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Research Report

Constitutive nitric oxide synthases are responsible for the nitric oxide production in the ischemic aged cerebral cortex

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Abstract

Aged brain shows reduced biological plasticity to meet emergency conditions such as ischemia, a process in which nitric oxide (NO) and apoptosis have been shown to play important roles. Using a model of transient global ischemia, we have analyzed the NO system and the p53, bax and bcl-2 response in the cerebral cortex of aged rats. Although immediately after ischemia the NO level is maintained, the reperfusion period increases NO concentrations together with the following: (i) greater bulk-protein nitration mainly due to a 50-kDa immunoreactive band; (ii) an increase in p53 protein; and (iii) an up-regulation of Bax together with a down-regulation of Bcl-2. These results match up with induced endothelial nitric oxide synthase expression immediately after ischemia and in neuronal nitric oxide synthase with the reperfusion. However, inducible nitric oxide synthase was not altered with ischemia/reperfusion. Altogether, these data suggest that NO production in cerebral cortex of aged ischemic animals is due to the constitutive NO synthase isoforms. This response is accompanied by the increased expression of pro-apoptotic proteins.

Theme: Disorders of the nervous system
Topic: Ischemia

Keywords: Nitric oxide; Ischemia/reperfusion; Aged cerebral cortex; Apoptosis

1. Introduction

Age is an important factor for the incidence and prevalence of stroke as well as for its outcome [34,40]. It has been suggested that nitric oxide (NO), the system of which we have previously studied in the aged brain [41], is a key factor in the pathophysiological response of the brain to hypoxia and/or ischemia [5,8,20].

NO is a gaseous chemical messenger biologically synthesized by at least three isoforms of nitric oxide synthase (NOS): neuronal (nNOS) and endothelial (eNOS), both constitutively expressed, and inducible (iNOS) [30].

Balanced NO production exerts a protective effect through vasodilatation and maintenance of cerebral blood flow [38]. On the other hand, cerebral ischemia, particularly during reperfusion, is accompanied by the increased formation of NO and other reactive species, generating deleterious peroxynitrite. These species cause cerebral damage either directly by interacting and destroying cellular proteins, lipids and DNA, or indirectly by interfering with cell signalling pathways and gene regulation [43].

Proteins such as p53 afford protection against DNA damage. This regulatory factor contributes to the preservation of tissue integrity by promoting either DNA repair or

Abbreviations: C_0, ischemia sham group; C_6, reperfusion sham group; eNOS, endothelial nitric oxide synthase; I_0, ischemia group; I_6, reperfusion group; iNOS, inducible nitric oxide synthase; NADPH-d, NADPH–diaphorase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NOx, nitrogen oxides; PB, phosphate buffer

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cell death by apoptosis. Emerging evidence has shown that p53 expression is augmented in response to brain ischemia, particularly before cell death [25,33]. Current literature indicates that NO can induce cell death by both necrosis and apoptosis, depending on its concentration, flux and cell type [22]. Apoptosis is crucial in nervous system development or injury and is regulated by, among others, the Bcl-2 family, which consists of pro-apoptotic (e.g., Bax) and anti-apoptotic (e.g., Bcl-2) proteins [23]. Members of this family are regulated by p53, which transactivates the expression of pro-apoptotic genes, whereas it down-regulates the expression of the anti-apoptotic protein Bcl-2 [32].

Although ischemic damages become more pronounced with advancing age [1,18,34], few studies have been made on the molecular response of the aged brain to ischemia. The aim of the present study is to analyze, in the aged cerebral cortex, the events underlying the early cellular response to a global ischemia, specifically the NO system and the p53, Bax and Bcl-2 response.

2. Materials and methods

2.1. Ischemic model

The study was performed on aged male albino Wistar rats (27–28 months old), kept under standard conditions of light and temperature and allowed ad libitum access to food and water.

