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Peroxynitrite-induced mitochondrial translocation of PKCα causes U937 cell survival

Liana Cerioni [a], Letizia Palomba [a], Bernhard Brüne [b], Orazio Cantoni [a,*]

[a] *Istituto di Farmacologia e Farmacognosia, Università degli Studi di Urbino "Carlo Bo" Via S. Chiara, 27-61029 Urbino, PU, Italy*
[b] *Institute of Biochemistry I, Johann Wolfgang Goethe-University, Theodor-Stern-Kai, 7-60590 Frankfurt, Germany*

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Abstract

Our previous work has shown that non-toxic concentrations of peroxynitrite nevertheless commit U937 cells to mitochondrial permeability-transition (MPT)-dependent necrosis that is however prevented by a parallel survival signaling pathway involving cytosolic phospholipase A_2 ($cPLA_2$)-dependent arachidonic acid release and PKCα activation associated with the cytosolic translocation of Bad. The present study provides evidence of an early mitochondrial translocation of PKCα. Inhibition of the survival signaling at the level of either $cPLA_2$, or PKC, was invariably associated with prevention of the mitochondrial localization of PKCα, with the mitochondrial translocation of Bad and Bax and with a very rapid lethal response. Collectively, the results presented in this study demonstrate that peroxynitrite, while committing U937 cells to necrosis, triggers a parallel signaling response leading to the cytosolic localization of two important members of the Bcl-2 family implicated in the onset of MPT.

Keywords: Peroxynitrite; Arachidonic acid; PKCα; Bcl-2; Bad; Bax; Survival; Mitochondrial permeability transition; Necrosis; U937 cells

In the context of the inflammatory response various cell types, including neutrophils, monocytes, and macrophages, produce an array of toxic molecules resulting in tissue damage. Peroxynitrite, the coupling product of nitric oxide and superoxide, is one of these species that, because of its extreme reactivity, is likely to generate extensive damage in the same cell in which it is being produced. How these cells cope with their own peroxynitrite and, more generally, how they can survive in an environment in which other cell types die remain unanswered questions. Our recent work using U937 cells as a monocyte cellular system [1–3], and confirming the critical results in various cell types belonging to the monocyte/macrophage lineage, including human monocytes and macrophages [4], provides some insights in this direction. We showed that non-toxic concentrations of peroxynitrite nevertheless commit cells to mitochondrial permeability transition (MPT), which is however prevented by a survival signaling driven by arachidonic acid (AA). Under these conditions, peroxynitrite promotes a prompt release of AA, via activation of the cytosolic phospholipase A_2 ($cPLA_2$) isoform, and pharmacological inhibition or genetic depletion of $cPLA_2$ prevents AA release and causes an extremely rapid, MPT-dependent necrotic response.

These results strongly suggest that monocyte survival in the presence of peroxynitrite requires an AA-dependent signaling pathway and that inhibition of the latter causes an immediate necrosis, which however presents features of a regulated mode of cell death. The ATP pool of these cells was indeed virtually unaffected and ATP was required to allow the survival response mediated by nanomolar levels of exogenous AA [5]. Subsequent findings revealed that a downstream target of AA is PKCα [6] and that Bcl-2, while not being a direct target of PKCα, is nevertheless involved downstream in this protective signaling [7]. We indeed found that exposure to an otherwise non-toxic concentration of peroxynitrite promotes the same toxicity in cells genetically depleted in Bcl-2 or $cPLA_2$ (or PKCα).

* Corresponding author: Fax: +39 0722 303521.
E-mail address: cantoni@uniurb.it (O. Cantoni).

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Furthermore, although the effects of Bcl-2 were downstream to $cPLA_2$ and PKCα, no changes in Bcl-2 phosphorylation at serine 70 were detected in untreated cells or in cells treated with peroxynitrite alone or associated with inhibitors of either PLA_2 or PKC. These findings therefore suggest that PKCα targets other proteins of the Bcl-2 family involved in the regulation of Bcl-2 anti-MPT activity.

