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Expression of *BCL-2* Inhibits Cellular Radical Generation

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Bcl-2 expression in neural cells has been shown to inhibit apoptotic death in association with a decrease in reactive oxygen species. We present the results of a study that used electron spin resonance (ESR) measurements to evaluate the level of hydroxyl radical production in *bcl-2* expressing GT1–7 cells and control cells. Incubation of cell monolayers with the spin trap N-t-α-phenylnitrone (PBN), and measurement of the hydroxyl radical production at different timepoints, revealed a higher radical production in control cells than in *bcl-2* expressing cells, even in the absence of insult. The ESR signal was suppressed by addition of ethanol, indicating that the trapped radical was indeed hydroxyl radical. The mechanism by which the expression of *bcl-2* leads to a decrease in cellular production of hydroxyl radical is unknown.

Key words: Free radical generation, ESR, ·OH-PBN adduct, apoptosis, Bcl-2, antiapoptotic gene

INTRODUCTION

Bcl-2 is a proto-oncogene that was discovered in studies of human B-cell lymphomas with the t(14; 18) chromosome translocation.[1] It has been shown that *bcl-2*-expression inhibits apoptotic, and in some cases necrotic, cell death in a number of neural cell lines, whether induced by serum withdrawal, calcium ionophores, decrease in glutathione, oxidative damage, or other means.[2-9] Using dichlorofluorescin (DCFH), it was previously demonstrated that the net cellular generation of reactive oxygen species (ROS) is decreased in *bcl-2* expressing neural cells. In contrast, Hockenbery *et al.*,[10] using hematopoietic cells rather than neural cells, detected no difference in ROS generation when using DCFH in cells expressing *bcl-2*. Nonetheless, expression of *bcl-2* was found to inhibit the lipid peroxidation associated with apoptosis. The authors of that study therefore concluded that, rather than inhibiting the net cellular generation of reactive oxygen species (ROS), *bcl-2* expression has no effect on

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cellular ROS production, but decreases membrane damage from the ROS.[10]

Data from several different laboratories have implicated ROS as mediators of apoptosis.[5,10–12] However, it is neither clear how such species would induce necrosis in some cases and apoptosis in others, nor how the relatively uncontrolled diffusion of ROS would lead to the tightly controlled process of apoptosis. It was hypothesized[12] that ROS induce necrosis and apoptosis in fundamentally different ways: that ROS act as effectors in necrosis, leading to oxidative damage of lipids, nucleic acids and proteins; in contrast, that ROS act as signaling molecules in apoptosis, inducing cell death via redox- sensitive cellular factors.

Electron spin resonance (ESR) measurement is a widely used method to observe free radicals that are trapped by nitroso or nitrone compounds. They form a relatively stable spin adduct with the short-lived radicals.[13–16] In previous studies the radical-adducts of N-t-α-phenylnitrone (PBN) were extracted into ethyl acetate, and the solution was saturated with air to reoxidize any adducts that had been reduced to hydroxylamines. Saturation of the ethyl acetate solution with nitrogen immediately before the measurements improved the lineshape by eliminating oxygen broadening. Since spin adducts were more stable in ethyl acetate than in aqueous solutions,[17] this technique was chosen for the study presented. Additionally, the use of lead dioxide (PbO_2) for reoxidation of reduced spin adducts was evaluated.

In the present study PBN was added to the supernatant of cell cultures to form a spin adduct with hydroxyl (·OH) radicals generated by immortalized hypothalamic GT1–7 cells that had been infected with the recombinant retroviral vector pBabe-puro-bcl-2.[5] GT1–7 cells infected with pBabe-puro, that differs only in the lack of the *bcl-2* open reading frame, were used as control.

The similarity of the hyperfine splitting constant to the previously published constants for a hydroxyl radical adduct in ethyl acetate[17] and the diminution of the signal following ethanol addition argue that the trapped species indeed was hydroxyl radical.

This study confirms that the net generation of hydroxyl radical is decreased in neural cells expressing *bcl-2*, and extends the previous work to show that this reduction is present even prior to cellular insult (other than the insult of growth in monolayer culture).