The perfusion model of global ischemia was carried out as previously published [37]. Briefly, rats ($n = 28$) were deeply anaesthetized with Ketolar (15 mg/100 mg i.p.) and perfused by means of a peristaltic pump via the left ventricle with an oxygenated, buffered plasma substitute (Hemoce, Hoechst Farma, Barcelona, Spain) enriched with 11 mM glucose. A 15-min protection period with a magnesium concentration of 10 mM in the perfusate was followed by a 15-min adaptation period at a physiological magnesium concentration (1.19 mM) and next by a 30-min ischemia period during which the perfusate, containing no glucose, was gassed with 95% N_2 and 5% CO_2. This was followed by a reperfusion period of up to 6 h, using the same perfusion solution as in the adaptation period. Brains collected immediately after the oxygen and glucose deprivation period formed the ischemia group (I_0), while those collected after the reperfusion period comprised the reperfusion group (I_6). The corresponding sham groups, C_0 and C_6, were perfused without oxygen or glucose deprivation.

All the experiments were conducted according to E.U. guidelines on the use of animals for biochemical research (86/609/EU).

2.2. Western blot analysis

For Western blot analysis, the cerebral cortex from 4 animals from each group was dissected and stored at −80 °C until used. Brain tissues were homogenized in 1/3 (w/v) of 30 mM Tris–HCl, pH 7.4, containing 0.5 mM DTT, 1 mM EDTA, 1% SDS and protease inhibitors. The resulting homogenates were centrifuged for 1 h at 100,000×g. All the procedures were performed at 0–4 °C. Protein concentrations in the supernatants were determined by the Bradford method [7].

Equal amounts of the denatured proteins per lane were loaded and separated on a 7.5% (NOS), 10% (Nitrotyrosine and p53) or 12% (Bax, Bcl-2) SDS–polyacrylamide gel (Mini Protean II, BioRad), as described by Laemmli [24]. Afterwards, proteins were transferred to a PVDF membrane (Immobilon P, Millipore). The membranes were blocked with 5% powdered non-fat milk in 25 mM Tris–HCl, pH 7.6; 137 mM NaCl, 2.6 mM KCl, 0.1% Tween-20, and incubated overnight at 4 °C with diluted monoclonal anti-eNOS (1:800, Transduction), rabbit polyclonal anti-nNOS (1:5000, gift from V. Riveros-Moreno of Welcome Research Laboratories, Berkenhem, UK), anti-iNOS [44], anti-nitrotyrosine (1:1000, produced by our group) [44], anti-p53 (1:1000, Novocastra), anti-Bax (1:1000, Santa Cruz Biotechnology, Inc.) and anti-Bcl-2 (1:800, Santa Cruz Biotechnology, Inc.) in blocking solution. Bound antibody was revealed by means of an enhanced chemiluminescence kit (Amersham) according to the manufacturer's instructions. After immunodetection, membranes were probed with anti-α-tubulin (Sigma) as a loading control. The relative amount of the proteins in each sample was quantified by densitometric scanning.

2.3. NADPH–diaphorase histochemistry

Three rats from each experimental group were perfused through the left ventricle with 500 ml of fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The cerebrum was then removed and postfixed with the same fixative for 4 h at room temperature. The organ was again rinsed and cryoprotected by immersion overnight at 4 °C, in 0.1 M PB containing 30% sucrose.

NADPH–diaphorase histochemical staining was used as a complementary method for the indirect visualization of NOS activity by light microscopy and for histological analysis. Free-floating sections (40 μm) were incubated for 4 h in PBS containing 0.1% Triton X-100. After several washes in 0.1 M Tris–HCl, pH 7.4 buffer, the sections were incubated in the dark, for 45 min at 37 °C, in 0.1 M Tris–HCl, pH 7.4, containing 1 mM β-NADPH and 2 mM nitroblue tetrazolium (in 70% dimethylformamide). The sections were then washed twice with 0.1 M Tris–HCl, pH 7.4, quickly dehydrated in a graded ethanol series, cleared and mounted in DPX (Fluka, Madrid, Spain). Control reactions were performed omitting or replacing β-NADPH with an equivalent amount of either α-NADPH or 0.1 M Tris–HCl.

2.4. NO production

The final products of NO in vivo are nitrite and nitrate, and thus the sum of both species was used as an index of total NO production [15]. Briefly, a portion of tissue specimens from the 4 animals of each group was homogenized in 3 volumes (w/v) of PBS (pH 7.6) at 4 °C. Homogenates were then sonicated and centrifuged at 100,000×g for 60 min at 4 °C. The nitrate plus nitrite (NOx) was determined in the supernatants using a colorimetric kit according to the manufacturer's instructions (Nitrate/Nitrite colorimetric Assay Kit, Cayman Chemical). Results were referred to protein concentration [7].

