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Up-regulation of Bcl-2 by redox signals in glomerular mesangial cells

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Abstract

The mediators nitric oxide (NO) and superoxide (O_2^-) are known to regulate cell death and survival. In mesangial cells (MC), NO induced apoptosis and in higher concentrations necrosis. Intriguingly, cogeneration of NO and O_2^- in a balanced ratio promoted cell protection. Under these conditions, we noticed the accumulation of the anti-apoptotic protein Bcl-2. Its up-regulation is based on an increase in mRNA and protein level. To investigate whether oxidative stress elicits Bcl-2 expression in general, we further used the chemically unrelated oxidative agents diamide and butyl hydroperoxide. Both stimulated mRNA and protein up-regulation of Bcl-2. But in contrast to diamide, butyl hydroperoxide evoked apoptosis and necrosis despite Bcl-2 accumulation. As diamide was non-toxic, we used diamide as a Bcl-2 activator to protect MC against a subsequent toxic dose of NO. We conclude that redox changes promote Bcl-2 up-regulation that may confer cellular protection towards apoptosis. *Cell Death and Differentiation* (2000) **7,** 118–125.

Keywords: Bcl-2; nitric oxide; superoxide; redox signals; apoptosis; protection

Abbreviations: NO, nitric oxide; O_2^- superoxide; GSNO, S-nitrosoglutathione; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; iNOS, inducible nitric oxide synthase; MC, mesangial cells; CHX, cycloheximide; BHP, butyl hydroperoxide; LDH, lactate dehydrogenase; RT–PCR, reverse transcriptase polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $ONOO^-$, peroxynitrite

Introduction

Apoptosis is an essential program in scavenging genetically modified or toxicological affected cells in an effort to maintain a homeostatic cell number. Its regulation is complex and especially decreased apoptosis is often associated with tumorgenesis. Apoptosis is characterized by distinctive morphological and biochemical features but the signal transduction pathways differ among cell types.[1] One of the key players is Bcl-2. Bcl-2, an acronym for the B-cell lymphoma/leukemia-2 gene, is a cell protective protein of the Bcl-2 family, that contains members with pro- and anti-apoptotic functions.[2] It is unquestioned that Bcl-2 over-expression or a high basal level found in cancer cells inhibit apoptosis although detailed mechanisms are still unclear. Considerations for the anti-apoptotic Bcl-2 action include (i) its antioxidant ability, (ii) the interaction and neutralization of pro-apoptotic proteins such as Bax, and (iii) inhibition of the mitochondrial permeability transition that participates in promoting apoptosis.[3–5] Expression of Bcl-2 varies among cell types, but transcriptional or translational regulatory elements of Bcl-2 are marginally examined. As a result of cytotoxic stimulation Bcl-2 is known to decline which may be a result of the negative regulation by the tumor suppressor p53.[6] Physiological accumulation of Bcl-2 have been shown for a few cases, only.[7–9] At least in neuronal cells the transcription factor Brn-3a which belongs to the POU family is able to up-regulate Bcl-2.[10] In addition, NF-κB and NF-AT have been discussed to influence Bcl-2 expression besides the Sp-1 binding site, the CAAT box, and the TATA element in the bcl-2 promotor[11–14] but a general mechanism for its synthesis has not been envisioned.

Reactive oxygen- and nitrogen species (ROS, RNS) such as superoxide (O_2^-) or nitric oxide (NO) are produced under divers conditions. NO is generated by NO-synthase isoenzymes which can be roughly distinguished as constitutively expressed versus cytokine inducible (iNOS).[15] O_2^- is generated by the mitochondrial respiration or by activation of NADP(H)-like oxidases. Production of large amounts of ROS and RNS over an extended period is often associated with cell destruction i.e. apoptosis whereas small concentrations are considered as physiological regulators. This is exemplified in the cardio-vascular system where NO provokes cGMP-dependent signaling,[16] whereas O_2^- is known to activate different transcription factors such as NF-κB or AP-1.[17,18] The action of NO is determined by its reaction with oxygen, O_2^- or transition metals which leads to the formation of different reactive nitrogen species with divers chemical properties.[19] This has been shown in previous studies in glomerular mesangial cells (MC). Stimulation with NO donors (spermine-NO, S-nitrosoglutathione) or O_2^--generating systems (xanthine oxidase/hypoxanthine, 2,3-dimethoxy-1, 4-naphtoquinone) induced apoptosis or in higher concentrations necrosis whereas a balanced and simultaneous generation of both radicals was non-destructive.[20] Initiation of apoptosis was accompanied by an increased p53 and Bax expression, caspase activation, and DNA fragmentation.[21] However, these alterations were abrogated under conditions of NO/O_2^--coadministration although NO/O_2^- has a highly oxidative potential measured as generation of oxidized

glutathione (unpublished data). Signaling mechanisms as a consequence of the NO/O_2^--interaction may redirect apoptotic initiating signals by NO towards cell protection.