MATERIALS AND METHODS

Cell Culture

GT1-7 cells that were stably infected with the recombinant retrovirus Babe-puro-bcl-2[5] and control GT1–7 cells infected with the retrovirus Babe-puro were grown on poly-L-lysine (Sigma) coated 10 cm plates (Nunc) in Dulbecco's modified Eagle's medium (Cellgro) with 10% fetal bovine serum (heat inactivated (Sigma)) and Penicillin/Streptomycin (Gibco). Cells were maintained at 37°C in an atmosphere of 5% CO_2.

ESR Study

Cell assay

Cells were washed three times with 5 ml modified Krebs Ringer (KR) solution containing 20 mM Hepes, 10 mM dextrose, 127 mM NaCl, 5.5 mM KCl, 1 mM $CaCl_2$, and 2 mM $MgSO_4$, pH 7.4.

For assessment of radical production 3 ml KR containing 1 mM PBN (Aldrich) was added to the cells and incubated at 37°C. The supernatant was collected at different timepoints from t = 0 to 90 minutes. Spin adducts were extracted by mixing the supernatant with 2 ml ethyl acetate (Sigma).[17] Centrifugation for 10 minutes at 1000 RPM at 22°C separated the organic from the aqueous phase. The organic phase, containing the spin adduct, was concentrated to 300 µl by passing argon over the solution. ·OH-PBN adducts that had been reduced to hydroxylamines during the incubations were reoxidized to ·OH-PBN nitroxides with 50 mg PbO_2 (Sigma). The solution was centrifuged for 30

seconds at 10 000 RPM at 22°C to pellet the PbO_2, and 250 µl of the ethyl acetate supernatant was transferred to a quartz tube. After degassing the sample with argon and capping it with aluminum foil, the quartz tube was immediately transferred to the ESR cavity (Varian E-109 with E-231 cavity).

For further experiments either 0.05% ethanol or 10% ethanol (Quantum) were added to 1 mM PBN in KR at the beginning of the incubation.

Chemical ·OH radical generation

5% H_2O_2 was added to 20 µM ferric ethylenediaminetetraacetate (FeEDTA) and 40 mM PBN in 30 mM KH_2PO_4. After 10 minutes the adducts were extracted into ethyl acetate and processed as described above. For some studies, 20 mM Hepes or 10% ethanol was added.

Protein Assay

Protein was isolated by freezing/thawing the plates three times and centrifuging the debris. Aliquots from the supernatant were compared to a bovine serum albumin (Sigma) standard curve using the Biorad DC Protein assay and a microplate reader (Molecular Devices) to determine the absorbance at 570 nm. ESR results were quantified by dividing the amplitude measured in mm as the distance between the highest and lowest peak of each triplet signal of the ESR spectra by the total protein amount of the plate (measured in mg). This value for each measurement was used in the statistical procedure. When cell number was used for the denominator, the significance of the results was unchanged.

Viability Test

Prior to the ESR assays, PBN, KR, and ethanol were tested for their effects on GT1–7 cells.

Bcl-2 expressing GT1–7 cells and control cells were counted and evenly plated on 96 well plates (Nunclon) and incubated for 90 min. with either KR instead of medium or different concentrations of PBN in KR (0.1 mM, 1 mM, or 10 mM) at 37°C in 5% CO_2 atmosphere. In other experiments they were incubated with either 1% ethanol or 10% ethanol in KR for 90 min. Viability of the cells was tested 24 hours later, as described, staining the dead cells with the fluorescent dye propidium iodide (Sigma).[5] This dye stains the nuclei (DNA) of cells that have lost membrane integrity (either due to apoptosis with secondary necrosis, or due to primary necrosis). The plates were examined by a cytofluor reader and the number of viable cells in the test plates compared to the viable cells of the controls. Differentiation of apoptosis from necrosis is accomplished by noting the presence or absence of nuclear fragmentation, homogeneous nuclear staining, and apoptotic bodies.[3]

RESULTS

Viability tests with the compounds used for ESR assays revealed that none of the compounds caused cell damage that led to increased cell death in GT1–7 *bcl-2*-expressing cells or GT1–7 control cells (Data not shown). These tests were performed to rule out the possibility of cell damage and free radical generation by the addition of the compounds used during the ESR experiments.