2.5. Statistical analysis

Data are expressed as means ± SD. Student's t test was performed to evaluate the significance of differences between groups, accepting $P < 0.05$ as the level of significance.

3. Results

3.1. Differential NOS isoform expression in ischemic cerebral cortex

To analyze the NOS response to an ischemic injury, we performed Western blot analysis using the nNOS, eNOS and iNOS antibodies on denatured homogenates from sham, ischemic and reperfused aged rat cerebral cortex. The nNOS, eNOS and iNOS antisera revealed a positive band, each occurring at the appropriate molecular weight (150, 140 and 130 kDa, respectively; Fig. 1).

A decrease in nNOS was found in the cerebral cortex of ischemic rats (I_0) compared with sham (C_0) values ($P < 0.01$). This expression augmented after 6 h of reperfusion (I_6 versus C_6 $P < 0.05$). However, eNOS significantly increased in the ischemic rats (I_0 versus C_0, $P < 0.01$) and declined after reperfusion (I_6 versus I_0, $P < 0.01$). Compared to the corresponding sham group, we detected no ischemia/reperfusion iNOS induction.

3.2. NADPH–diaphorase histochemistry

To evaluate NOS enzymatic activity in situ, as well as to make a histological analysis, we used the NADPH–diaphorase (NADPH-d) histochemical technique [11,17] (Fig. 2). The highest density of NADPH-d staining was detected in neuronal soma and processes, and in the wall of the blood vessels of all experimental groups. In ischemic animals (I_0), the staining for NADPH-d was predominant in the blood vessels. However, after a 6-h reperfusion (I_6), the labeling was located mainly in neurons. The histological analysis showed that ischemic animals (I_0) presented a morphological structure of cortical tissue similar to that of the sham group (C_0); however, in the reperfused group (I_6) neurodegenerative signs were found (see in Fig. 2 neuron with varicose processes inserted in I_6).

3.3. Measurements of total nitrogen oxides (NOx)

We detected no increase in the NOx level during the ischemic period (I_0), although a significant increase ($P < 0.001$) was found after 6 h of reperfusion (I_6) (Table 1).

3.4. Protein nitration

The nitrotyrosine antiserum revealed three immunoreactive bands in all the experimental groups corresponding to proteins of 70, 50 and 40 kDa (Fig. 3), although the first one was very faint.

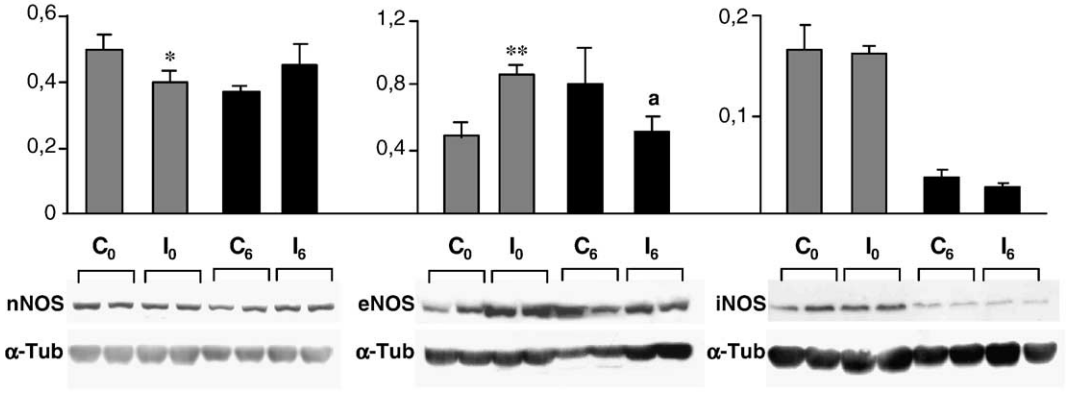

Fig. 1. Western blot analysis of nNOS, eNOS and iNOS expression in the ischemic rat cerebral cortex. Top panel: densitometric quantification of the NOS isoform protein in the ischemic (I_0), reperfusion (I_6) and the corresponding sham (C_0, C_6) groups. Results are average values of 4 experimental animals in each group. Bottom panel: representative autoradiographies of the corresponding nNOS, eNOS and iNOS band. α-Tubulin immunodetection was also included as a protein-loading control. *Protein expression significantly lower than in sham group ($P < 0.05$). **Protein expression significantly greater than in sham group ($P < 0.01$). [a]Protein expression significantly lower than in I_0 group ($P < 0.01$).