As cell protection is often correlated to Bcl-2 regulation, we analyzed Bcl-2 expression using the NO donor S-nitrosoglutathione (GSNO) and/or the redox cycler 2,3-dimethoxy-1,4-naphtoquinone (DMNQ). Whereas Bcl-2 was down-regulated by GSNO, up-regulation of Bcl-2 mRNA and protein was noted as a result of NO/O_2^--coformation. This was reproduced by the oxidizing compounds diamide and butyl hydroperoxide (BHP). A prestimulation with diamide was used to protect MC against a toxic dose of GSNO. This leads to the conclusion that oxidative signals trigger the accumulation of Bcl-2. Once Bcl-2 is present it can confer cell protection to an otherwise lethal insult.

Results

In corroboration with previous studies we noted NO-induced apoptotic DNA fragmentation in rat MC with some accompanying signs of cellular necrosis such as lactate dehydrogenase (LDH) release (Table 1). DNA degradation in controls was around 9% that increased to roughly 25% in response to 250 μM of the NO donor GSNO. Correspondingly, LDH release increased from 6% in controls to 16% following NO treatment. The impact of oxygen radicals on cell integrity was checked by exposing MC to the superoxide generating compound DMNQ, which penetrates the cell membrane followed by redox-cycling to continuously form O_2^-. DMNQ at a concentration of 5 μM was not toxic, as revealed by the absence of DNA fragmentation and/or LDH release.

Interestingly, costimulation of MC with 250 μM GSNO in the presence of 5 μM DMNQ significantly attenuated NO-evoked cell damage. Evidently, the degree of apoptosis as well as necrosis was around control values under conditions of NO/O_2^--coformation. We conclude that formation of O_2^- interferes with NO-derived apoptosis in rat MC and that the shift towards cell protection is not the result of compensatory cellular necrosis.

DNA fragmentation is considered a late apoptotic phenomenon that results from differential expression of various pro- and anti-apoptotic proteins. Among these, the level of Bcl-2 is of considerable importance for cell protection. Therefore, we evaluated Bcl-2 expression by Western blot analysis followed by quantitative densitometric calculations under conditions of cellular damage versus protection (Figure 1A,B). In unstimulated cells we observed basal expression of Bcl-2 (3.9×10^4 relative density) that decreased over a period of 4 h in response to 250 or 500 μM GSNO (1.4×10^4 and 1.1×10^4 relative density). In contrast, DMNQ had no statistical significant impact on the Bcl-2 signal (4.3×10^4 relative density).

Extending experiments revealed an increased Bcl-2 expression under conditions of NO/O_2^--cogeneration (Figure 1B). Relative to controls, protein levels were elevated within 4 h to a relative density of 7.1×10^4 and 8.7×10^4 following the addition of 5 μM DMNQ/250 μM GSNO and 5 μM DMNQ/500 μM GSNO.

To further gain insights into molecular mechanisms of Bcl-2 expression under conditions of NO/O_2^--coformation we used the protein synthesis inhibitor cycloheximide (CHX) (Figure 2A). Blocking protein *de novo* synthesis under conditions of DMNQ/GSNO-coapplication largely attenuated up-regulation of Bcl-2, thus implying new protein expression rather than inhibition of its degradation to account for increased appearance of Bcl-2 in Western blots.

To verify *de novo* protein synthesis under the impact of NO/O_2^--coformation we performed RT–PCR analysis of bcl-2 mRNA (Figure 2B) in order to identify a possible transcriptional regulatory pathway. Relative to the appearance of GAPDH, the mRNA of bcl-2 time-dependently increased when MC were exposed to DMNQ/GSNO. Strongest expression was noticed at 1 and 2 h that

Table 1 GSNO-induced DNA fragmentation and LDH release is abrogated by DMNQ costimulation

	% DNA fragmentation	% LDH release
Control	9±2	6±2
GSNO [250 μM]	24±5*	16±3
DMNO [5 μM]	11±2	8±2
GSNO [250 μM]+DMNQ [5 μM]	9±2**	13±2

Mesangial cells (2.5×10^5 cells/assay) were stimulated with vehicle (control), 5 μM DMNQ, 250 μM GSNO, or the combination of DMNQ and GSNO as indicated. DNA fragmentation and LDH release were analyzed after 24 h as outlined in Materials and Methods. Data are mean values±S.D. of at least five separate experiments. *$P<0.01$ *vs* control, **$P<0.01$ *vs* GSNO

