CMLS, Cell. Mol. Life Sci. 59 (2002) 894–901
1420-682X/02/050894-08 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Protein kinases mediate nitric oxide-induced apoptosis in the insect cell line IPLB-LdFB

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Received 15 March 2002; accepted 25 March 2002

Abstract. The involvement of protein kinases (PKA, PKC and PKB) in nitric oxide (NO)-induced apoptosis with sodium nitroprusside plus N-acetyl-L-cysteine in the IPLB-LdFB cell line from the insect *Lymantria dispar* was investigated. The presence of protein kinase-like molecules was demonstrated by Western blot analysis. The role of the kinases in programmed cell death was analysed in cytofluorimetric experiments by incubating the insect cells with H-89 (a specific inhibitor of PKA), calphostin C (an inhibitor of PKC) or wortmannin (an inhibitor of phosphatidylinositol 3-kinase). The results show that PKA is correlated with the induction and PKC and PKB with the prevention of NO-induced insect cell death. Moreover, NO-induced apoptosis involves the release of cytochrome c.

Key words. Insect cell line (IPLB-LdFB); nitric oxide; nitroprusside; S-nitroso-N-acetylpenicillamine; N-acetyl-L-cysteine; apoptosis; protein kinases; cytochrome c.

Apoptosis induced by nitric oxide (NO) donors such as nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP) and S-nitroglutathione has been investigated in several mammalian cell types [for a review see ref. 1]. However, the modalities by which NO induces apoptosis are still controversial, and different mechanisms have been proposed: (i) NO directly damages DNA by deamination and mutagenesis [2]; (ii) NO interacts with superoxide anion generating peroxynitrite, an apoptotic inducer [3]; (iii) NO disactivates different antioxidant enzymes including catalase and glutathione peroxidase [4]; (iv) NO provokes apoptosis by triggering mitochondrial permeability transition [5]; (v) the apoptotic effect may be mediated via the stimulation or inhibition of protein kinases [1].

In invertebrates, the relationship between NO and apoptosis is an unexplored field, with few studies in the literature [6–8]. In a previous paper, we found that the NO donor SNP induced both NO synthase-like molecules and apoptosis in the IPLB-LdFB cell line from the insect *Lymantria dispar* [9]. Cell death was observed after 48 h. The co-incubation of the cells with SNP plus N-acetyl-L-cysteine (NAC) not only increased the percentage of apoptosis, but also reduced the time before cell death (24 h) [9]. Interestingly, the programmed cell death of this cell line is not regulated by Bcl-2 [10, 11].

To define the possible pathways of the apoptotic signalling cascade, we examined here the role of protein kinases (PKA, PKC, PKB) in NO-induced cell death using SNP plus NAC in the insect cell line IPLB-LdFB. The following protein kinase inhibitors were used: H-89, an inhibitor of PKA [12, 13], calphostin C (CC), an inhibitor of PKC [14, 15] and wortmannin (Worth), an inhibitor of phosphatidylinositol (PI) 3-kinase [16]. The enzymatic activity of PI 3-kinase produces PI 3,4-bisphosphate and PI 3,4,5-trisphosphate, which activate PKB/Akt and some PKC iso-

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forms. In in vitro experiments, this process has been seen to occur by means of a protein kinase called PDK_1 [17]. However, these kinases show several multistep activation mechanisms, and the in vivo involvement of PDK_1 and 3-phosphoinositides has not yet been established [18].

Materials and methods

Cell cultures

The IPLB-LdFB cell line from the fat body of the insect *L. dispar* (Lepidoptera) was used [19]. The cells were cultured in Ex-Cell 405 medium at 26 °C.

Detection of apoptosis

Apoptosis in the insect cell line was analysed by light microscopy and flow cytometry, as reported in detail in previous papers [9, 20, 21]. Morphological studies were performed with May-Grünwald and Giemsa staining, and the cytofluorimetric analysis was detected by reduced fluorescence of the DNA-binding dye propidium iodide in the apoptotic nuclei, following the procedure of Nicoletti et al. [22]. The cells were centrifuged at 1800 rpm, and the pellet resuspended in 1 ml hypotonic fluorochrome solution containing 50 µg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100. The cytofluorimetric evaluations were performed after 30 min cell incubation. Results were obtained using a FACScan (Becton-Dickinson, Mountain View, Calif.) equipped with a single 488-nm argon laser. A total of 10,000 cells was acquired in list mode and analysed with the Lysys II software program.

