

# Bcl-2 prevents nitric oxide-mediated apoptosis and poly(ADP-ribose) polymerase cleavage

Zora Mělková<sup>\*1,a</sup>, Sean Bong Lee<sup>2,b</sup>, Dolores Rodriguez<sup>c</sup>, Mariano Esteban<sup>a,b,c,\*</sup>

<sup>a</sup>Department of Biochemistry, SUNY, Health Science Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, NY 11203, USA

<sup>b</sup>Department of Microbiology and Immunology, SUNY, Health Science Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, NY 11203, USA

<sup>c</sup>Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, 28049 Madrid, Spain

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**Abstract** Toxic effects of nitric oxide (NO) were suggested to be mediated by its metabolite peroxynitrite, a strong oxidizing agent. To determine if antioxidative effects of Bcl-2 proto-oncogene can prevent NO-mediated apoptosis, we used vaccinia virus recombinants expressing mouse *inducible NO-synthase*, *iNOS*, or human *bcl-2* genes. Expression of *iNOS* in HeLa G cells induces apoptosis which can be prevented by co-expression of *bcl-2* or by addition of reduced glutathione or *N*-acetylcysteine. We demonstrate that this NO-induced apoptosis proceeds through the activation of interleukin-1 $\beta$ -converting enzyme-like proteases and cleavage of the poly(ADP-ribose) polymerase, an effect which is also prevented by Bcl-2.

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**Key words:** Nitric oxide; Apoptosis; Bcl-2; PARP

## 1. Introduction

Although extensive biochemical and morphological studies reveal several different mechanisms involved in the induction of apoptosis, the key events underlying development of apoptosis remain largely unknown. The *bcl-2* protooncogene has been shown to prevent apoptosis in multiple contexts: it protects hematopoietic [1,2] and neuronal cell lines [3,4] from apoptosis caused by growth factor withdrawal, and lymphoid cells from apoptosis induced by  $\gamma$ -irradiation, calcium ionophores, or corticosteroids [5,6]. Bcl-2 prevents apoptosis caused by anti-cancer drugs [7,8] and protects infected cells from virus-induced apoptosis [9–11]. Bcl-2 was also shown to prevent H<sub>2</sub>O<sub>2</sub>-mediated lipid peroxidation and apoptosis [12] in which the hydroxyl free-radical is thought to be the most

toxic species, thus suggesting that Bcl-2 contributes to the regulation of cellular levels of reactive oxygen intermediates (ROI).

Hydroxyl radical-like species also originate from the decomposition of peroxynitrite, a product of nitric oxide (NO) reaction with superoxide radical [13,14]. Chemistry of NO seems to be dominated by its interaction with superoxide, and peroxynitrite has been suggested to mediate many of the inhibitory effects of NO, such as DNA damage [15], modification of protein thiols [16] or lipid peroxidation [17]. NO was reported to cause adverse effects like inhibition of ribonucleotide reductase, inactivation of mitochondrial enzymes of the respiratory chain and Krebs cycle [18], as well as oxidation of protein thiol groups mediated by nitrosonium ion (NO<sup>+</sup>) [19]. Nitrosonium ion- or peroxynitrite-mediated oxidation of protein thiol groups leads to the formation of disulfide bridges and changes in protein properties [16,19]. All these reactions are likely to significantly contribute to the apoptosis induced by NO [20].

NO-mediated DNA damage and neurotoxicity leading to apoptosis were shown to involve activation of poly(ADP-ribose) polymerase (PARP) which catalyses addition of poly(ADP-ribose) residues to several nuclear proteins, including histones, PARP itself or Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonuclease [21]. The significance of this modification of nuclear proteins for the DNA repair process has not been clearly defined; however, synthesis of poly(ADP-ribose) causes a depletion of intracellular NADH and ATP which has been suggested as a possible cause of apoptosis [21,22]. Recently, accumulating evidence defines PARP also as a death substrate for CPP32/apopain, member of the ICE-like family of aspartate-specific cysteine proteases [23,24].