Bcl-2 expressing cells undergo little apoptosis and therefore increase their number more rapidly than control cells when plated in tissue culture plates. Experiments with cell monolayers were carried out exactly as described for experiments shown in Figure 2 with different cell densities to rule out the possibility that the number of cells per plate had an effect on the amplitude of the ESR signal per mg protein. No significant deviation from the average result could be seen when puro-control cells were plated more densely than *bcl-2* cells (Data not shown). Furthermore, when cell number, rather than protein concentration, was used as the denominator, the significance of the results was unchanged.

It was previously shown that ·OH-PBN adducts prepared from 5% H_2O_2 and 20 µM Fe EDTA that was extracted into ethyl acetate are a triplet

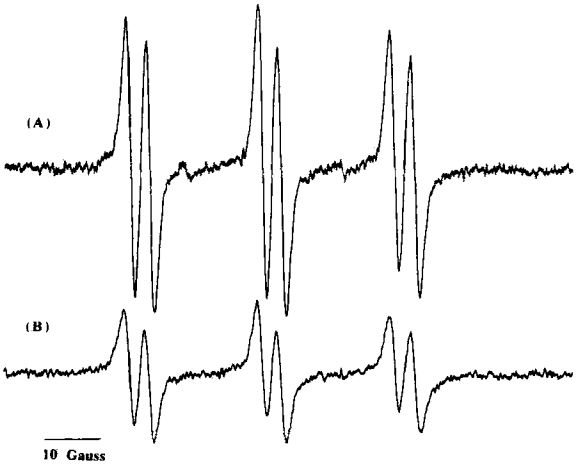

FIGURE 1a ESR spectra of the hydroxyl radical trapped by PBN obtained from (A) puro GT1–7 (control) and (B) *bcl-2* expressing cells. Cells were incubated with 1 mM PBN in KR for 60 minutes. Protein per 10 cm plate: (A): 2.3 mg (B): 1.7 mg. ESR spectra were recorded at room temperature. Conditions were: microwave power 10 milliwatts, modulation amplitude 1 Gauss, time constant 10 ms, sweep width 60 Gauss, sweep time 8 × 30 seconds, receiver gain 1000.

of doublets.[17] This spectrum was shown to result from the following coupling constants: a_N = 13.71 G and a_H = 2.1 G.[18] The corresponding coupling constants of the radical adducts measured in the present study are a_N = 13.7 G and a_H = 1.9 G.

HEPES rapidly forms a stable adduct with ·OH as reported by Hicks and Gebicki.[19] Since we used Hepes in the buffer, we tested its effect on radical trapping. Chemically prepared ·OH-radicals from KH_2PO_4 and FeEDTA in phosphate buffer solution were trapped with PBN. Their EPR spectra are compatible with the spectra recorded from the cell supernatants. Addition of the same concentration of Hepes that was used in cell supernatants to the chemically prepared sample led to a new signal pattern, that was not obtained from the cell supernatants (Figure 1b).

Therefore, we conclude that PBN entered the cells, formed an adduct with the intracellular ·OH-radicals, the adduct left the cell and was then recorded in the supernatant. ·OH-radical that would have been formed outside the cell should