It is well established that proteins belonging to the Bcl-2 family can positively or negatively control MPT, at least in part through protein–protein interaction. The anti-apoptotic proteins Bcl-2 and Bcl-X_L inhibit MPT [8,9] and their activity is lost upon heterodimerization with Bad, an additional member of this family of proteins [10,11]. The latter reaction is tightly regulated by the degree of Bad phosphorylation on two serine residues, an event associated with the inactivation of Bad that now resides in the cytosol complexed to the 14-3-3 protein [8,11]. Survival factors prevent MPT-dependent apoptosis by phosphorylating Bad at Ser^{136}, via a pathway involving phosphatidylinositol 3-kinase and Akt [12,13], as well as at Ser^{112}, via a PKC/$p90^{RSK}$-dependent mechanism [14,15]. Several PKC isoforms, including PKCα, can support the latter pathway [14]. We indeed provided experimental evidence indicating that the non-toxic concentration of peroxynitrite promotes the cytosolic translocation of Bad via a $cPLA_2$/PKC-dependent mechanism [7].

We herein report results indicating that the survival signaling mediated by peroxynitrite involves the AA-dependent mitochondrial translocation of PKCα, an event associated with the mitochondria to cytosol translocation of Bad. In addition, we provide experimental evidence indicating that, under these conditions, Bax is retained in the cytosol. Upstream inhibition of the survival signaling inhibits the mitochondrial translocation of PKCα and leads to a delayed cell death preceded by the mitochondrial translocation of Bad and Bax.

Materials and methods

Cell culture and treatment conditions. U937 cells and EGFP/PKCα-stably transfected U937 cells were cultured in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Sera-Lab, Crawley Down, England), at 37 °C in T-75 tissue culture flasks (Corning, Corning, NY) gassed with an atmosphere of 95% air–5% CO_2. EGFP/PKCα-stably transfected U937 cells were obtained as previously described [16]. Peroxynitrite was synthesized by the reaction of nitrite with acidified H_2O_2, as described [1,17]. Treatments were performed in pre-warmed saline A (8.182 g/L NaCl, 0.372 g/L KCl, 0.336 g/L $NaHCO_3$, and 0.9 g/L glucose) containing 2.5×10^5 cells/ml.

Subcellular fractionation and Western blot analysis. After treatments, the cells were processed to obtain the cytosolic and mitochondrial fractions, as described by Bossy-Wetzel et al. [18] and Majumder et al. [19], respectively. Western blot analysis was next performed using antibodies recognizing PKCα (BD Transduction Laboratories, Lexington, KY), Bad (BD Transduction Laboratories, Lexington, KY), Bax (Santa Cruz, Santa Cruz, CA), actin (Sigma–Aldrich, Milan, Italy), and HSP-60 (Santa Cruz, Santa Cruz, CA). Details on Western blotting apparatus and conditions are reported elsewhere [6]. Antibodies against actin or HSP-60 were used to assess the purity of the fractions and equal loading of the lanes.

Subcellular distribution of PKCα in intact cells. U937 cells were first incubated (20 min) with CMXRos (Molecular Probes Europe, Leiden, The Netherlands), a fluorescent probe that specifically stains mitochondria, treated as detailed in the text, and then processed for PKCα immunocytochemical determination, as previously described [6]. EGFP/PKCα stably transfected cells were loaded with CMXRos, treated, and analyzed by fluorescence microscopy to determine the localization of the EGFP/PKCα fusion protein. Cells were observed with a BX-51 microscope (Olympus, Milan, Italy) equipped with a SPOT-RT camera unit (Diagnostic Instruments, Sterling Heights, MI) and the images were digitally acquired on a personal computer using Scion Image software (Scion, Frederick, MD).