Since Bcl-2 was reported to exert antioxidative effects and ROI play a significant role in the chemistry of NO, it was of interest to determine if Bcl-2 could prevent apoptosis caused by NO. In this report, using vaccinia virus (VV) recombinants expressing either mouse *inducible NO-synthase* gene [25] or human *bcl-2* gene [26], we show that overexpression of *bcl-2* inhibits NO-mediated apoptosis. Further, we demonstrate that this NO-mediated apoptosis proceeds through the activation of ICE-like proteases and cleavage of PARP, a process which is also prevented by Bcl-2.

## 2. Materials and methods

### 2.1. Cells

Human cervical carcinoma HeLa G (Glasgow strain) and African green monkey kidney BSC-40 cell lines were grown in Dulbecco's modified Eagle medium (DMEM; glucose 4.5 g/l) supplemented with 10% neonatal calf serum (NCS). The cells were maintained at 37°C, in a 5% CO<sub>2</sub> atmosphere with 95% humidity. All the media and

\*Corresponding authors. Fax: (42) 2 2491-2834; (34) 1 585-4506.

<sup>1</sup>Current address: Department of Pathological Physiology, 1st Medical Faculty, Charles University, Unemocnice 5, 128 53 Prague 2, Czech Republic.

<sup>2</sup>Current address: MGH Cancer Center, Molecular Genetics, CNY7, Charleston, MA 02129, USA.

**Abbreviations:** NO, nitric oxide; iNOS, inducible nitric oxide synthase; VV, vaccinia virus; WRNOS, VV recombinant expressing *iNOS*; WRbcl2, VV recombinant expressing *bcl2*; CAT, chloramphenicol acetyltransferase; WRCAT, VV recombinant expressing *CAT*; ROI, reactive oxygen intermediate; PARP, poly(ADP-ribose) polymerase; ICE, interleukin-1 $\beta$ -converting enzyme; HeLa G, human cervical carcinoma cell line; BSC-40, African green monkey kidney epithelial cell line; DMEM, Dulbecco's modified Eagle medium; NCS, neonatal calf serum; IPTG, isopropyl- $\beta$ -thiogalactoside; PFU, plaque forming unit; h.p.i., hours postinfection; TBARS, thiobarbituric acid reactive substance; GSH, reduced glutathione; NAC, *N*-acetylcysteine

growth supplements were purchased from Gibco BRL, unless otherwise specified.

## 2.2. Viruses

VV recombinants WRNOS [27] and WRbcl-2 [28] that express either mouse *inducible NO-synthase* gene [25] or human *bcl-2* gene [26], respectively, were used. Another VV recombinant expressing the *chloramphenicol acetyltransferase* gene (WRCAT) [29] was used as a control. All the recombinants were generated by homologous recombination into the thymidine kinase locus of VV genome using VV insertional vector pPR35 [30]. In this vector, the expression of the recombinant gene is regulated by *E. coli* LacI operator/repressor elements and is induced by isopropyl- $\beta$ -thiogalactoside (IPTG) [30].

The viruses were propagated in BSC-40 cells and their titers were determined by serial dilutions and plaque assays in the same cells. For the infections of HeLa G cells, crude virus stocks were used at multiplicity of infection (PFU/cell) specified in each experiment. Virus was adsorbed to the cells for 1 h in the presence or absence of IPTG. Virus inoculum was then removed, cells were washed with DMEM, and supplemented with DMEM containing 2% NCS, 1 mM arginine, with or without 1.5 mM IPTG. At 36 h postinfection (h.p.i.), an aliquot of medium was removed for nitrite and nitrate determination and the cells were collected in medium, pelleted, and processed as indicated below. For virus titration, the cells were resuspended directly in the culture medium, lysed by two cycles of freezing-thawing and sonication, and virus yields were determined by plaque assays in BSC-40 cells.