Cell preparations for cytofluorimetric experiments

The cytofluorimetric detection of the apoptotic effects of NO donors on insect cells was carried out as follows: 300-µl cell suspensions (10^6 cells) were placed into plastic dishes and a final volume of 1000 µl was obtained by adding Ex-Cell 405 medium or the different substances either alone (10 µM H-89, 0.1 µM CC, 10 and 100 nM Worth, 10 mM NAC, 10 mM SNP or 0.5 mM SNAP) or in combination (10 mM SNP + 10 mM NAC or 0.5 mM SNAP + 10 mM NAC).

The role of protein kinases (PKA, PKC, PKB) in NO apoptosis induced by SNP + NAC was studied by adding the following inhibitors: 10 µM H-89, a PKA inhibitor; 0.1 µM CC, a PKC inhibitor; or 100 nM Worth, a PI 3-kinase inhibitor. The experiments were performed by incubating the cells for 24 h at 26 °C.

With the exception of SNAP, the concentrations of the different substances utilized in the present experiments have already been found to be effective without producing any cytotoxic effect in the same and in other invertebrate models [21, 23–25]. For SNAP, the concentration tested in the human leukaemia HL-60 cell line [26] was used. With regard to the incubation time, we reported in a previous paper that SNP alone induces apoptosis after 48 h incubation, whereas in combination with NAC, this phenomenon is seen after 24 h [9].

NO-induced apoptosis: immunoblot analysis of protein kinases

One-millilitre cell suspensions were placed into two plastic flasks and a final volume of 5 ml (10^6 cells/ml) was obtained by adding either Ex-Cell 405 medium (control) or 10 mM SNP + 10 mM NAC. The cells were incubated for 24 h at 26 °C. The presence of protein kinases (PKA, PKC, PKB) in treated samples and controls was detected by Western blot analysis, as reported in detail elsewhere [10]. Briefly, the insect cell line was lysated in lysis buffer. Total cell lysates (15 µl for each lane) were separated by SDS-PAGE (12%) and electrophoretically transferred to PVDF membranes (0.2-µm pore size). The following primary antibodies were used: anti-PKAα, anti-Akt1 and anti-cPKCα polyclonal antibodies (pAbs) (1:1000). Immunoreactive bands were visualized using a NBT/BCIP detection system.

NO-induced apoptosis: immunoblot analysis of cytochrome c and protein assay

Samples (10^6 cells/ml) were obtained by incubating the cells with 10 mM SNP + 10 mM NAC, or with 10 mM SNP + 10 mM NAC + 10 µM H-89, or with Ex-Cell 405 medium (control) for 24 h at 26 °C. The three samples were centrifuged at 200 g for 8 min, lysated and electrophoretically separated (15 µl for each lane). The immunoblot was performed as described above using an anti-cytochrome c pAb (1 : 500) as primary antibody. For protein determination, the cells of each sample were lysed in 5 vol. of a lysis buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 10 µg/ml antipain, 10 µg/ml bestatin, 1 mM phenylmethanesulphonyl fluoride brought to pH 7.0 at 20 °C with HCl and centrifuged at 13,000 g for 30 min at 4 °C. The supernatant and pellet were collected separately. Protein quantification was performed both on soluble and on particulate fractions according to Bradford [27] with bovine serum albumin as standard by using a Helios β spectrophotometer (Spectronic Unicam, Cambridge, UK).

Densitometric quantification by computerized image analysis

The cytochrome c immunoblot was acquired using a Sony MAVICA digital camera with the default settings and analysed with Matrix analysis software (Quanta Vision, Madison, Wisc.).

Statistical analysis

Each experiment was repeated in duplicate three times and statistical analysis was performed by Student's t test.