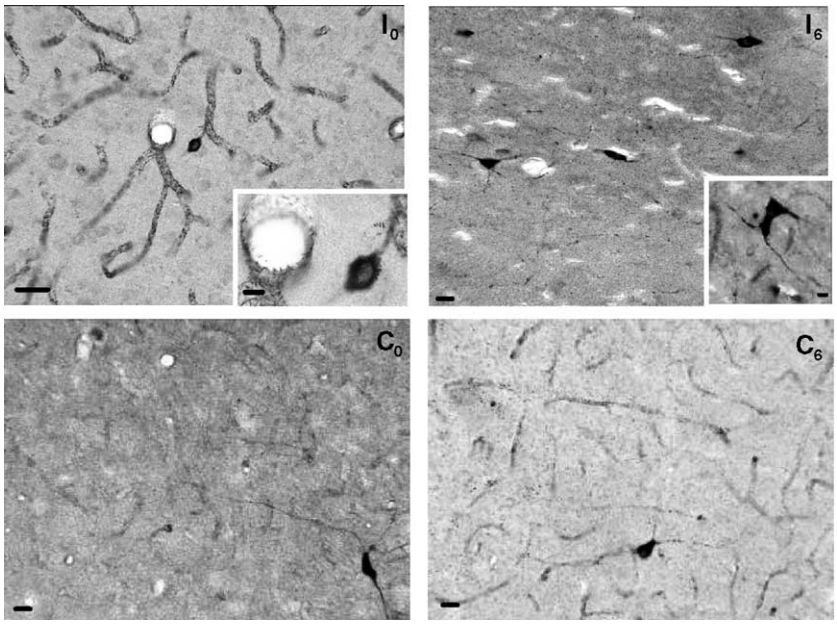

Fig. 2. Photomicrographs of NADPH diaphorase staining in the rat cerebral cortex immediately after transient global ischemia (I_0), after 6 h of reperfusion (I_6) and the corresponding sham groups (C_0, C_6). NADPH diaphorase-positive blood vessels and neurons can be seen. Inserts in I_0 and I_6 show NADPH diaphorase-positive neurons in both experimental groups. Scale bars: I_0, I_6, C_0 and C_6 20 μm; inserts 10 μm.

The quantitative evaluation of bulk-nitrated proteins demonstrated a significant increase in the nitration only when the ischemia was followed by 6 h of reperfusion ($P < 0.001$). This increase was due mainly to the 50-kDa protein.

3.5. The p53 response to ischemic/reperfusion injury

To investigate the effect of ischemia/reperfusion on p53, we analyzed the p53 protein level in the different experimental groups (Fig. 4). Although p53 does not respond to the ischemic injury (I_0), when the ischemia is followed by a 6-h reperfusion, its expression is significantly increased (I_6 versus C_6 $P < 0.02$).

3.6. Does the ischemic injury induce apoptosis?

We studied the expression of Bcl-2 and Bax, two proteins which display anti-apoptotic or pro-apoptotic function, respectively. Fig. 5 shows a representative Western blot of both proteins. Immediately after the ischemic injury (I_0), only Bax protein level fell ($P < 0.01$). However, after 6 h of reperfusion (I_6) Bax expression increased ($P < 0.02$) while Bcl-2 decreased ($P < 0.02$), indicating a predisposition to apoptosis.

Table 1
Nitrate/nitrite levels in the ischemic rat cerebral cortex

NOx level (μmol/mg)			
C_0	I_0	C_6	I_6
0.760 ± 0.077	0.734 ± 0.131	0.686 ± 0.408	1.551 ± 0.174*

Data are the means (±SD) of four determinations.
* NOx levels significantly greater than in sham group ($P < 0.001$).