## 2.3. Isolation of low molecular weight DNA

Low molecular weight DNA was isolated as described [31]. Cells were lysed in a buffer containing 20 mM Tris-HCl, 10 mM EDTA, pH 8, and 1% Triton X-100, and high molecular weight DNA was removed by centrifugation at  $10000\times g$  for 10 min. The supernatant was extracted with phenol/chloroform/iso-amyl alcohol (25:24:1), and low molecular weight DNA was precipitated with ethanol, resuspended in 10 mM Tris-HCl and 5 mM EDTA, pH 8, and treated with RNaseA (5  $\mu$ g/ml) at 37°C for 1 h. DNA was resolved by 2% agarose gel electrophoresis in 1 $\times$ TBE buffer, stained with ethidium bromide, and visualized by UV.

## 2.4. NO determination

Production of NO by iNOS was assayed by measuring the accumulation of nitrite and nitrate, NO oxidation products, in the culture medium by the diazotization assay with Griess reagent [32]. Nitrite was determined in an aliquot of each sample by adding an equal volume of Griess reagent (0.5% sulfanilamide, 0.05% naphthylethylenediamine, 2.5%  $H_3PO_4$ ), and by reading absorbance at 550 nm within 10–15 min. Total nitrite and nitrate were determined after conversion of nitrate to nitrite with nitrate reductase as described [33]. Sodium nitrite was used as a standard. Sulfanilamide, naphthylethylenediamine dihydrochloride, and sodium nitrite were purchased from Sigma, and 85% *o*-phosphoric acid from Fisher. Nitrate reductase from *Aspergillus Niger* was from Boehringer.

## 2.5. Other methods

Western blot analysis was performed as described [34], using enhanced chemiluminescence (ECL kit, Amersham). iNOS was detected using a rabbit polyclonal antibody against mouse macrophage NOS (from Transduction Laboratories, Kentucky; dilution 1:200); PARP was detected using the mouse monoclonal antibody C2-10 (from Centre de Recherche du Chul, Quebec, Canada; dilution 1:1000). Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) [35] using chemicals from Sigma, and by decrease of fluorescence of *cis*-parinaric acid [36] from Molecular Probes, USA.

## 3. Results and discussion

### 3.1. Bcl-2 prevents NO-mediated apoptosis

To evaluate the induction of apoptosis by iNOS expression, HeLa G cells were infected with 1 PFU/cell of WRNOS recombinant virus [27] containing mouse *iNOS* gene [25]. At various times postinfection, low molecular weight DNA was

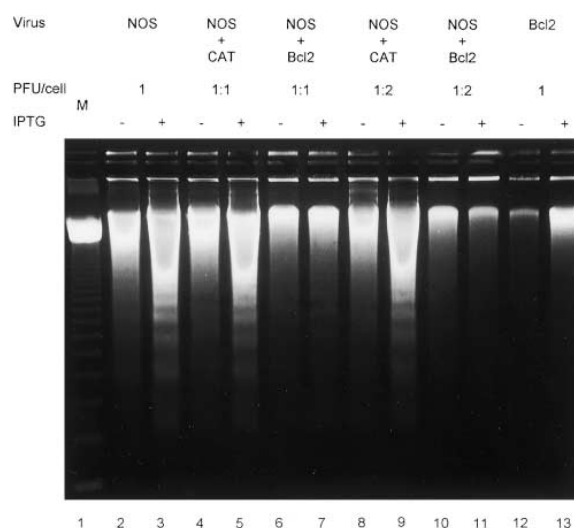


Fig. 1. Bcl-2 prevents NO-induced DNA fragmentation.  $7.5\times 10^6$  of HeLa G cells in 60 mm plates were infected with 1 PFU/cell of WRNOS alone (lanes 2 and 3) or in combination with 1 or 2 PFU/cell of WRCAT (lanes 4, 5 and 8, 9) or WRbcl2 (lanes 6, 7 and 10, 11), in the absence or presence of 1.5 mM IPTG. At 36 h.p.i., cells were collected, low molecular weight DNA was isolated and resolved by 2% agarose gel electrophoresis [29]. Top denotes the virus origin, multiplicity of infection and IPTG treatment. M, molecular weight markers.