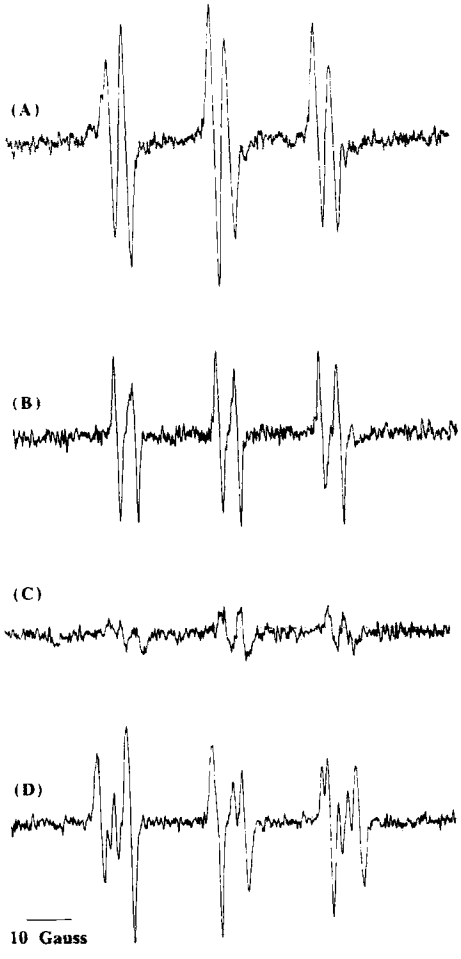

FIGURE 1b ESR spectra chemically generated (see Materials and Methods) and trapped with 40 mM PBN. (A) 5% H_2O_2 and 20 μM FeEDTA in 30 mM KH_2PO_4, spin adducts were reoxidized with 50 mg PbO_2. (B) as (A) without reoxidation with PbO_2. (C) as (A) 20 mM Hepes added. (D) as (A) 10% ethanol added. ESR setting as described in legend 1b. except receiver gain 2000.

have given an extra signal because of the presence of Hepes in the supernatant.

In control experiments we tested the efficacy of PbO_2 for reoxidation of the hydroxylamines formed during the incubations. Ethyl acetate extracts from Babe-puro retrovirally infected control cells that had been incubated for 60 min with PBN were degassed with nitrogen, and their ESR

spectra were recorded. The extracts were then treated with 50 mg of PbO_2 for 5 min. After the PbO_2 was removed by centrifugation, the ethyl acetate extracts were degassed with nitrogen, and their ESR spectra were recorded again. A greater than two-fold increase in signal intensity was observed after reoxidation (data not shown). In an experiment with chemically generated ·OH-PBN signal the addition of PbO_2 almost doubled the signal (see Figure 1b). Solutions of pure PBN in ethyl acetate did not produce an ESR spectrum after treatment with PbO_2.

To measure the hydroxyl radical production of the cells, GT1–7 *bcl-2* expressing cells and controls were incubated with 1 mM PBN for different time periods from 1 minute to 90 minutes. Figure 1a shows the triplet of doublets formed by the hydroxyl radical adducts from the cell supernatants.

Figure 2 shows a typical example of the different amounts of radical generation in *bcl-2* expressing and control cells. Similar differences were detected in five to seven repetitions of the experiments at each timepoint. In this example, after incubation periods of either 1 minute or 15 minutes with 1 mM PBN, the control cells showed only a slightly different level of radical production

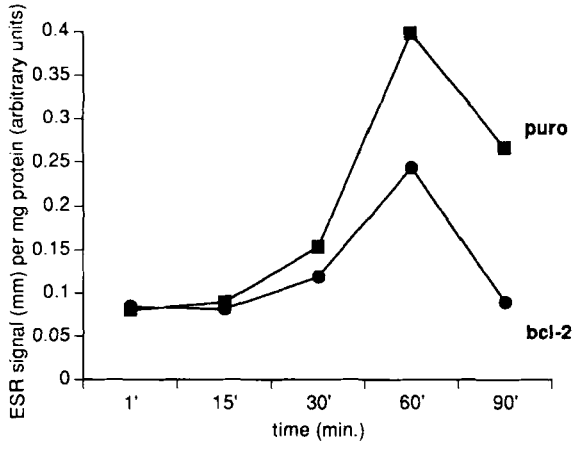

FIGURE 2 Amount of hydroxyl radical trapped with PBN at different timepoints from one typical experiment (arbitrary units). 1 mM PBN in KR was applied to each 10 cm cell plate. All experiments were executed 5 to 7 times.