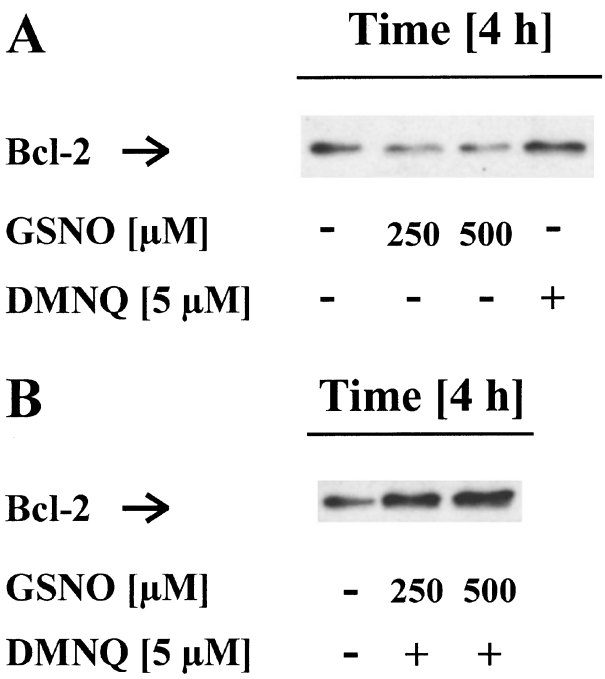

Figure 1 Changes of Bcl-2 protein in response to NO and/or O_2^-. Mesangial cells (2×10^6 cells/assay) were treated with vehicle (control), 5 μM DMNQ, 250 or 500 μM GSNO (**A**), or 5 μM DMNQ in combinations with 250 and 500 M GSNO (**B**). Bcl-2 (26–28 kDa) was detected after a 4 h incubation period by Western blot analysis as outlined under Materials and Methods. Blots are representative for three similar experiments

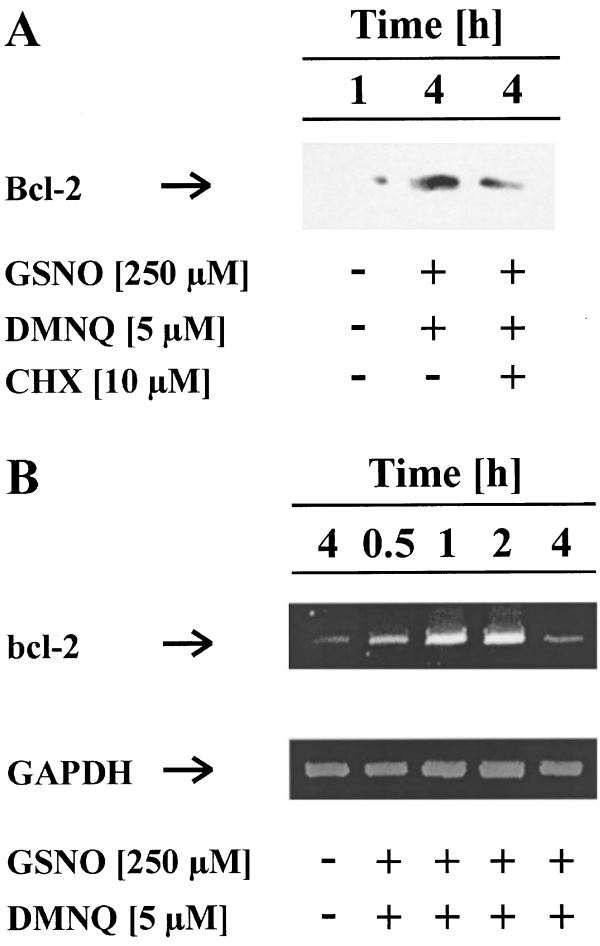

Figure 2 Bcl-2 mRNA and protein level after NO/O_2^--coadministration. Mesangial cells (2×10^6 cells/assay) were treated with vehicle (control), 250 μM GSNO/5 μM DMNQ alone or in combination with 10 μM CHX. Bcl-2 (26–28 kDa) was detected after a 4 h incubation period by Western blot analysis as outlined under Materials and Methods (**A**). For bcl-2 and GAPDH mRNA analysis, MC were stimulated with vehicle or 250 μM GSNO/5 μM DMNQ for 0.5, 1, 2, and 4 h. Total mRNA was isolated and RT–PCR was performed as described under Materials and Methods. Blots are representative for three similar experiments