Chemical reagents

SNP and NAC were purchased from Sigma (USA), SNAP from Alexis Biochem, San Diego (USA), wortmannin (Worth), H-89 and CC from Biomol Biomolecules Res. (USA), anti-PKAα cat (C-20): *sc-903* pAb, anti-Akt1 (C-20): *sc-1618* pAb, anti-cPKCα (C-20): *sc-208* pAb and anti-cytochrome c (H-104): *sc-7159* pAb from Santa Cruz Biotechnology (USA), and Ex-Cell medium from JRH Biosciences (USA). All the reagents for western blot analysis were purchased from Bio-Rad (USA), except the blocking reagent and NBT/BCIP which came from Boehringer Mannheim (Germany).

Results

Cytofluorimetric analyses showed that both SNP and SNAP induced insect cell line apoptosis, and the percentage of cell death increased significantly ($p < 0.05$) after incubation of the cell with NO donors plus NAC (table 1, fig. 1). In contrast, NAC alone showed values in the same range as controls (table 1, fig. 1). Note that both NO donors showed similar results (table 1, fig. 1). Furthermore, the percentage of cell death in the presence of the different protein kinase inhibitors used alone was comparable to that of controls (fig. 2). The cytofluorimetric results were also confirmed by morphological observations (data not shown).

The Western blot analyses carried out on the insect cells incubated with SNP + NAC showed a different involvement of protein kinases in the NO-induced apoptosis. Both in treated cells and controls, a single immunoreactive PKA band with a molecular weight (MW) of 49 kDa was observed (fig. 3). Two bands were detected for PKC (94 and 79 kDa, respectively) and PKB (64 and 54 kDa, respectively). The PKC immunoreactive band corresponding to a MW of 94 kDa was absent in the treated samples (fig. 3).

Flow cytometry with the various protein kinase inhibitors revealed a dose-correlated action and a different behaviour of the kinases in the cell apoptotic phenomenon. As

Table 1. Percentage of apoptotic cells in the IPLB-LdFB cell line from *Lymantria dispar*.

Substances	Mean ± SD
Control	3.82 ± 0.14
NAC	3.90 ± 0.08
SNP	18.46 ± 0.51*
SNP + NAC	31.62 ± 0.99*
SNAP	17.06 ± 0.36*
SNAP + NAC	33.16 ± 0.43*

The mean ± SD of six experiments is shown. Statistical analysis was performed by Student's t-test (* $p < 0.05$ vs control).