Results and discussion

Non-toxic concentrations of peroxynitrite nevertheless commit U937 cells to MPT, that is however prevented by a parallel survival signaling in which $cPLA_2$-dependent AA release [1,3], activation of PKCα [6], and mitochondria to cytosol translocation of Bad [7] are sequentially involved. These events were readily observed after addition of 100 μM peroxynitrite and their prevention, via pharmacological inhibition or genetic depletion of either $cPLA_2$ [3,6] or PKCα [6], was invariably associated with a very rapid onset of MPT-dependent necrosis. Likewise, toxicity was observed in cells genetically depleted in Bcl-2 [7]. Collectively, our previous studies indicate that U937 cells survive to peroxynitrite by reducing the fraction of mitochondrial Bad. This notion is also established by the experimental results illustrated in Fig. 1. The observation that a significant amount of Bad is localized in the mitochondria from untreated cells is consistent with the possibility that, under these conditions, Bcl-2/Bad heterodimers are being formed. Since this event causes loss of the anti-MPT activity of Bcl-2 [10,11], it does indeed make sense that these cells counteract commitment to MPT via a signaling cascade leading to the cytosolic translocation of Bad. The latter reaction is tightly regulated by the degree of Bad phosphorylation mediated by various kinases [13,15], including PKCα [14].

The above information, along with our previous findings indicating that peroxynitrite promotes the

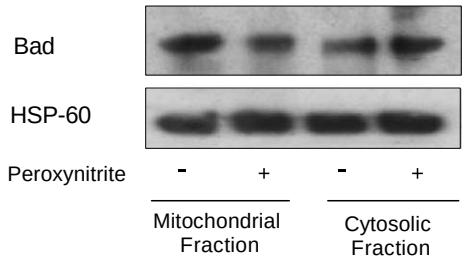

Fig. 1. Peroxynitrite promotes the translocation of Bad from mitochondria to the cytosol. Cells were exposed for 10 min to 100 μM peroxynitrite and then processed to obtain the mitochondrial and cytosolic fractions for Western blot analysis using an anti-Bad antibody. The results shown are representative of three separate experiments with similar outcomes.

mitochondria to cytosol translocation of Bad via a PKC-dependent mechanism [7], would therefore imply that activation of PKCα involves its translocation to the mitochondrial compartment. Such an event was indeed detected 10 min after exposure to 100 μM peroxynitrite by Western blot (Figs. 2A and B) and immunocytochemistry (Fig. 2D), using a PKCα antibody, as well as by fluorescence microscopy using U937 cells stably transfected with an EGFP-PKCα expression plasmid (Fig. 2E). Evidence of mitochondrial translocation of PKCα was also obtained upon exposure of U937 cells to tetradecanoylphorbol acetate (TPA, 100 ng/ml) (Figs. 2C and E).

The mitochondrial translocation of PKCα was prevented by pharmacological inhibition of PLA_2 and promptly re-established by exogenous AA. This notion was established by Western blot analysis of PKCα in the mitochondrial fraction (Fig. 2A) as well as by immunocytochemistry (Fig. 2D). It should be noted that the second experimental approach utilized 5,8,11,14-eicosatetraenoic acid (ETYA, 50 μM) instead of the more specific arachidonyl trifluoromethyl ketone ($AACOCF_3$, 50 μM) used in Western blot studies, since the latter is intrinsically fluorescent. Our previous work [1], however, clearly demonstrated that these two inhibitors can be interchangeably used, since they produce identical effects on toxicity and other parameters measured following the addition of peroxynitrite. The specificity of the above effects is emphasized by the observation that mitochondrial translocation of PKCα is insensitive to $AACOCF_3$, when induced by TPA (Fig. 2C), and suppressed by the PKC inhibitor Gö6850, regardless of whether induced by peroxynitrite (Fig. 2A) or TPA (Fig. 2C).