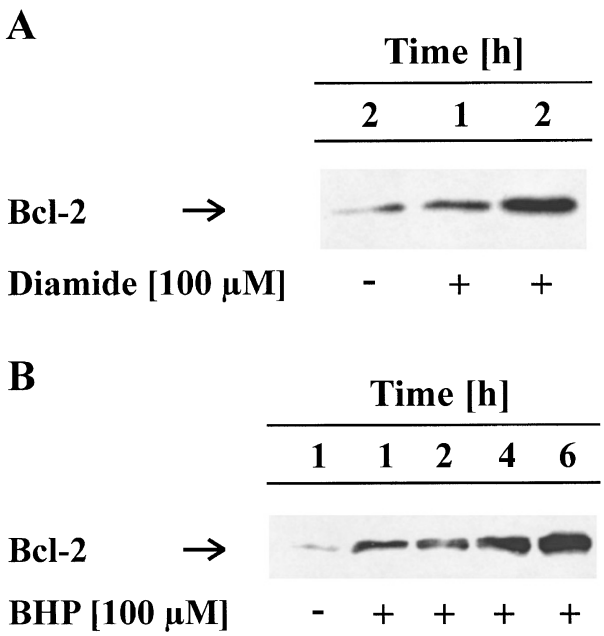

Figure 3 Bcl-2 accumulation induced by diamide and butyl hydroperoxide. Mesangial cells (2×10^6 cells/assay) were incubated with vehicle (control), 100 μM diamide (**A**), or 100 μM BHP (**B**). Incubations were terminated after 1, 2, 4, or 6 h as indicated and Bcl-2 was detected by Western blot analysis as described under Materials and Methods. The blot is representative for three similar experiments

vanished after 4 h. Taking into consideration that coformation of NO and O_2^- initiated a substantial and long oxidative stress in MC, as based on oxidized glutathione (GSSG) formation (unpublished data), we followed the intention that an oxidative signal may account for transduction thus culminating in Bcl-2 expression. To obtain experimental evidence for our hypothesis we used the oxidating agents diamide and butyl hydroperoxide (BHP) and examined Bcl-2 protein expression relative to their application (Figure 3A,B). Diamide up-regulated Bcl-2 within 1 h and promoted even stronger Bcl-2 expression within a 2 h exposure period after which no further increase became apparent.

Qualitatively, BHP behaved similarly, although the time response was slower. BHP evoked Bcl-2 expression within 1 h and progressively promoted stronger expression during a 6 h lasting incubation period. Again, we verified an increase in bcl-2 mRNA in response to diamide and BHP (Figure 4A,B). Diamide elicited a transient, however, significant RT–PCR signal within 30 min, while BHP promoted increased transcription of bcl-2 between 30 min and 4 h.

Conclusively, oxidizing conditions such as NO/O_2^--coformation, diamide- or BHP-application signal towards enhanced transcriptional and translational regulation of the well known anti-apoptotic protein Bcl-2.

Since oxidative conditions are often characterized to initiate apoptosis and/or necrosis the question arises whether diamide- or BHP-treatment challenge toxic conditions despite Bcl-2 up-regulation. Based on DNA fragmentation and LDH release we analyzed how rat MC responded towards 100 μM of the individual agonists (Figure 5A,B). During these experiments diamide and BHP were exposed for various times as indicated, followed by their removal that was achieved by exchanging the incubation medium. Thereafter, incubations went on to reach a total incubation period of 24 h that was required to determine quantitative DNA fragmentation by the diphenylamine assay.

Diamide at the concentration and times being used was non-toxic because we neither observed DNA fragmentation nor LDH release. BHP behaved differently. Exposure periods of 2 to 4 h promoted significant DNA fragmentation that was not further enhanced during a 6 h lasting exposure period. In addition, BHP induced the release of the necrotic marker LDH. Necrosis became evident after 1 h and steadily increased with incubation times up to 6 h.