isolated and resolved by agarose gel electrophoresis. The induction of iNOS expression with IPTG resulted in a degradation of cellular DNA into typical internucleosomal fragments by 36 h.p.i. (Fig. 1, lane 3). A limited DNA damage occurred also in the uninduced cells (Fig. 1, lane 2) due to a leaky expression of the *iNOS* gene, as noted previously with other inducible genes [37]. A similar induction of apoptosis was observed also in the cells infected simultaneously with WRNOS (1 PFU/cell) and a control VV recombinant expressing the *CAT* gene, WRCAT (1 or 2 PFU/cell) [29], (Fig. 1, lanes 4, 5, and 8, 9). In contrast, when the cells were infected simultaneously with WRNOS and a VV recombinant expressing the human *bcl-2* gene [26], WRbcl2 [28], a significant inhibition of DNA degradation was observed with 1 PFU/cell (Fig. 1, lanes 6 and 7) or 2 PFU/cell of WRbcl2 (Fig. 1, lanes 10 and 11). The pattern of DNA was similar to that observed in cells infected with WRbcl2 alone (lanes 12 and 13). Bcl-2-mediated inhibition of apoptosis induced by NO was also confirmed by fluorescence microscopy after staining the infected cells with the Hoechst DNA intercalating dye [38]. Using this analysis, we estimated that about 40–50% of the cells infected with WRNOS underwent apoptosis by 36 h.p.i.; Bcl-2 completely prevented the NO-mediated cell death.

### 3.2. Bcl-2 prevents development of apoptosis without any effect on NO-mediated inhibition of VV growth

We have previously shown that induction of iNOS expression and NO production resulted in the inhibition of VV replication at the level of DNA synthesis [27]. Therefore, we examined whether Bcl-2 affects VV growth, expression of iNOS, and/or levels of nitrite and nitrate. The levels of iNOS expression in cells infected simultaneously with WRNOS and WRbcl2 or with WRNOS and WRCAT were analyzed by Western blotting. The results indicated that expression of *bcl-2* did not affect expression of iNOS (data not

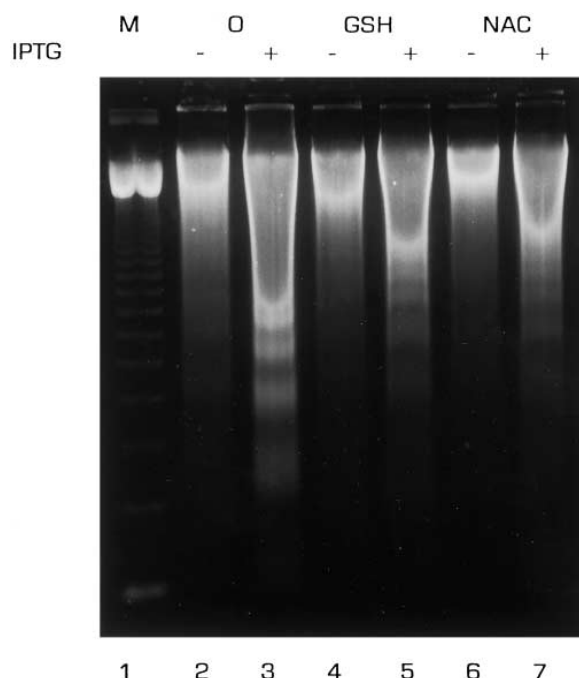


Fig. 2. Glutathione and *N*-acetylcysteine partially prevent NO-induced DNA damage.  $6.6 \times 10^6$  of HeLa G cells in 60 mm plates were infected with 1 PFU/cell of WRNOS in the presence or absence of IPTG. The culture medium was supplemented with 25 mM reduced glutathione (GSH) or *N*-acetylcysteine (NAC). At 36 h.p.i., cells were collected, low molecular weight DNA was isolated and resolved by 2% agarose gel electrophoresis. O, untreated cells; M, molecular weight markers.