4. Discussion

Most experimental models only use healthy young animals to study the molecular mechanisms underlying the ischemic process. Using aged rats, we found in the ischemic cerebral cortex changes in NO production due to the constitutive NOS isoforms, accompanied by an increase in

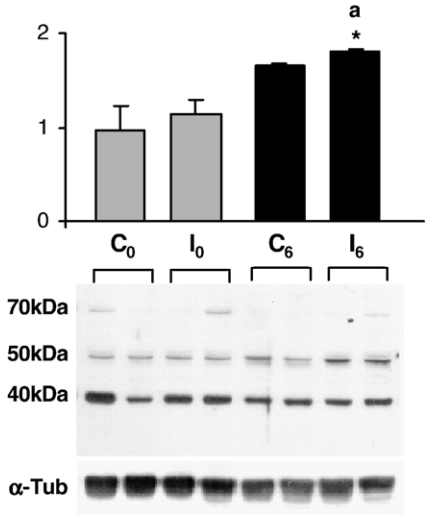

Fig. 3. Western blot analysis of the nitrotyrosine modified proteins in the ischemic rat cerebral cortex. Top panel: densitometric quantifications of bulk nitrotyrosine-modified protein expression in the ischemic (I_0), reperfusion (I_6) and corresponding sham (C_0, C_6) groups. Results are average values of 4 experimental animals in each group. Bottom panel: representative autoradiography of the different immunoreactive bands (70, 50 and 40 kDa); α-tubulin was also included as a protein-loading control. *Protein expression significantly greater than in sham group ($P < 0.001$). [a]Protein expression significantly greater than in I_0 group ($P < 0.001$).

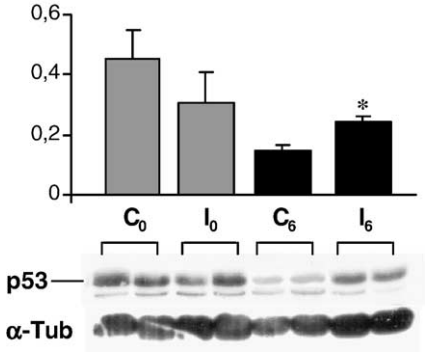

Fig. 4. The p53 response in the rat ischemic cerebral cortex. Top panel: densitometric analysis of p53 protein level in the ischemic (I_0), reperfusion (I_6) and corresponding sham (C_0, C_6) groups. Results are average values of 4 experimental animals in each group. Bottom panel: representative autoradiography of the p53 immunoreactive band; α-tubulin immunodetection was also included as a protein-loading control. *Protein expression significantly greater than in sham group ($P < 0.02$).

protein nitration and in the expression of pro-apoptotic proteins in reperfused animals.

Ischemic models usually involve invasive procedures, particularly those employing the isolated brain or brain slices. Although we have used a preparation of the whole brain, maintained and perfused in the cranial cavity [3,37], it undoubtedly involves some additional injury, particularly in old animals. To overcome this obstacle, we compared all the results to those of the corresponding sham group (C_0 and C_6), so that the ischemia/reperfusion effect could be clearly discerned.

In the present model of global ischemia, the early changes in NO production appear to be due to the constitutive NOS, according to the expression of NOS isoenzymes and to the location of the activity observed by means of the NADPH–diaphorase histochemistry. Particularly during the ischemia eNOS expression is augmented, while nNOS increases during reperfusion. It has been suggested that eNOS-derived NO may protect against ischemia by contributing to vasodilatation and by inhibiting aggregation and adherence of platelets or leukocytes [2,12,27]. In fact, eNOS knockout mice or rats treated with a selective eNOS inhibitor suffer greater damage than do controls, after reversible ischemia [19,21]. On the other hand, NO overproduction by nNOS could lead to neurotoxicity [16,38]. Our results in ischemic aged rats reinforce this hypothesis, as increased nNOS protein but not eNOS correlates with higher levels of either nitrative stress or pro-apoptotic proteins.

In addition, we have detected no ischemia/reperfusion iNOS induction. This absence of iNOS response in aged animal contrasts with the results found by others in young animals using this same experimental model [3]. Although, we have previously reported that aging does not affect the basal iNOS level in rat cerebral cortex [41], the present results suggest an age differential response of this isoenzyme to this type of ischemic insult.