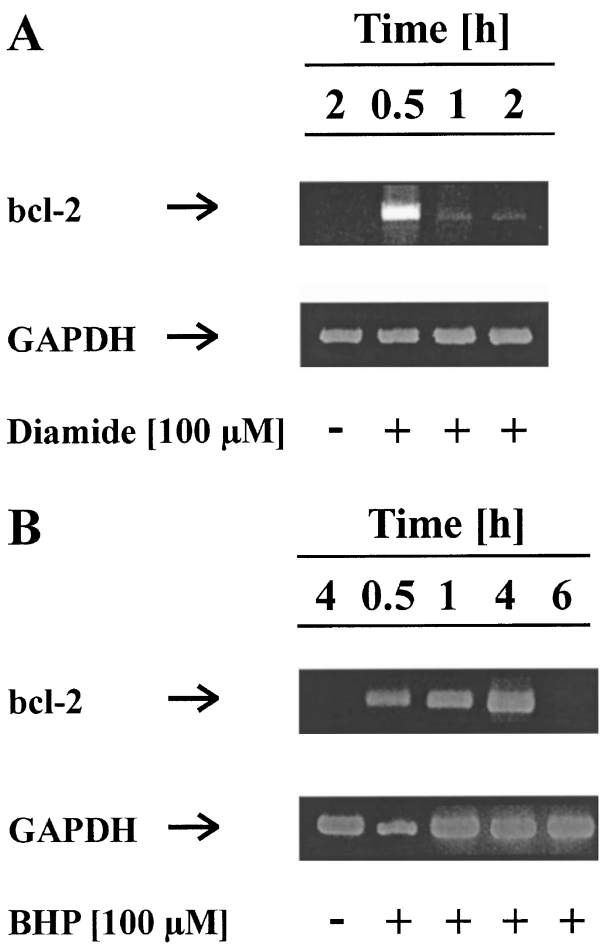

Figure 4 mRNA changes of bcl-2 by diamide and butyl hydroperoxide. Mesangial cells (2×10^6 cells/assay) were incubated with vehicle (control), 100 μM diamide (**A**), or 100 μM BHP (**B**). Incubations were terminated after 0.5, 1, 2, 4, or 6 h as indicated and bcl-2 and GAPDH was detected by RT–PCR as described under Materials and Methods. The blot is representative for three similar experiments

Although diamide and BHP up-regulated Bcl-2, only BHP evoked cell damage. As it is known that Bcl-2 overexpression confers cellular protection the question arises whether oxidative conditions protect cells from a subsequent toxic challenge. For these experiments we chose diamide as BHP was cytotoxic to preactivate MC followed by removal of the oxidant and subsequent addition of the pro-apoptotic agonist GSNO (Figure 6). Prestimulation of MC with 100 μM diamide for 1 or 2 h was non-apoptotic, whereas 250 μM GSNO produced around 22% DNA fragmentation.

In MC that had been preexposed with diamide for 1 h we observed reduced DNA damage in response to NO, while GSNO-induced fragmentation was substantially attenuated with a 2 h lasting preexposure period of diamide. Under these conditions DNA fragmentation was reduced to control values with no signs of cellular necrosis (data not shown). As a result of these examinations we conclude that preactivation of rat MC with a classical oxidizing compound attenuates apoptotic cell death that normally occurs in response to the NO-delivering compound GSNO.

Discussion

Radicals such as NO or O_2^- contain the ability to react with divers targets thereby acting as effector molecules or modulators thus changing the cellular answer towards a stimulus. In small concentrations they are considered as physiological regulators whereas high amounts are cytotoxic in general. Therefore, cells are equipped with self-defense mechanisms such as superoxide dismutase, catalase, or glutathione among others to detoxify radicals and to keep their concentration low. In this and previous studies we established NO-mediated apoptotic cell death in rat MC with the notion that NO donors such as GSNO or spermine-NO dose-dependently evoked apoptosis[22] (Table 1). DNA fragmentation and chromatin condensation appeared as late apoptotic events, whereas p53 and Bax up-regulation as well as caspase activation occurred much earlier.[21] The correlation between apoptosis and NO production is also established *in vivo* for the early phase of Thy1.1 glomerulonephritis.[23,24] Damaging effects of O_2^- have been described for diseases i.e. ischemia-reperfusion injuries. However, O_2^- can also enhance cell growth under some conditions[25] and tumor cells for example are highly resistant against O_2^- and oxidative stress.[26] In addition, radicals react with each other and in case of NO and O_2^- they form peroxynitrite ($ONOO^-$) in a diffusion-controlled reaction.[19] $ONOO^-$ is a strong oxidant but nevertheless some cell types are not toxicologically affected.[27] Glutathione is discussed to detoxify $ONOO^-$ but detailed mechanisms are unknown. However, in this and earlier experiments we have shown that NO/O_2^--coadministration by employing GSNO or spermine-NO together with DMNQ or hypoxanthine/xanthine oxidase is non-destructive for rat MC[20] (Table 1) although an increase of oxidized glutathione, an indicator for oxidative stress, was noticed (unpublished data). We conclude that NO/O_2^--costimulation redirect an apoptotic response elicited by NO rather than by O_2^- to a protective but still unknown pathway.

Increased expression of several proteins such as heat shock proteins,[28] cyclooxygenase-2,[29] as well as Bcl-2 family members,[30] among others are associated with protection. Overexpression of Bcl-2 prevented or delayed apoptosis brought about by NO, ceramide, or cytokine withdrawal.[30,31] In addition, tumor cells with their disturbed apoptotic signaling pathways often display high Bcl-2 expression. During apoptosis Bcl-2 expression generally decreases whereas pro-apoptotic proteins, i.e. Bax increase as seen after NO-treatment (Figure 1A).[21] Negative regulation of Bcl-2 by the tumor suppressor p53 has been described[6] and might be the mechanism behind but Bcl-2 can also be cleaved by caspases.[32] Under conditions of oxidative stress generated by NO/O_2^--coadministration, we observed accumulation of the anti-apoptotic protein Bcl-2 which correlates with cell survival. Experiments with CHX implied and RT–PCR shows that up-regulation of Bcl-2 is most likely based on an increased transcriptional and/or translational mechanism although nuclear run-on experiments would be necessary to verify