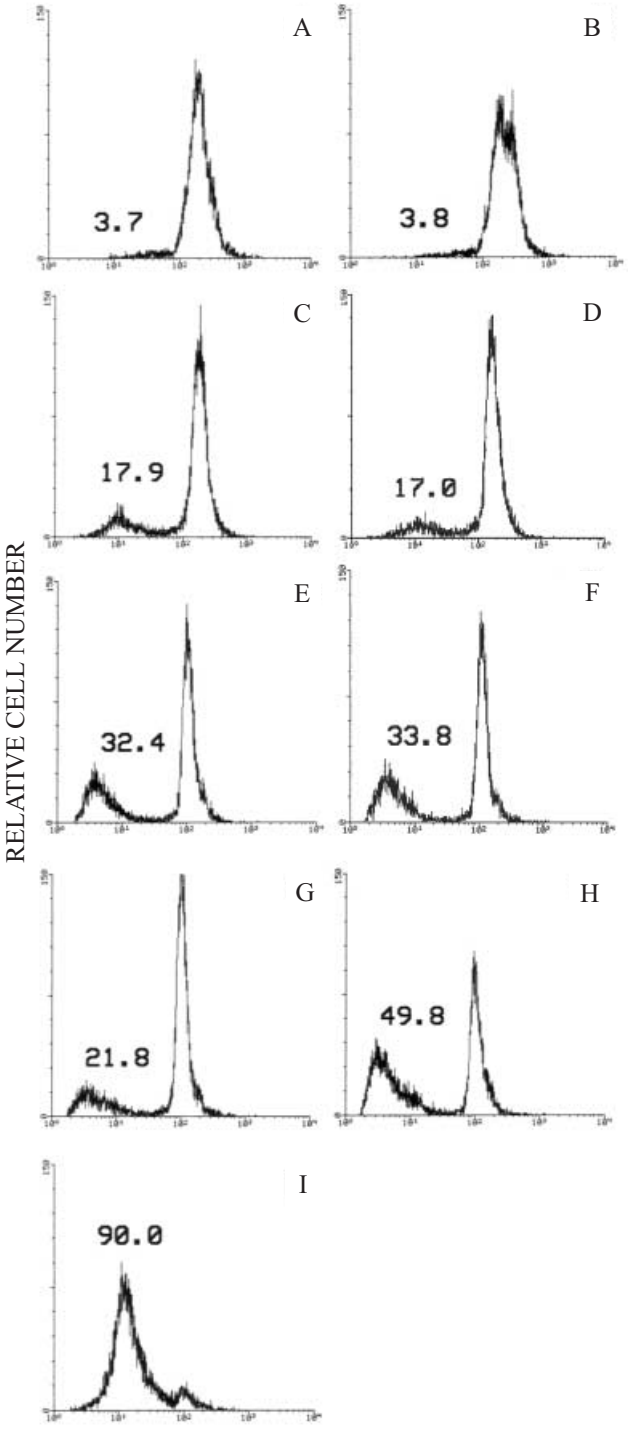

Figure 1. Cytofluorimetric analysis of apoptosis in the insect cell line IPLB-LdFB after 24 h incubation with different substances: medium (control) (*A*); 10 mM NAC (*B*); 10 mM SNP (*C*); 0.5 mM SNAP (*D*); 10 mM SNP + 10 mM NAC (*E*); 0.5 mM SNAP + 10 mM NAC (*F*); 10 mM SNP + 10 mM NAC + 10 µM H-89 (*G*); 10 mM SNP + 10 mM NAC + 0.1 µM calphostin C (CC) (*H*); SNP + 10 mM NAC + 100 nM wortmannin (Worth) (*I*). The numbers in each panel refer to the percentage of apoptotic cells. One experiment representative of a set of six is shown.

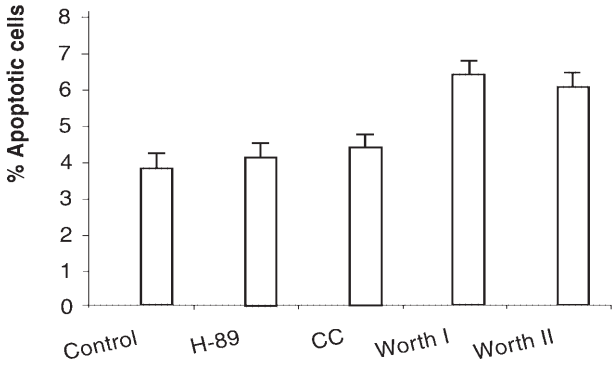

Figure 2. Percentage of cell death in insect cell line IPLB-LdFB in the presence of the different protein kinase inhibitors: 10 µM H-89, 0.1 µM CC, 10 nM Worth I and 100 nM Worth II.

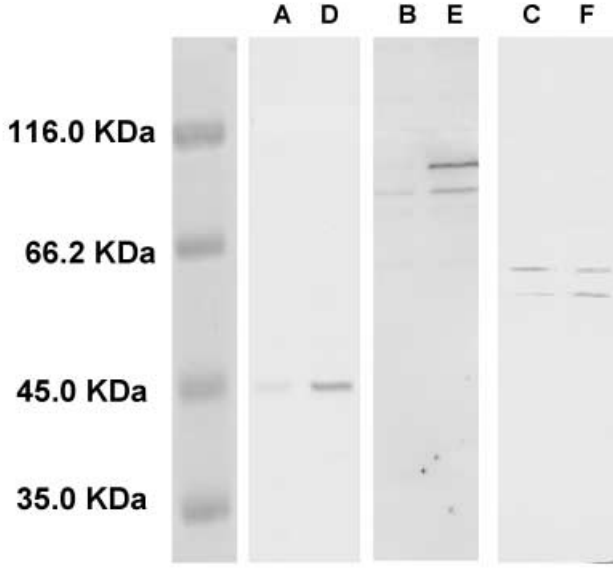

Figure 3. Western blot analysis of the protein kinases from the insect cell line IPLB-LdFB before (PKA, lane A; PKC, lane B; PKB, lane C) and after 24-h incubation with 10 mM SNP + 10 mM NAC (PKA, lane D; PKC, lane E; PKB, lane F).