Taken together, these results strongly suggest that a non-toxic concentration of peroxynitrite causes an early mitochondrial translocation of PKCα via an AA-dependent mechanism. This event is associated with the translocation of Bad from the mitochondria to the cytosol [7] and with cell survival [6]. Indeed, cells exposed to 100 μM

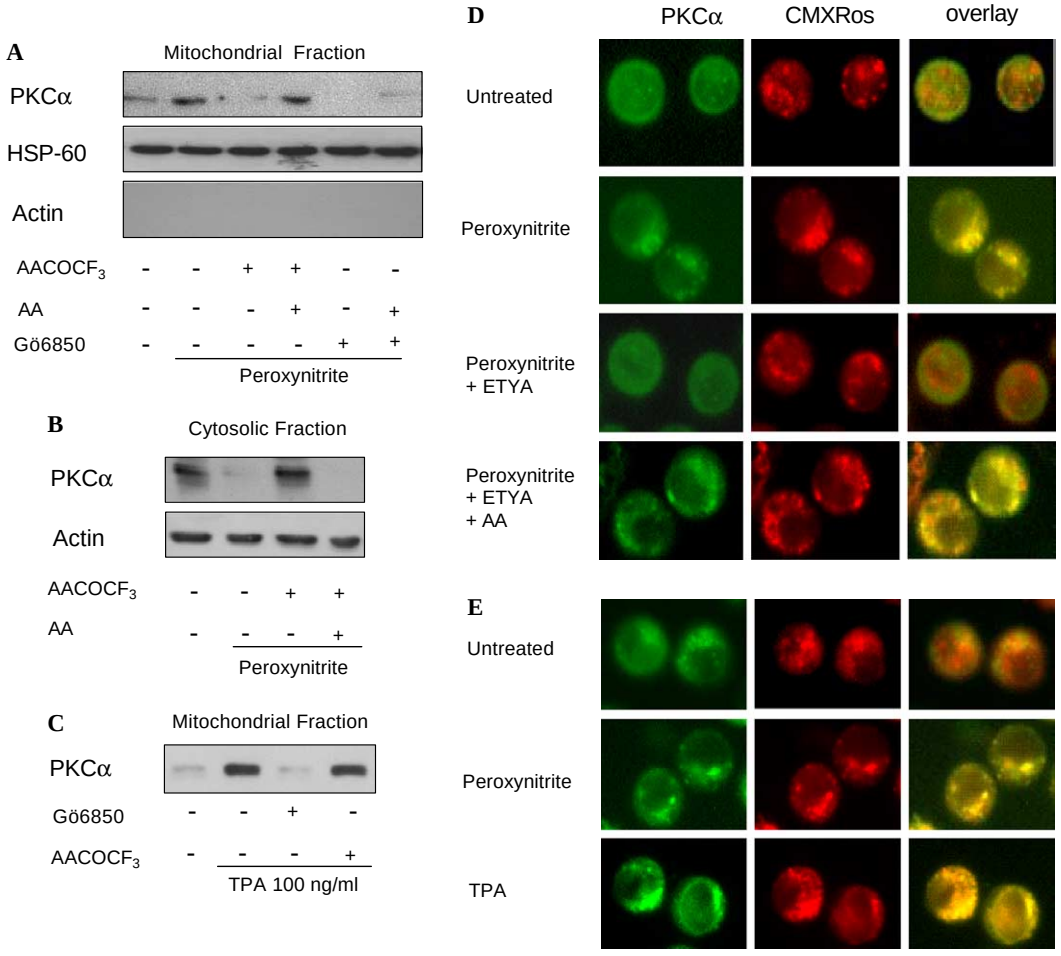

Fig. 2. Peroxynitrite promotes the mitochondrial translocation of PKCα via an AA-dependent mechanism. Cells were exposed for 3 min to 100 μM peroxynitrite and incubated for a further 7 min in the absence or presence of various additions, as indicated in the figure. In some experiments, the cells were treated for 20 min with 100 ng/ml TPA. Cells were then processed to obtain the mitochondrial and cytosolic fractions for Western blot analysis using an anti-PKCα antibody (A–C). In other experiments, U937 cells (D) or EGFP/PKCα-stably transfected U937 cells (E) were first incubated for 20 min in complete culture medium with a fluorescent probe that specifically stains mitochondria (500 nM CMXRos) and then treated as described above. Cells were then processed for the immunocytochemical assessment of PKCα (D) or analyzed by fluorescence microscopy to determine the localization of the EGFP/PKCα fusion protein (E). The results shown are representative of four separate experiments with similar outcomes.

peroxynitrite did not show signs of toxicity that was however readily observed after addition of inhibitors of either PLA_2 or PKC (52.7 ± 3 and $49.9 \pm 5\%$ reduction in the number of viable cells, respectively). Supplementation of AA (0.1 μM), or activation of PKC with TPA (100 ng/ml), prevented toxicity elicited by PLA_2 inhibition and failed to affect the lethal response evoked by the PKC inhibitor. It is important to note that viability was measured 60 min after addition of peroxynitrite, while toxicity was never detected after 10 min (i.e., the time at which translocation of Bad and PKCα was measured).