shown). The analysis of the levels of nitrite and nitrate, NO oxidation products, revealed that Bcl-2 did not alter NO production either (Table 1). Nevertheless, in cells infected with either combination of the two viruses (WRNOS and WRbcl2 or WRNOS and WRCAT), the levels of nitrite and nitrate as well as levels of iNOS protein were decreased when compared

to the cells infected with WRNOS alone. This decrease was due to the competition of the two viruses. However, both cells infected with WRNOS and WRbcl2 or with WRNOS and WRCAT produced similar high levels of NO upon induction by IPTG, compared to cells infected only with WRCAT. High levels of NO produced early in VV growth cycle cause inhibition of its growth [27,39], and Bcl-2 could not reverse this antiviral effect of NO (Table 1). The results of Fig. 1 and Table 1 demonstrate that Bcl-2 prevents NO-mediated apoptosis without affecting NO-mediated inhibition of VV growth or the production of nitrite and nitrate.

### 3.3. Glutathione and *N*-acetylcysteine reverse NO-mediated apoptosis and rescue VV growth

Peroxynitrite, a product of reaction of NO with superoxide radical [13,14], can yield hydroxyl radical-like species causing DNA and protein damage, as well as lipid peroxidation [13,17]. Both ROI-mediated lipid peroxidation and apoptosis can be prevented by overexpression of *Bcl-2* or by addition of *N*-acetylcysteine (NAC), a precursor in glutathione synthesis (GSH) [12]. To examine whether GSH and NAC can prevent NO-mediated apoptosis, HeLa G cells were infected with WRNOS in the presence or absence of IPTG and the culture medium was supplemented with 25 mM reduced glutathione or *N*-acetylcysteine. As shown in Fig. 2, both GSH (lanes 4 and 5) and NAC (lanes 6 and 7) reduced cellular DNA degradation caused by NO. At the same time, NO-mediated inhibition of VV growth was prevented completely by GSH and partially by NAC (data not shown). This result is in contrast to the effect of Bcl-2 which does not rescue VV growth, thus suggesting that the mechanism of action of Bcl-2 is different from GSH or NAC.

### 3.4. Lipid peroxidation is not enhanced during NO-mediated apoptosis

High production of NO is a known cause of cell-mediated toxicity and apoptosis [20,21], but the exact mechanism has not been elucidated. As mentioned before, hydroxyl radical-

Table 1  
Bcl-2 does not affect NO production nor prevent NO-mediated inhibition of vaccinia growth

Virus	PFU/cell	IPTG	Nitrite		Nitrite+Nitrate		$\text{NO}_2^- : \text{NO}_2^- + \text{NO}_3^-$	Titer	
			nmol/ $1.15 \times 10^6$ cells/36 h (Mean)	(SEM)	nmol/ $1.15 \times 10^6$ cells/36 h (Mean)	(SEM)		PFU/ml $\times 10^7$ (Mean)	(SEM)
0	0	—	1.43	0.24	5.78	0.88	0.25	—	—
		+	1.32	0.21	2.74	0.11	0.48	—	—
NOS	1	—	23.72	1.07	77.42	5.52	0.31	14.10	0.28
		+	95.73	1.37	214.55	6.62	0.45	4.30	0.59
CAT	1	—	1.07	0.27	3.28	0.77	0.33	16.38	1.27
		+	1.37	0.32	3.24	0.42	0.42	13.30	0.85
Bcl-2	1	—	1.17	0.26	3.60	0.35	0.32	14.81	0.58
		+	1.37	0.30	5.08	0.90	0.27	10.20	0.98
NOS+CAT	1+1	—	13.02	0.18	38.05	1.99	0.34	14.30	1.03
		+	71.46	1.32	185.21	9.69	0.39	4.85	0.66
NOS+Bcl-2	1+1	—	14.88	0.53	44.86	3.74	0.33	16.20	1.38
		+	80.45	1.38	189.57	7.87	0.42	4.55	0.17
NOS+CAT	1+2	—	7.89	0.60	25.86	1.29	0.31	15.90	0.85
		+	63.9	1.44	170.47	6.46	0.37	7.60	0.37
NOS+Bcl-2	1+2	—	10.76	0.14	26.79	1.22	0.40	17.80	1.14
		+	69.96	0.62	171.44	9.63	0.41	6.13	0.67

