained after subtraction of the spectrum of carboxy-PTIO from spectrum 7. Spectra 4 and 9 represent computer simulations of the spectra of carboxy-PTIO and IMND, respectively. *C*- EPR-monitored kinetics of IMND formation by HVTP, CL-

containing DOPC liposomes, cyt c and H_2O_2 as a function of CL. In *B*, arrow indicates the spectral line used to monitor the formation of IMND. *D*- Oligomerization of cyt c by H_2O_2 (25 μM) and CL(125 μM)-containing liposomes in the absence and in the presence of HVTP (*a*, *b*, *c*, *d* and *e*- 25, 50, 100, 200 and 400 μM, respectively).

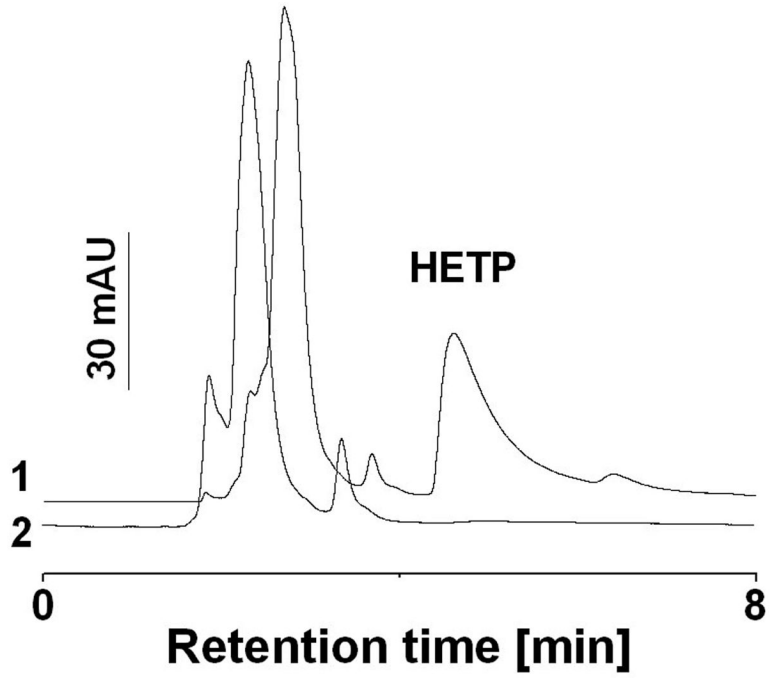


Figure 2.
HVTP compartmentalizes in mitochondria. HPLC analysis of mitochondrial (chromatogram 1) and cytosolic (chromatogram 2) content of HVTP in MEC +/+. Additional experimental details are provided in Methods.

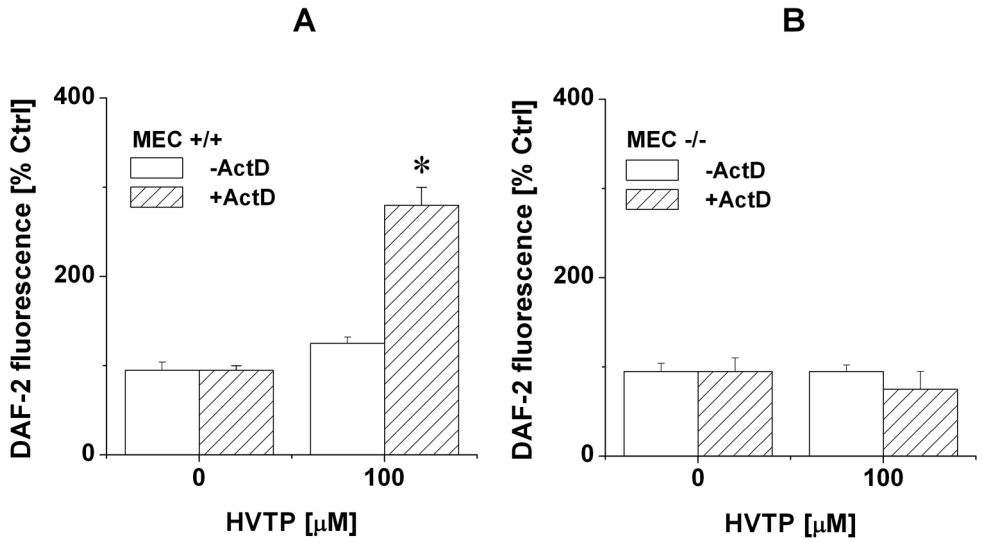


Figure 3.