Bax, an additional member of the Bcl-2 family, can form heterodimers with Bcl-2 or Bcl-X_L [20]. Although physical interaction between these proteins neutralizes each other [20,21], Bax can nevertheless function independently and directly cause [22], or participate [10,23] in events provoking MPT. Bax normally resides in the cytosol of healthy cells but translocates to membrane sites, including mitochondria, in response to death signals [24,25]. Furthermore, the cytosolic localization of Bad prevents Bax translocation to the mitochondria [26], whereas the mitochondrial translocation of Bad prevents the interaction of Bax with Bcl-2/Bcl-X_L and allows the formation of Bax oligomers causing permeabilization of the outer mitochondrial membrane [24].

The results reported in Fig. 3 show that Bax is preferentially localized in the cytosol of untreated cells as well as of cells that received the non-toxic treatment with peroxynitrite. Interestingly, pharmacological inhibition of $cPLA_2$ or PKC, conditions resulting in the mitochondrial localization of Bad, promoted mitochondrial translocation of Bax via AA-sensitive and AA-insensitive mechanism, respectively. Thus, cells survive to peroxynitrite under conditions in which Bax is localized in the cytosol, whereas MPT-dependent necrosis is preceded by the mitochondrial translocation of Bax.

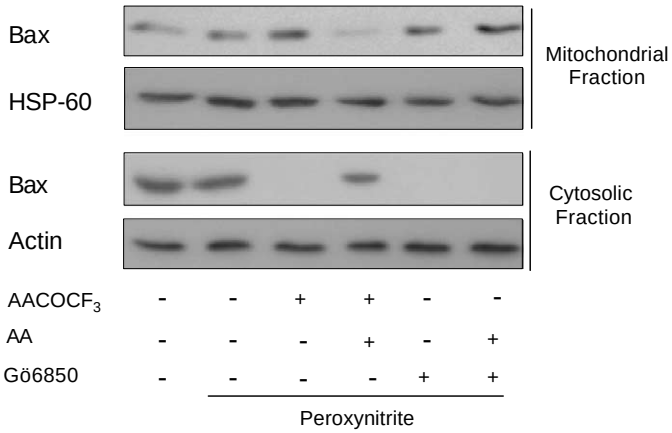

Fig. 3. Inhibition of the PLA_2/PKC-dependent survival pathway is associated with an early mitochondrial translocation of Bax. Cells were exposed for 3 min to 100 μM peroxynitrite and incubated for a further 7 min in the absence or presence of various additions, as indicated in the figure. Cells were then processed to obtain the mitochondrial and cytosolic fractions for Western blot analysis using an antibody recognizing Bax. The blots shown are representative of three separate experiments with similar outcomes.

Collectively, the results herein presented extend our previous findings on the mechanism whereby monocytes cope with peroxynitrite (Fig. 4). While committed to MPT, possibly via direct effects on the adenine nucleotide translocator in conjunction with alterations in their redox state [27], these cells can nevertheless survive in the presence of peroxynitrite because of an upstream signal driven by $cPLA_2$-released AA [1,2] resulting in the activation of PKCα [6]. We now report evidence indicating that AA causes the mitochondrial translocation of PKCα, an event causally linked to the translocation of Bad from the mitochondria to the cytosol and with a preferential localization of Bax in the cytosol. Inhibition of the survival signaling at the level of either PLA_2 or PKC prevents the early mitochondrial translocation of PKCα, as well as the inactivation of Bad [7], and enforces the translocation of Bax to