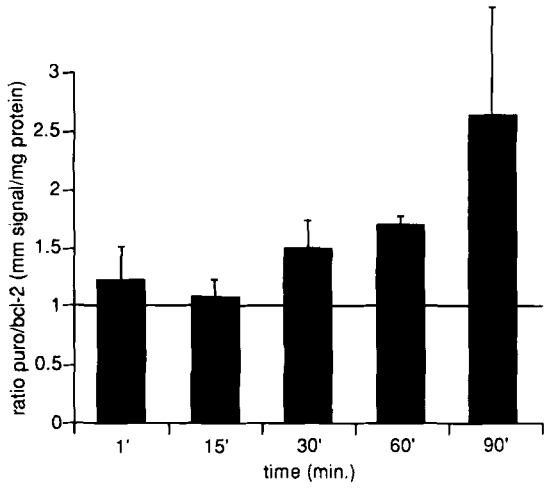

FIGURE 3 Ratio of ESR signal of the ·OH-PBN adduct per mg protein in puro compared to *bcl-2* expressing cells. Summary of all experiments conducted 5 to 7 times. No significant differences between control and *bcl-2* expressing cells were obtained at timepoints 1 min. and 15 min. using the one-sample t-test (see text). At 30 min. and 90 min. p equals 0.08, which shows a tendency towards a statistically significant higher ratio. At 60 min. p equals<0.001 and is significantly different from 1 with an average ratio of 1.73.

than the *bcl-2* expressing cells (Figure 2). The ratio of signal per protein (mm/mg) for puro cells compared to *bcl-2* expressing cells, however, varied when the experiments were repeated several times, as indicated by the error bars on Figure 3. Even more differing results were found after incubation for 90 min; a difference of up to 16 times more radical in puro cells was seen (Figure 3). At time points 30 and 60 min the signals increased compared to the short incubation periods, since PBN trapped more radical (Figure 2). The difference between *bcl-2* expressing cells and controls became more significant. At the 60 min timepoint almost twice as many radicals were trapped in the control cells than in *bcl-2* expressing cells. The assumption that PBN enters the cells to form an ·OH-PBN-adduct could explain the finding that significant differences in radical production of the two cell types appear after a prolonged incubation time (note that after only 1 minute of incubation with PBN, the concentration of ·OH-PBN adducts is already higher, but not significantly higher, for

the control cells). Within 90 minutes of incubation, the signals in this experiment decreased in both cell lines (Figure 2). The decrease might be due to a toxic effect of PBN, or to reduction of the ·OH-PBN adducts to compounds that could not be reoxidized to nitroxides by PbO_2. Another explanation might be the destruction of the ·OH-PBN adducts.

In order to verify statistically the observation that the 60 min timepoint shows the strongest difference between *bcl-2* expressing cells and puro control cells, we used the one-sample t-test to compare the obtained ratios to the ratio 1. Since a ratio that equals 1 indicates that there is no difference in radical production between the compared cells at a certain timepoint, any significant higher ratio for control cells versus *bcl-2* expressing cells compared to the ratio 1 will indicate a significant difference between the level of radical production of control cells compared to *bcl-2* cells.

Since consistently significantly different levels of radical production could be observed for the timepoint 60 min, we chose it for further investigation.

Figure 4 shows the results of one typical experiment of an incubation with 1 mM PBN and 10% ethanol or 0.05% ethanol of GT1–7 *bcl-2* expressing cells and control cells compared to addition of 1 mM PBN only. The addition of ethanol decreases the hydroxyl radical signal per protein amount by forming hydroxylethyl radical (see Figure 1b).