Cyt c-dependent oxidation of HVTP to NO in MEC. Apoptosis in HVTP-pretreated cells was induced with ActD as described in Methods. Thereafter, the cells were treated with DAF-2-DA, trypsinized, fixed with formaldehyde, and green fluorescence reflecting NO-dependent formation of DAF-2-triazole was recorded by flow cytometry. Data are presented as means ± SD (n=3; *p<0.01 *vs*. non-treated cells).

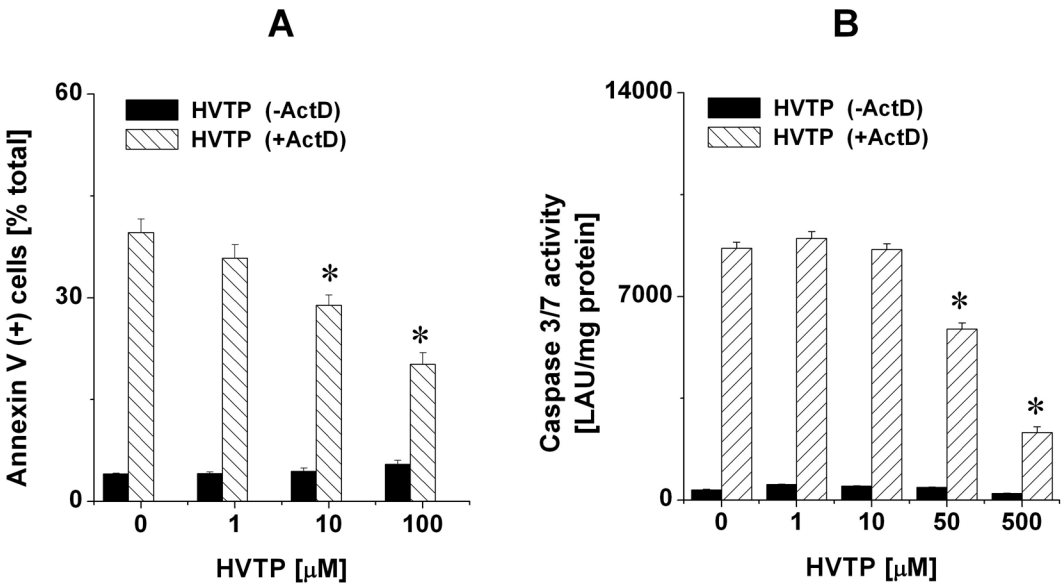
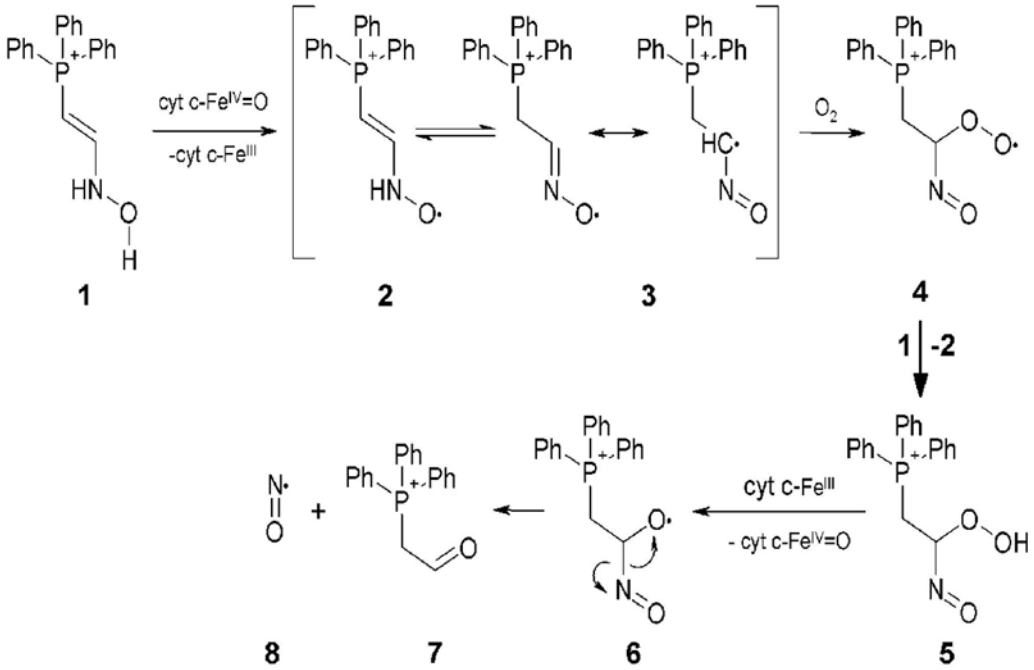


Figure 4.

HVTP impedes PS externalization and caspases 3/7 activation in ActD-treated MEC+/+. Data are means ±SD (n=3), *p < 0.01 *vs*. non-treated cells.



Scheme 1.