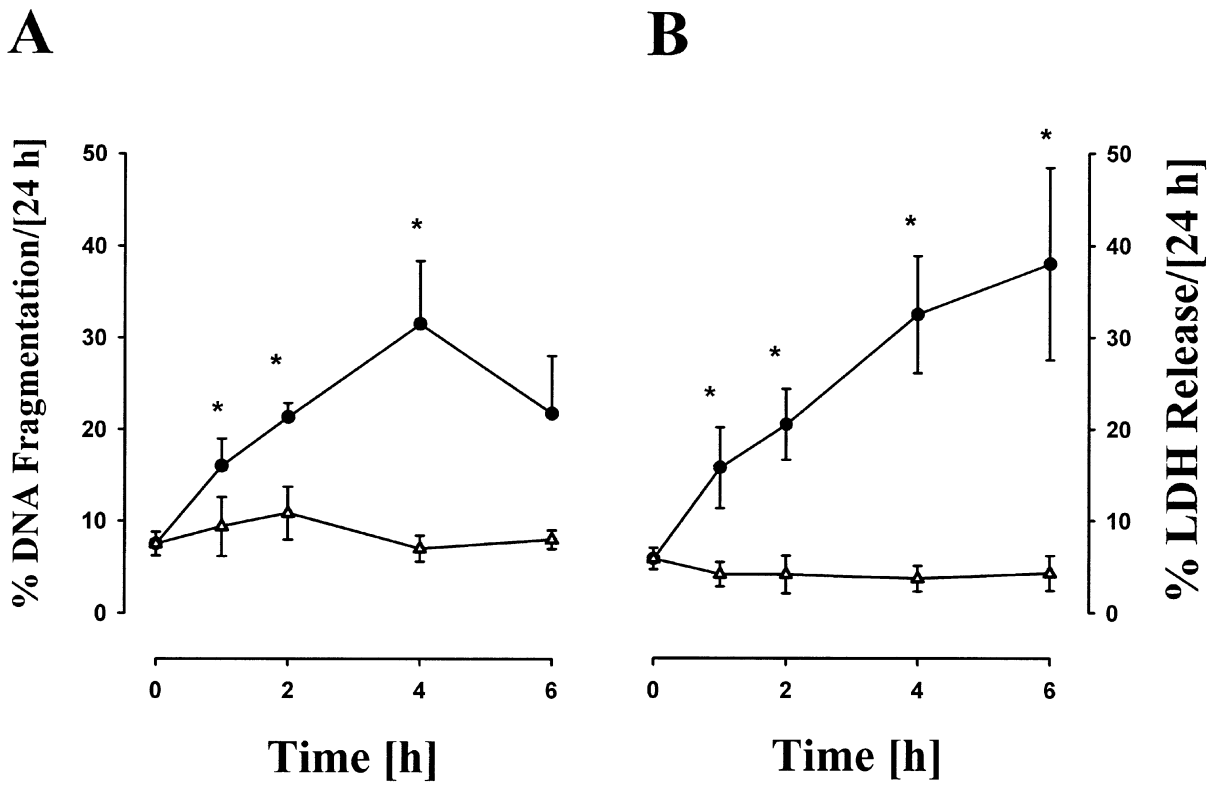

Figure 5 DNA fragmentation and LDH release in response to diamide and butyl hydroperoxide. Mesangial cells (2.5×10^5 cells/assay) were stimulated with vehicle (control), 100 μM diamide (Δ), or 100 μM BHP ($\bullet$) for 1, 2, 4, and 6 h followed by exchanging the incubation medium. DNA fragmentation and LDH release was analyzed after a total incubation time of 24 h as outlined in Materials and Methods. Data are mean values $\pm$ S.D. of at least four separate experiments. *$P < 0.01$ *vs* control