Figure 4. Immunoblot analysis of cytochrome (cyto) c in cell lysates from the insect cell line IPLB-LdFB: (1) control; (2) cells incubated with 10 mM NAC + 10 mM SNP for 24 h; (3) cells incubated with 10 mM NAC + 10 mM SNP + 10 µM H-89 for 24 h.

reported in table 2 and in figure 1, the incubation of the cells with SNP + NAC and H-89 (a PKA inhibitor) showed a significant decrease in the percentage of apoptotic cells with respect to values obtained with SNP + NAC. An opposite effect was observed for CC (a PKC inhibitor), which showed a pronounced increase in cell death. This trend was again more evident in the presence of Worth, an inhibitor of PI 3-kinase. Indeed, the percentage of apoptotic cells rose about three times in comparison to the results with SNP + NAC. With SNP + NAC + CC, the increase was of the order of one and a half times compared to SNP + NAC (table 2).

The immunoblot analysis performed on the total cell lysate pellets to reveal the presence of cytochrome c showed three bands of different intensity (fig. 4). The band corresponding to control cells was the most pronounced (lane 1), while that obtained from samples incubated with SNP + NAC was the least evident (lane 2). The samples incubated with SNP + NAC + H-89 showed an intermediate intensity (lane 3). The densitometric quantification of the cytochrome c immunoblot of lanes 2 and 3 showed an intensity of 18.9% and 64.3%, respectively, with respect to lane 1 (100%, control) (fig. 5A). These findings are in agreement with protein quantification according to the Bradford method carried out both on the particulate and the soluble fractions of the three samples. The particulate fraction of the three samples showed the same protein amount (80 µg/µl), while different quantities were found in the soluble fractions of the three samples: 36 µg/µl (control), 12.75 µg/µl (cells incubated with SNP + NAC) and 16.44 µg/µl (cells incubated with SNP + NAC + H-89) (fig. 5B). A corresponding relationship exists between the densitometric results and the protein measurement of the soluble fraction of the three samples (fig. 5A, B).

Table 2. Percentage of apoptotic cells in the IPLB-LdFB cell line from *L. dispar*: dose-response of the different protein kinase inhibitors.

Substances	Mean ± SD
SNP + NAC	31.62 ± 0.99
SNP + NAC + 1 µM H-89	31.04 ± 0.52
SNP + NAC + 10 µM H-89	22.77 ± 0.95*
SNP + NAC + 50 µM H-89	cytotoxic
SNP + NAC + 0.01 µM CC	31.80 ± 0.21
SNP + NAC + 0.1 µM CC	48.17 ± 1.47*
SNP + NAC + 1 µM CC	cytotoxic
SNP + NAC + 1 nM Worth	31.75 ± 0.33
SNP + NAC + 10 nM Worth	88.78 ± 0.19*
SNP + NAC + 100 nM Worth	89.01 ± 1.61*

The mean ± SD of six experiments is shown. Statistical analysis was performed by Student's t-test (* $p < 0.05$ vs SNP + NAC).

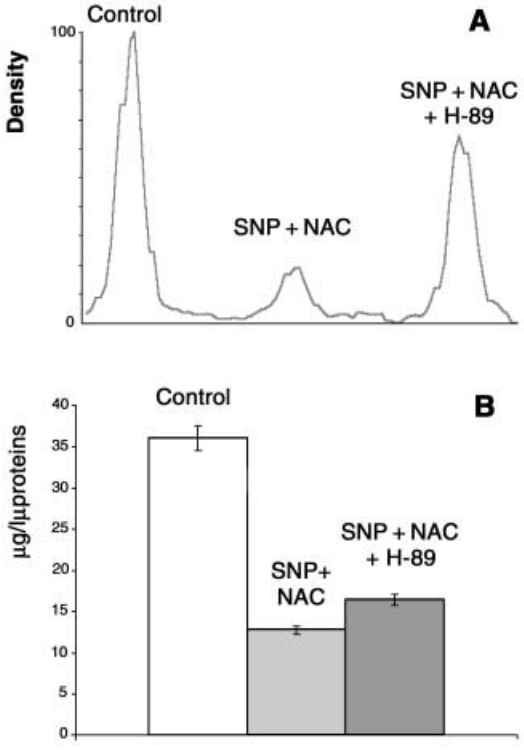

Figure 5. Correlation between the densitometric quantification of the cytochrome c immunoblot bands (*A*) and the protein assay of the soluble fractions (*B*) of the lysate insect cell line IPLB-LdFB.

