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(54) Title: NITROSYLATION TO INACTIVATE APOPTOTIC ENZYMES

(57) Abstract

S–nitrosylation (reaction of nitric oxide [NO] species with critical cysteine sulfhydryl groups of a caspase [RS] to form RS–NO) inhibits caspase activity and thereby ameliorates apoptosis not only in neuronal cells, but also in other tissues. Additionally, ICE–like (caspase–like) sequence ICARG is used to protect from excitoxic neuronal damage and neurological as well as non–neurological and non–ophthalmological indications characterized by undesired apoptosis.

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NITROSYLATION TO INACTIVATE APOPTOTIC ENZYMES

Cross Reference to Related Application

This application claims benefit from provisional application Serial No. 60/042,144, filed March 31, 1997.

Background of the Invention

This application is in the general field of treating diseases characterized by apoptosis.

Apoptosis is a programmed cell death which occurs not only in natural development but also in disorders of many tissues incident to certain insults, such as growth factor deprivation and exposure to reactive oxygen species. Apoptosis is implicated, for example in chronic neurodegenerative disorders such as Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, and AIDS dementia, as well as in the penumbra of acute focal cerebral infarcts and after spinal chord injury or other forms of central nervous system trauma. Schwartz and Milligan, *Trends in Neurosci.* 19:555-562 (1996).

The family of cysteine proteases related to interleukin 1β-converting enzyme (ICE) has been generally found to be essential to apoptosis. Patel et al. *FASEB. J.* 10:587-797 (1996); Schwartz and Milligan, *Trends in Neurosci.* 19:555-562 (1996); Troy et al., *Proc. Nat'l Acad. Sci. (USA)* 93:5635-5640 (1996). The term caspase is now generally used to designate this ICE family of enzymes. Alnemri et al. *Cell* 87:171 (1996). A conserved cysteine-containing sequence characteristic of caspases is essential for their activity. Patel et al. *FASEB. J.* 10:587-797 (1996). For all known caspase enzymes, this sequence is QACRG. Patel et al. FASEB. J. 10:587-797 (1996). An apoptotic-like neuronal cell death process induced by growth factor deprivation or reactive oxygen

species exposure of a neuronal-like cell line (PC12 cells) can be ameliorated by a pseudo-caspase enzyme, a fragment of the natural substrate (IQACRG) which contains that critical sequence and is believed to complex with and thus protect the natural substrates from degradation by caspases. Troy et al., Proc. Nat'l. Acad. Sci. (USA) 93:5635-5640 (1996).

Summary of the Invention

S-nitrosylation (reaction of nitric oxide [NO] species with critical cysteine sulfhydryl groups of a caspase [RS] to form RS-NO) inhibits caspase activity and thereby ameliorates apoptosis. Such inhibition takes place throughout the body, in both neuronal and non-neuronal tissue and in ophthalmological and non-ophthalmological tissues. Accordingly, one aspect of the invention features methods of treating diseases characterized by apoptosis, by administering an S-nitrosylating compound to the patient in an amount effective to reduce caspase activity.

Another aspect of the invention features the use of caspase pseudo-enzymes to treat all apoptotic indications, neurological, ophthalmological, and others. Specifically, apoptotic-like neuronal cell death of cerebrocortical neurons induced by mild excitotoxic injury [see, Bonfoco et al. Proc. Nat'l Acad. Sci. (USA) 92:7162-7166 (1995)] can be ameliorated by pseudo-caspase enzymes -- peptides containing the sequence QACRG, particularly those containing IQACRG and most particularly, IQACRG itself. These peptides may be linked to an antennapedia sequence (see Troy et al., cited above, which is hereby incorporated by reference) or they may be incorporated into liposomes to enhance transport across the blood-brain barrier and/or entry into neurons.

Finally the two approaches (nitrosylating therapies and pseudocaspase therapies) may be combined to treat apoptotic indications.

Description of the Preferred Embodiments

Among the non-neuronal medical indications that can be treated according to the invention are: autoimmune diseases, including diseases of lymphocytes, systemic lupus erythematosus (SLE), synovial cells of rheumatoid arthritis (RA), fibroblasts (scleroderma), defective hematopoiesis, atherosclerosis, gastrointestinal diseases associated with cell death, including hepatobiliary disease, cell-mediated cytotoxicity, drug and chemical toxicity, carcinogenesis, viral disease, T-cell depletion associated with AIDS, oxidative stress, glomerulonephritis, cystic renal disease, renal tubular injury, atherosclerosis, myocardial ischemia or infarction, diabetic nephropathies, Chagas' disease polycystic kidney disease, glomerulonephritis, hypocellular end-stage kidney disease, kidney disease associated with diabetes mellitus, Sjögren's syndrome, fulminant hepatitis (hepatitis B and C), red cell pathology; polycythemia, thalassemia, deficiencies in folate, vitamin B12, iron, glucose-6-phosphate dehydrogenase abnormalities, bone marrow failure, myelodysplasia, and chronic inflammatory disease.