$1.15 \times 10^6$  of HeLa G cells in 12 well plates were infected with 1 PFU/cell of WRNOS, WRbcl2 or WRCAT, or with combination of 1 PFU/cell of WRNOS and 1 or 2 PFU/cell of WRCAT or WRbcl2, in the absence or presence of 1.5 mM IPTG. At 36 h.p.i., cells were collected and aliquots of the medium removed for nitrite and nitrate determination [32,33]. The ratio of nitrite ( $\text{NO}_2^-$ ) versus total nitrite+nitrate ( $\text{NO}_2^- + \text{NO}_3^-$ ) was also calculated. Virus yields were determined by titration in BSC-40 cells. The results represent mean of four samples+standard error of mean (SEM).

like species, produced by protonation and decomposition of peroxynitrite, causes DNA and protein damage together with lipid peroxidation [13,15,17]. Lipid peroxidation that results from the increased generation of ROI due to inhibition of electron transport in mitochondria often accompanies cell death, and Bcl-2 was implicated in prevention of both apoptosis caused by ROI and lipid peroxidation [12,40,41]. The correlation of lipid peroxidation and cellular damage was, therefore, evaluated in HeLa G cells infected with WRNOS, WRbcl2 and WRCAT alone or in combinations. Lipid peroxidation was evaluated by determining thiobarbituric acid reactive substances (TBARS) [35] and by measuring changes in fluorescence of a naturally occurring polyunsaturated fatty acid, *cis*-parinaric acid [36]. We could not detect any TBARS upon expression of *iNOS*. A more sensitive assay using fluorescence of *cis*-parinaric acid revealed increased fluorescence upon IPTG induction of expression of *iNOS* or *bcl-2* alone or together (data not shown). Thus, NO-mediated apoptosis is not accompanied by an increase in lipid peroxidation, suggesting that NO-damaging effects do not involve generation of hydroxyl free-radical. Alternatively, radical chain propagation reactions can be terminated by NO [42]. Additionally, this result confirms reports of others about antioxidative effects of Bcl-2 [12,41].