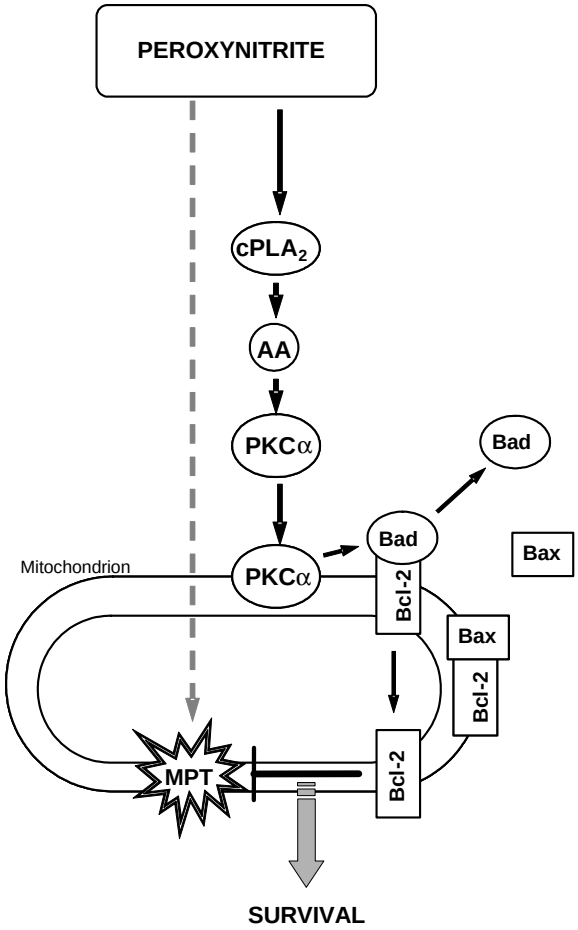

Fig. 4. Survival signaling preventing toxicity in U937 cells committed to MPT by peroxynitrite. A non-toxic, but nevertheless MPT-committing, concentration of peroxynitrite stimulates $cPLA_2$ activity and leads to AA release, which in turn promotes the mitochondrial translocation of PKCα. The latter, directly or indirectly phosphorylates Bad and causes its cytosolic localization, an event preventing the heterodimerization of Bad with Bcl-2. Under these conditions Bax is also kept in the cytosolic compartment. Bcl-2 can then homodimerize and prevent MPT (and necrosis).

the mitochondria. This condition is followed by a very rapid necrotic response associated with immediate cell lysis. Addition of AA to PLA_2 inhibitor-supplemented cells always prevented death and allowed the localization of PKCα to mitochondria and that of Bad [7] and Bax to the cytosol. Preventing the mitochondrial localization of these pro-MPT proteins will then allow the full expression of the anti-MPT function of Bcl-2 thereby leading to survival. The observation that MPT-dependent necrosis does not take place under conditions in which Bax is kept away from the mitochondria may also suggest a direct involvement of this protein in opening of permeability transition pores.

In conclusion, the results presented in this study, along with those previously obtained in our laboratory, provide a mechanism explaining how monocytes cope with peroxynitrite at the inflammatory sites. Under these conditions, endogenous as well as exogenous AA triggers an ATP-dependent [5] signaling response leading to survival. These cells, even when committed to MPT, can thus survive in the same environment in which other cells die. Finally, it is interesting to note that pharmacological inhibition of the survival signaling causes an extremely rapid necrosis, although the ATP pool is virtually unaffected [5]. Hence, these results put more weight on the emerging notion that necrosis is not always a passive response to an overwhelming damage associated with ATP depletion. Rather, at least under the conditions utilized in this study, necrosis appears to be a highly regulated mode of death, taking place as a consequence of the inhibition of a survival signaling. While the same signaling was previously described to prevent apoptosis induced by an array of different agents and conditions, it remains to be determined why monocytes (and macrophages) die by necrosis (and not by apoptosis). It is tempting to speculate that necrotic death represents an ultimate strategy of these cells to promote/sustain inflammation.

Acknowledgment

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