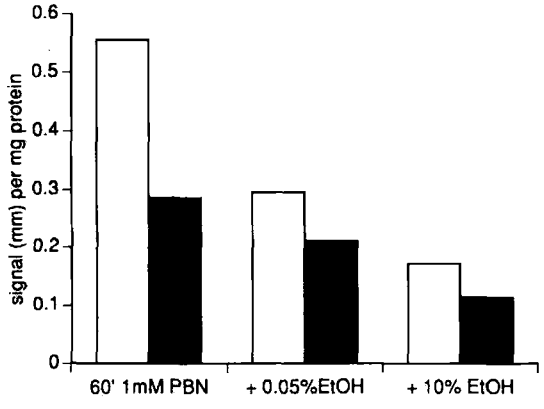

FIGURE 4 Concentration of ·OH-PBN adducts derived from control and *bcl-2* expressing cells (arbitrary units). Both cell lines show a decreased signal following the addition of 0.05% ethanol and 10% ethanol in KR and 1 mM PBN. Addition of 10% ethanol decreases the signal in control cells (blank bars) more than 7 times. The signal decreases by the factor 5 in *bcl-2* expressing cells (solid bars). Measurements were made after 60 min of incubation with 1 mM PBN.

DISCUSSION

The present study uses the ESR method to confirm that the *bcl-2*-infected neural GT1–7 cell line generates less hydroxyl radical that can be trapped by PBN than a control cell line. Previous results, using the less sensitive DCFH method, demonstrated a difference between the control and *bcl-2* GT1–7 transfectants' radical generation following the addition of diethylmaleate. The results presented here show that even in the absence of an insult (other than growth in monolayer culture), the cells expressing *bcl-2* have a lower rate of hydroxyl radical production.

To prove that the initially formed radical was hydroxyl radical, ·OH-PBN adducts were chemically generated, and the signals derived by the different methods were compared. As noted above, the chemically generated signals and signals from cell supernatants were similar, which supports the conclusion that the trapped radical was hydroxyl radical. As further confirmation, ethanol was added, because of its ability to scavenge hydroxyl radical.[22] The expected decrease of the ·OH-PBN signal, and the appearance of a new signal compatible with hydroxylethyl radical adduct, provide further support for the conclusion that hydroxyl radical was trapped by PBN in these experiments.[21]

Stability of the ·OH-PBN adduct for time periods over 20 minutes has been reported.[22] The present study shows that the radical adduct accumulates for 60 minutes in the supernatant of a GT1–7 cell monolayer. Measurements that were executed for more than 60 minutes, however, revealed a declining amount of the spin adduct.

Prior studies suggested that *bcl-2* plays a role in preventing cell death associated with reactive

oxygen species.[3,5] Although the site and mechanism of the protective effect of *bcl-2* are not clear, the present study shows that hydroxyl radical is one species whose production is reduced in GT1–7 neural cells in association with *bcl-2* expression. It is noteworthy that recent reports[23,24] have shown that cell death induced by anoxia is also inhibited by the expression of *bcl-2*, suggesting that *bcl-2* expression has additional cellular effects, and that, at least in one paradigm, its effect on reactive oxygen species is not required for its inhibition of cell death. Nonetheless, manipulation of the cellular generation of reactive oxygen species has been shown in numerous studies to affect both apoptotic and necrotic cell death (summarized in ref. 12).

How might the expression of *bcl-2* affect cellular hydroxyl radical generation? *Bcl-2* expression could conceivably affect Fenton-related cellular generation of hydroxyl radical,[25] cellular antioxidant concentrations, the redox status of the cell, or other cellular parameters that determine the net cellular generation of hydroxyl radical.[5] Previous work demonstrated that *bcl-2* expression increases cellular glutathione concentration in at least some cell types.[5] Glutathione may play an important role in adjusting the cellular 'apostat' (the likelihood of the cell to undergo apoptosis), since (1) blastocysts become more sensitive to hydrogen peroxide-induced apoptosis when cellular glutathione is depleted,[11] and (2) overexpression of glutathione peroxidase inhibits apoptosis.[10]

The results presented here support the conclusion that *bcl-2* rescues cells from different types of damage, at least in part by down-regulating ROS production, thus diminishing oxidative stress. This does not exclude the possibility that *bcl-2* has additional cellular effects, nor does it distinguish between direct and indirect effect of *bcl-2* on cellular radical production.

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