Discussion

As reported in previous papers [9, 20, 21], the present cytofluorimetric and morphological data on apoptosis in the IPLB-LdFB cell line from the fat body of the insect *L. dispar* confirm that this type of cell death is well conserved during evolution. Indeed, not only does it occur naturally, but it can also be induced by different substances. We have found that both 2-deoxy-D-ribose (dRib) [20] and SNP induce apoptosis [9]. With regard to the latter, the exposure of cells to SNP + NAC generates higher time- and concentration-dependent rates of cell death than SNP alone. In agreement with Ignarro et al. [28], this behaviour could be explained as follows. SNP spontaneously releases NO which, in turn, reacts with cysteine or, in our case, NAC to form active intermediates such as S-nitrosothiols, resulting in a subsequent increase in the bioavailability of NO.

Recently, Terwel et al. [29] have claimed that SNP provokes apoptosis by the production of hydrogen peroxidase rather than NO. Our results with SNP are, however, substantiated by SNAP, giving further support for the involvement of NO in IPLB-LdFB cell line apoptosis.

This pharmacological approach to the study of one of the possible mechanisms in NO-induced cell death, i.e. the involvement of protein kinases, has demonstrated that all the protein kinases examined (PKA, PKC and PKB) are involved in the insect cell line apoptosis. Inhibition of the various protein kinases in the specific signal transduction pathways modulates the apoptotic effects of NO in different ways. We have obtained data in support of pro- and anti-apoptotic effects, suggesting that PKA enhances the NO-related apoptosis, while PKC and PKB have an opposite effect. The high percentage of apoptotic cells observed in the presence of the inhibitor of PI 3-kinase supports the hypothesis that both PKB and PKC are a downstream target of PI 3-kinase activation [17, 18].

Generally speaking, PKA is associated with the induction and PKC and PKB with the prevention of NO-induced insect cell death. The literature reveals some dispute in defining the relationship between protein kinases and apoptosis in both vertebrates and invertebrates. With regard to mammalian cells, PKA and PKC play a protective role in NO-induced apoptotic cell death in murine RAW 264.7 macrophages [30, 31]. In contrast, the PKC activator phorbol ester causes an enhancement of NO-related apoptosis in HL-60 human promyelocytic leukaemia cells [26]. Moreover, SNAP cell death is prevented by PKC and PKA inhibitors in smooth muscle cells [32]. In human cancer cell lines (IPC-81, MIA PaCa-2, MCF-7, A-172), the activation of PKA by increasing cAMP levels or directly by cAMP analogues is correlated with the induction of apoptosis [33–36], while CC, a specific PKC inhibitor, induces cell death [37, 38]. The opposite result is reported in thymocytes: the apoptosis induced by hydrocortisone is prevented by adding the PKC inhibitor H-7 to the cells [39]. The inhibition of PKA does not significantly affect cell death in HL-60 cells [40], whereas raising cAMP levels by forskolin increases apoptosis in human B precursor cells [41]. Recently, Kanaoka et al. [42] reported the involvement of PKA in the prevention of apoptosis by calcitonin in murine osteoclast-like cells following treatment with NO-releaser NOC18. The activation of PKA seems to regulate the main step in cell death commitment. Indeed, PKA was detected in a site upstream of the activation of pro-caspase-9 and -3 enzymes that could be involved in osteoclast apoptosis by NO. Finally, the findings for PKB are more concordant. Most studies report an involvement of this kinase in protecting cells from apoptosis [43, 44]. In invertebrates, we have previously found that the survival effect elicited by PDGF-AB and TGF-β1 against the apoptotic inducer dRib in an insect cell line (IPLB-LdFB) requires the activation of PKA, PKC and PKB [21]. The protein kinase inhibitor H-7 induces cell death in the *Drosophila* cell line ML-DmBG2-c2, while specific protein kinase inhibitors, such as H-89, CC, ML-9 or CKI-7, do not affect apoptosis [45].