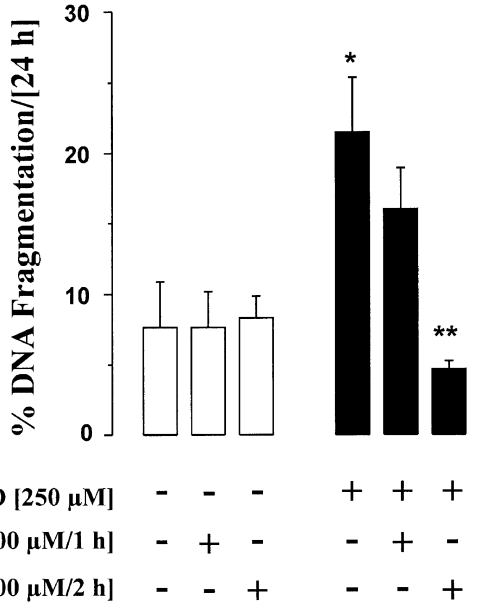

Figure 6 Prestimulation with diamide protects MC against GSNO. Mesangial cells (2.5×10^5 cells/assay) were prestimulated with vehicle, or 100 μM diamide for 1 or 2 h followed by a medium exchange. Afterwards, cells were treated for 24 h with 250 μM GSNO as indicated. DNA fragmentation was analyzed by the dipenylamine assay as outlined in Materials and Methods. Data are mean values $\pm$ S.D. of at least four separate experiments. *$P < 0.01$ *vs* control, **$P < 0.01$ *vs* GSNO

between increased transcription/translation or stabilization of the mRNA or protein (Figure 2A,B). However, stimulation with the oxidative agents diamide and BHP underline that oxidative stress in general can trigger Bcl-2 accumulation (Figures 3 and 4). Nevertheless, expression of Bcl-2 not necessarily confers cellular resistance as seen in case of BHP (Figure 5). Both, diamide and BHP, up-regulate Bcl-2 but in contrast to diamide increased DNA fragmentation and LDH release can be detected after BHP application. The signal transduction leading to Bcl-2 expression induced by oxidative stress is still unknown. In addition, the transcriptional and/or translational regulatory elements of Bcl-2 are marginal examined. Despite oxidative stress, only a few published data describe Bcl-2 up-regulation. For example, nerve growth factor rescues PC12 cells from apoptosis induced by serum withdrawal via bcl-2 up-regulation,[7] interleukin-2 abrogates T-cell apoptosis by Bcl-2 reexpression,[8] vascular endothelial growth factor-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2,[33] and the Epstein-Barr virus latent membrane protein LMP1 enhances B-cell survival through Bcl-2 up-regulation.[9] In neuronal cells, the long form of the transcription factor Brn-3a has been identified to activate the proximal p2 promoter for Bcl-2. In addition, Brn-3a function can be suppressed by p53 as they bind to adjacent sites in the p2 promoter and directly interact with one another.[10,34] This would explain the antagonistic effects of Bcl-2 and p53 on

the rate of apoptosis. Positive regulation of Bcl-2 has been described for the transcription factors NF-AT and aiolos in T-cells[11,35] and tumor necrosis factor induced Bcl-2 expression through NF-κB activation in primary hippocampal neurons.[12] Besides, several consensus binding sites for the transcription factor Sp1 are located on the p1 promoter but its structure more resembles promoters of constitutively expressed genes. The p2 promoter is located approximately 1.3 kb downstream of p1 and includes a CCAAT box and a TATA element and seems to be more important for Bcl-2 up-regulation.[13,14] However, it is still unclear how redox changes as described in this paper influence Bcl-2 expression. As NF-κB can be activated by redox modifications achieved by O_2^- or a disturbed reduced/oxidized glutathione ratio[17] this might be a rational pathway and needs to be studied in more detail.

In several cases, cells can be triggered by mild stress to up-regulated protective proteins which then prevent cell death by a subsequent toxic stimulus. In line, the artificial situation of Bcl-2 overexpression which protects several cell types is well known. Therefore, we used the mild oxidant diamide to up-regulate Bcl-2 and to trigger MC to protect themselves against a cell destructive dose of GSNO (Figure 6). The use of Bcl-2 antisense oligonucleotides for establishing any cause-effect relationship under these experimental conditions cannot be of any help as Bcl-2 antisense oligonucleotides would promote cell death by favoring pro-apoptotic signals.

In conclusion, redox changes regulate the Bcl-2 level in rat MC either as a consequence of NO/O_2^--coincubation or as a result of diamide- or BHP-treatment (Figures 1 and 3). Herein, the accumulation of Bcl-2 protein is most likely based on an increase of its mRNA (Figures 2B and 4). Nevertheless, high levels of Bcl-2 do not necessarily attenuate apoptosis and/or necrosis as seen in case of BHP (Figure 5) but prestimulation with diamide protects MC against a toxic concentration of NO (Figure 6). It seems that Bcl-2 can confer cellular protection under conditions of low oxidative stress only, whereas a stronger oxidative stimulus induces apoptotic/necrotic cell death despite Bcl-2 expression. Signal strength and the onset of anti-apoptotic Bcl-2 actions relative to operating pro-apoptotic signaling pathways may be an important determinant. However, our observations might explain why tumor cells often display high Bcl-2 levels and resist O_2^- and oxidative stress.