### 3.5. NO-induced DNA degradation is inhibited by $Zn^{2+}$

The specific ladder-type degradation of DNA due to the

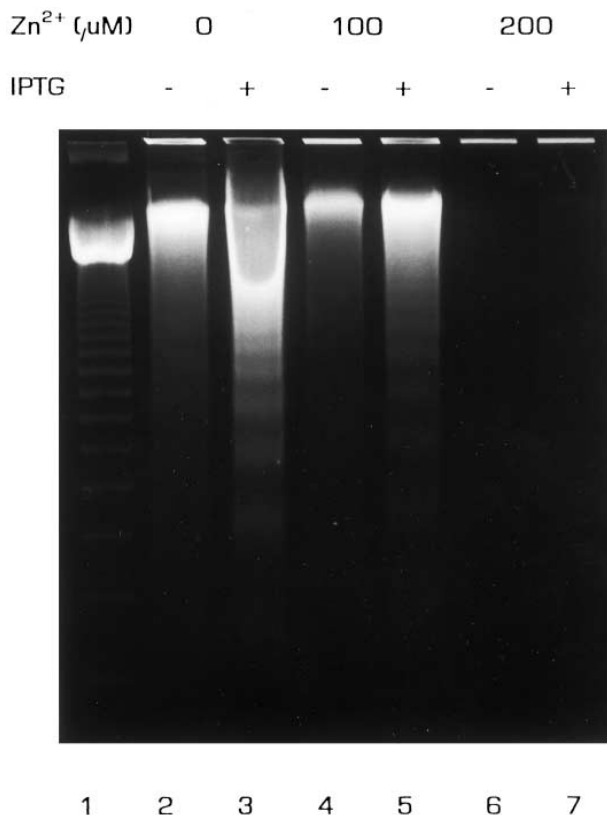


Fig. 3. Zinc ions inhibit NO-induced DNA fragmentation.  $6 \times 10^6$  of HeLa G cells in 60 mm plates were infected with 1 PFU/cell of WRNOS in the presence or absence of IPTG and increasing concentrations of  $ZnCl_2$ . At 36 h.p.i., cells were collected, low molecular weight DNA was isolated and resolved by 2% agarose gel electrophoresis. Lane 1, molecular weight markers.

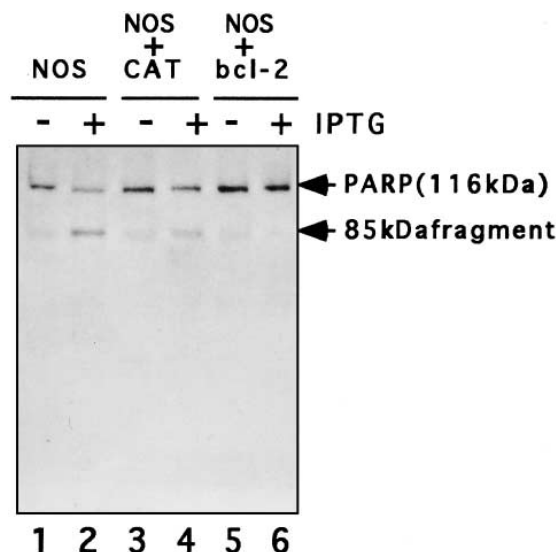


Fig. 4. Expression of *iNOS* causes cleavage of PARP and this cleavage is prevented by Bcl-2.  $1.15 \times 10^6$  of HeLa G cells in 12-well plates were infected with 1 PFU/cell of WRNOS alone (lanes 1 and 2) or in combination with 1 PFU/cell of WRCAT (lanes 3 and 4) or WRbcl2 (lanes 5 and 6), in the absence or presence of 1.5 mM IPTG. At 36 h.p.i., cells were collected, lysed in Laemmli sample buffer, and the proteins were resolved by 8% SDS-PAGE [34]. PARP was detected by Western blot analysis using mouse anti-PARP monoclonal antibody C2-10 (Centre de Recherche du Chul, Quebec, Canada), dilution 1:1000, and enhanced chemiluminescence (ECL kit, Amersham).

internucleosomal cleavage is considered as one of the hallmarks of apoptosis. However, the nature of the endonuclease has not been clearly elucidated. At least two different types of endonuclease activity can be distinguished:  $Ca^{2+}/Mg^{2+}$ -dependent endonuclease which is sensitive to inhibition by  $Zn^{2+}$  ions, and the acid endonuclease which is weakly inhibited by divalent cations, such as  $Ca^{2+}$ ,  $Mg^{2+}$ , and  $Zn^{2+}$  [43]. In order to determine which kind of endonuclease is activated in HeLa G cells by NO, the cells were infected by WRNOS in the presence of several divalent cations. Upon induction of *iNOS* expression by IPTG, NO-mediated DNA degradation could be completely prevented by 200  $\mu M$   $ZnCl_2$  (Fig. 3), while 100  $\mu M$   $MgCl_2$  significantly increased the extent of DNA cleavage (data not shown). This result suggests that a  $Ca^{2+}/Mg^{2+}$ -dependent endonuclease is responsible for DNA degradation during NO-mediated apoptosis in HeLa G cells.