The results of Western blotting underline an important rise in bulk-protein nitration during the reperfusion period, parallel to the higher NO production described above. It is known that cerebral ischemia and reperfusion increase the production of reactive species in the brain and that peroxynitrite production depends on the availability of NO in the presence of superoxide [13]. Once formed, peroxynitrite nitrates tyrosine residues, among others, thereby affecting the function of many proteins [6,14,28]. The nitrated protein pattern detected in the aged ischemic cerebral cortex points to the 50-kDa immunoreactive band as the main factor responsible for the nitrative change observed. Identification of the different nitrated proteins would enable a fuller understanding of the functional implications of these modifications.

Overproduction of NO by neurons during the early stages of ischemia may be detrimental to neuronal survival by accelerating cell metabolism and/or damaging the DNA [26]. Under these circumstances, p53 expression has been reported to be up-regulated, inducing a complex signalling cascade, which probably differs according to the type of tissue examined [36]. Most published data suggest that p53 aggravates brain injury, and in fact p53 knockout mice are more resistant to the ischemic insult [10]. On the contrary, other authors propose a protective effect of p53 through mechanisms unrelated to its pro-apoptotic properties [29]. It is also known that NO increases p53 expression, which in turn inhibits NOS activity in a negative feedback loop [4]. On the other hand, little has been published concerning the p53 role in the aged brain. Chung et al. [9] described an up-regulation of p53 in the Purkinje cells of aged rats, although another report suggests that, at least in senescence-accelerated mice, there are no significant differences in the p53 cerebral content values [35]. In addition, the p53 response to ischemic injury has not been reported in aged animals. In this sense, our results show that, in the aged cerebral cortex, p53 levels do not rise immediately after ischemia but rather with reperfusion, when the nitrative stress is higher.

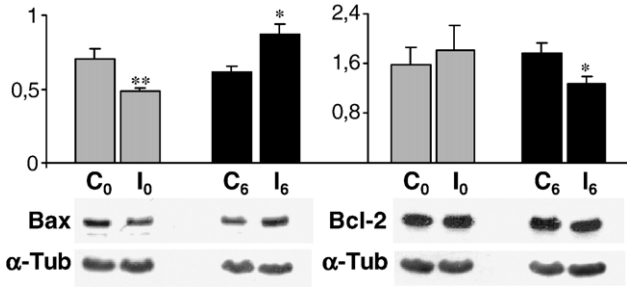

Fig. 5. Western blot analysis of Bax and Bcl-2 expression in the rat ischemic cerebral cortex. Top panel: densitometric quantification of Bax and Bcl-2 protein level in the ischemic (I_0), reperfusion (I_6) and corresponding sham (C_0, C_6) groups. Results are average values of 4 experimental animals in each group. Bottom panel: representative autoradiographies of the Bax and Bcl-2 immunoreactive bands. α-Tubulin immunodetection was also included as a protein-loading control. *Protein expression significantly different from sham group ($P < 0.02$). **Protein expression significantly lower than in sham group ($P < 0.01$).

Neuronal death after brain ischemia has been traditionally ascribed to necrotic mechanisms, although a role of apoptotic mechanisms has been proposed in acute neuronal death following brain ischemia [39]. In this sense, we have examined the changes of Bcl-2 and Bax contents, which inhibit or promote apoptosis, respectively. The decrease in Bcl-2 expression and the increase in Bax protein detected after 6 h of reperfusion suggest the occurrence of apoptosis in our model. As mentioned above, reperfusion boosts NO production, and it has been reported that NO-induced apoptosis is accompanied by down-regulation of Bcl-2, which is linked to the release of apoptotic factors such as cytochrome *c* from mitochondria into the cytosol [42]. Also, the increased p53 regulates gene expression of bax [31]. Thus, after ischemia, p53- and NO-controlled mechanisms may be activated in regions of the aged cerebral cortex with extensive damage, triggering apoptosis (via elevation of Bax and down-regulation of Bcl-2).

In conclusion, our results indicate that in the aged cerebral cortex, the changes in NO production after global cerebral ischemia are due to the constitutive NOS isoforms. In particular, reperfusion promotes a higher nNOS level, which is accompanied by the increased expression of pro-apoptotic proteins.

Acknowledgments

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