Materials and Methods

Reagents

Insulin, diphenylamine, triethanolamine, butyl hydroperoxide, cycloheximide, and diamide were purchased from Sigma, Deisenhofen, Germany. Pyruvate and NADH were bought from Boehringer Mannheim, Mannheim, Germany. RPMI 1640 and medium supplements were ordered from Biochrom, Berlin, Germany. FCS was purchased from Life Technologies, Berlin, Germany; Bcl-2 antibody from Immunotech, Hamburg, Germany; and secondary antibodies from Santa Cruz, Ismaning, Germany. S-nitrosoglutathione was synthesized as described previously.[36] All other chemicals were of the highest grade of purity commercially available.

Culture of mesangial cells

Rat MC were cultured, cloned and characterized as described previously.[37] Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and bovine insulin (5 μg/ml). One day before and during the experiments controls and stimulated cells were kept in medium with 0.5% fetal calf serum. For the experiments, passages 10–25 of mesangial cells were used.

Quantification of DNA fragmentation

DNA fragmentation was assayed as reported.[38] Briefly, following incubations, cells (2.5×10^5 cells/assay) were centrifuged, resuspended in 250 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and lysed by adding 250 μl cold lysis buffer (2 mM EDTA, 0.5% Triton X-100 (v/v), 5 mM Tris-HCl, pH 8.0). After 30 min at 4°C, disintegrated cells were centrifuged (14 000 $\times$ *g*, 15 min) to separate intact chromatin (pellet) from DNA fragments (supernatant). Pellets were resuspended in 500 μl TE buffer and the DNA content of pellets versus supernatants were measured using the diphenylamine reagent.

Bcl-2 quantification

Bcl-2 was quantified by Western blot analysis. Briefly, 2×10^6 cells were incubated for the times indicated, scraped off, lysed in 150 μl lysis buffer (50 mM Tris/HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0), and sonicated. After centrifugation (14 000 $\times$ *g*, 15 min) the protein content in the supernatant was analyzed. Finally, 100 μg protein was resuspended in the same volume of 2$\times$ sample buffer (125 mM Tris/HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromphenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 15% SDS-polyacrylamide gels, and blotted onto nitrocellulose sheets. Molecular weights were calibrated in proportion to molecular weight rainbow marker. Transblots were washed twice with TBS (140 mM NaCl, 50 mM Tris/HCl, pH 7.2) containing 0.1% Tween-20 before blocking unspecific binding with TBS/2% skim milk. The Bcl-2 (1 : 2000 in TBS/0.2% milk) antibody was added and incubated overnight at 4°C. Nitrocellulose sheets were washed five times and unspecific binding was blocked as described. For protein detection, blots were incubated with goat anti-mouse secondary antibodies conjugated with peroxidase (1 : 10 000 in TBS/0.2% milk) for 1 h, followed by ECL detection.

Determination of lactate dehydrogenase release

Following incubations, medium of 2.5×10^5 MC was collected and cells were supplemented with 0.2% (v/v) Triton X-100 (in PBS). Cells were lysed for 4 h at 4°C. Five-hundred μl reaction mix containing 50 mM triethanolamine dissolved in 5 mM EDTA, pH 7.6, 127 mM pyruvat and 14 mM NADH in 1% $NaHCO_3$ was added to 300 μl cell medium and lysed cells. LDH activity was monitored by the oxidation of NADH following the decrease in absorbance at 334 nm. The percentage of LDH release was defined as the ratio of LDH activity in the supernatant to the total amount of LDH (released plus the activity measured in the cell lysate). Apoptotic cells retain their LDH, which was released only after Triton X-100 addition.

RNA extraction and semiquantitative reverse transcriptase-polymerase chain reaction (RT – PCR)

RNA was extracted using RNA Clean (AGS, Heidelberg, Germany). Reverse transcription reactions and PCR for rat bcl-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed using the SuperScript RNase H^- Reverse Transcriptase (Gibco BRL, Karlsruhe, Germany) and recombinant Taq DNA Polymerase (Gibco BRL, Karlsruhe, Germany). The sequence of the primers was as follows: Rat bcl-2 (235 – 926) (16;22), T_A=60°C: 5′ > 3′ ATG GCG CAA GCC GGG A; 3′ > 5′ A CGT ATG GAC CCG GTG TTC ACT. GAPDH (murine 135 – 717, human 155 – 759) (34;35), T_A=60°C: 5′ > 3′ GAA GGC CAT GCC AGT GAG CTT CC; 3′ > 5′ CCA TCA ACG ACC CCT TCA TTG ACC.

The number of amplification cycles (25 for GAPDH; 36 for rat bcl-2) was necessary to achieve exponential amplification where product formation appeared proportional to starting cDNA. A H_2O sample served as a negative control. Products were run on 1% agarose gels and visualized by ethidium bromide staining.

Statistical analyses

Each experiment was performed at least three times and statistical analysis were performed using the two tailed Student's *t*-test. Normal distribution of data is ensured. Otherwise representative data are shown.

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