### 3.6. Bcl-2 prevents poly(ADP-ribose) polymerase cleavage induced by NO

NO-mediated DNA damage and neurotoxicity was shown to involve activation of PARP [21]. Synthesis of poly(ADP-ribose) adducts causes significant depletion of intracellular NADH and ATP which has been suggested as a possible cause of apoptosis [21,22]. In contrast, we could not prevent NO-mediated apoptosis by addition of 100 or 200  $\mu M$  benzamide, a reported inhibitor of PARP [21] (data not shown). Recently, PARP was demonstrated as a substrate for CPP32/apopain, member of the ICE/CED-like family of proteases [23,24]. To determine if NO-induced apoptosis in HeLa G cells also proceeds through the activation of the ICE/CED-like protease cascade, we evaluated the cleavage of PARP. As

shown in Fig. 4, *iNOS* expression and NO production result in the PARP cleavage, yielding an 85 kDa fragment typical for the ICE/CED-like protease activity (lanes 2 and 4) [24]. Co-expression of *Bcl-2* and *iNOS* prevented the cleavage of PARP (lane 6) while co-expression of *CAT* and *iNOS* had no effect (lane 4). The decrease in the intensity of the cleavage in lane 4 compared to lane 2 is due to the competition between the two viruses and lower *iNOS* expression as described above. These findings show that NO-induced apoptosis is mediated by ICE/CED-like protease cascade and that *Bcl-2* blocks an event in the apoptosis before this cascade can be activated or before cleavage of PARP.

Although NO has been shown to induce apoptosis through the expression of the tumor suppressor gene *p53* [44], it seems unlikely that NO-mediated apoptosis in HeLa G cells is connected to *p53* since this cell line does not contain an active *p53* [45].

Our results demonstrate that *Bcl-2* prevents NO-mediated apoptosis without any effect on NO-mediated inhibition of VV growth. *Bcl-2* does not abolish formation of NO or its direct interaction with the targets (one of them is ribonucleotide reductase, a key enzyme in DNA synthesis [18]). *Bcl-2* did not cause changes in the ratio of nitrite and nitrate either. *Bcl-2* was shown to decrease intracellular levels of hydroxyl free-radical [46], which can originate from the superoxide and  $H_2O_2$  produced by mitochondria as well as from peroxynitrite [13,14]. Recently, peroxynitrite itself was shown to induce apoptosis [47]. The damaging effects can be mediated by protonation and decomposition of peroxynitrite yielding hydroxyl radical-like and nitrogen dioxide-like species or directly by peroxynitrite itself [13,14,16,17]. Peroxynitrite reacts with iron-sulfur centers [48] or with protein thiols [16]. Peroxynitrite as well as nitrosonium ions mediate oxidation of protein thiols and formation of disulfide bridges, thus abolishing target protein function [16,19]. Such a protein might be protected or renatured, e.g. by a reduced glutathione, the levels of which are increased upon overexpression of *bcl-2* [41]. Also, *Bcl-2* could decrease peroxynitrite formation by decreasing superoxide generation by respiratory chain enzymes or by affecting mitochondrial function (manuscript in preparation).

In conclusion, in the inducible VV expression system described, NO production leads to the inhibition of virus growth, DNA synthesis [27] and induction of apoptosis. This NO-mediated apoptosis is initiated upstream of *Bcl-2* and requires the activation of the ICE/CED-like protease cascade as demonstrated by cleavage of PARP and by *Bcl-2*-mediated inhibition of both apoptosis and PARP cleavage. Since *Bcl-2* did not interfere with NO-induced inhibition of VV growth but completely prevented activation of the cell death cascade, it can be suggested that *Bcl-2* might prevent NO-mediated apoptosis by redirecting NO to different targets or by restoring a vitally important process inhibited by NO, e.g. mitochondrial function.

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