The controversial findings on the role of protein kinases in the regulation of NO-induced cell death could be as-

cribed to a combination of various factors, e.g. type of signal received from the cell, type of cell and target. This would lead to different protein kinase behaviour. Nevertheless, these findings suggest that both in invertebrates and vertebrates, apoptosis may also be regulated by the activity of signal transduction pathways involving PKA, PKC and PKB.

In the present insect model, the findings further show the involvement of cytochrome c in the apoptotic signalling cascade provoked by NO donor. The immunoblot analysis of the cytochrome c with the related densitometric analysis demonstrated the maximum intensity in controls, while the lowest positivity was observed in the cells incubated with SNP plus NAC. An intermediate value was detected in the cells incubated with SNP plus NAC plus H-89. The protein quantification of the soluble fraction of controls, of cells incubated with SNP plus NAC or with SNP plus NAC plus H-89 showed the same trend as that observed in the densitometric analysis, while the protein content of the particulate fractions was constant. This suggests that the different degree of immunoblot intensity is due to a different amount of cytochrome c in each sample. Considering that each experiment was performed using the same number of cells and showing the same amount of protein in the particulate fraction at the end of the experiment, these findings indicate that a part of the soluble fraction lost as a result of the apoptotic processes includes cytochrome c. Bearing in mind its usual location and the unmodified quantity of particulate-associated proteins, cytochrome c loss during apoptosis could suggest its release from mitochondria to cytosol. The less pronounced densitometric value observed in the presence of the PKA inhibitor H-89 suggests a link between the cytochrome c release and the PKA action. Furthermore, these findings are also in line with the cytofluorimetric results, i.e. that NO through PKA induces apoptosis. As discussed by Goldstein et al. [46], we are aware that the detection of cytochrome c release from an entire cell population raises various questions, but, in our case, we are only interested in verifying its involvement in a model in which Bcl-2 does not regulate programmed cell death [10, 11].

It should be underlined that the PKA inhibitor H-89 only partially prevents NO-mediated cell death, suggesting that apoptotic pathways other than that involving PKA occur. Recently, Chung and colleagues [47] in reviewing the pro-death pathways followed by NO donors reported a common final step, i.e. the release of cytochrome c which, in turn, activates the caspase family. Four different mechanisms provoking cytochrome c release were described. In one case, NO directly provokes mitochondrial membrane depolarization (MMD), while in the others, NO indirectly acts on the upregulation of p53, on the activation of JNK/SAPK and p38 MAPK and on ceramide generation, respectively.

While we have the same downstream pattern in our insect cell line, i.e. cytochrome c release, a slightly different situation is seen upstream. Ceramide is not involved, since we found no inhibition of PKB, a mechanism described in the TF-1 and COS-7 cell lines, respectively [48, 49]. However, the other suggested pathways cannot be excluded in our insect cells. With regards the effect provoked by the direct action of NO, it should be remembered that one of the candidates in the control of cytochrome c release from mitochondria, i.e. Bcl-2, is not involved in apoptosis in IPLB-LdFB, since blocking this molecule resulted in MMD without any apoptotic phenomena [10]. Furthermore, we found another pathway that involves a pro-death role for PKA.

Two types of PKA are described, PKA-I and PKA-II, which present a common C subunit and different regulatory subunit, RI and RII, respectively. These, in turn, show four isoforms, RIα, RIβ, RIIα and RIIβ [50]. PKA has been seen to induce Bcl-2 hyperphosphorylation and apoptosis [51]. Yang et al. [52] have reported details on the link between PKA-I and the apoptotic mechanism, showing that the PKA-I subunit (RIα isoform) is involved in the regulation of cytochrome c oxidase activity and in cytochrome c release. Interestingly, the present study also shows a correlation between PKA action, cytochrome c release and the induction of apoptosis, but in our insect cell model, Bcl-2 does not affect programmed cell death [10, 11].

Acknowledgements. The authors are grateful to Dr. D. E. Lynn (USDA/ARS, Insect Biocontrol Lab. BARC-West, Beltsville, Md.) who kindly supplied the cell line, and Prof. A. Cossarizza (Dept. Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy) who allowed us access to the cytofluorimeter in his laboratory. This work was supported by MIUR (Italy) grant to E. O.

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