Neuronal medical indications include Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis, autoimmune inflammation of the nervous system, multiple sclerosis, demyelinating diseases, autoimmune encephalomyelitis, status epilepticus and other seizure disorders, neurological mechanical trauma, hypoxia hypoglycemia, and ischemia, optic neuropathies, glaucoma, AIDS dementia, stroke, neuropathic pain, Huntington's disease, metabolic disorders (including

homocyst(e)inemia) Tourette's syndrome, and withdrawal from drug addiction, drug tolerance or drug dependency.

The S-nitrosylating therapeutics that can be used to effect treatment according to the invention include any compound which produces a sufficient amount of NO (most probably a related redox species such as an NO^+ equivalent or NO^- donor) upon administration to a mammal to decrease apoptotic damage or injury. For convenience, I have also used the less precise term "NO-generating compound" to include compounds that produce the above described NO-related redox species (e.g., RS-NO, an NO^+ equivalent, or NO^-) or a physiologically acceptable salt thereof.

Verification that a particular compound nitrosylates a caspase can be accomplished by the experiments provided below.

The two preferred compounds (nitroglycerin and sodium nitroprusside) provide the advantage of a proven record of safe human administration (i.e., for treatment for cardiovascular disorders). Other nitroso-compounds that can be used in the method of the invention include: isosorbide dinitrate (isordil); S-nitroso captopril (SNOCAP); Serum albumin coupled to nitric oxide ("SA-NO"); Cathepsin coupled to nitric oxide (cathepsin - NO); tissue plasminogen activator coupled to NO (tPA-NO); SIN-1 (or molsidomine) cation-nitrosyl complexes, including Fe^{2+}-nitrosyl complexes; Nicorandil; S-nitrosoglutathione; NO coupled to an adamantine derivative, such as memantine (see U.S. 5,614,650 hereby incorporated by reference); S-nitrosothiols including S-nitrosocysteine; quinones, including pyrroloquinoline quinone (PQQ), ester

derivatives of PQQ, or ubiquinone; sydnonimines or NONOates having the formula

$$X\text{-}[N(O)NO]^-$$

where X is any nucleophile including an amine; and agents which generate an oxidizing cascade similar to that generated by NO such as α-lipoic acid (thioctic acid and its enantiomers); dihydrolipoate; glutathione; ascorbate; or vitamin E. Alternatively, the NO donor can be a nitroxyl (NO^-) generator such as Piloty's acid, Angeli's salt (Oxi-NO), or sulfi-NO. See generally the list of NO compounds described in Chapter 7 of Feelisch and Stamler, Methods in Nitric Oxide Research, Wiley and Sons, Chichester, UK, (1996), pp 71-115, which is hereby incorporated by reference. Without wishing to be bound to a specific theory, the NO group in various redox forms can be transferred or react with the critical cysteine at the active site of caspases to decrease enzymatic function and thus provide protection against apoptosis.

Any of the above described nitroso-compounds may be combined with other redox compounds that facilitate production and maintenance of NO. For example, direct NO-generators can be combined with pyroloquinoline quinone (PQQ) (see U.S. Patent 5,091,391), or PQQ's derivative esters, or other quinones such as ubiquinone.

The ability of NO to be transported to and cross cell membranes facilitates therapies according to the invention.

My earlier U.S. patent U.S. 5,455,279 discloses that it is possible to build tolerance to undesired cardiovascular side effects of NO compounds (e.g., hypotension), without losing the desired protective effect. Accordingly, nitroso compounds capable of protecting against apoptosis can be administered continuously over an extended period with gradually escalating dosage, beginning at a dosage level which does

not substantially reduce the patient's blood pressure, and, later, increasing gradually to higher dosage levels desirable for achieving the anti-apoptotic effect. The later dosage level is high enough to substantially reduce a naive patient's blood pressure, but, due to the tolerance that has been achieved in the patient, the compound's blood-pressure lowering effect is reduced to tolerable levels.

An alternative way to offset the hypotensive effects of NO donors such as nitroglycerin is to co-administer with the NO-donating compounds, agents such as phenylephrine, dopamine, or yohimbine. See, e.g., Ma et al. *Cardiovasc. Pharmacol.* 20: 826-836 (1992). These agents may be given parenterally (e.g. IV) or orally depending on the drug.

Nitroglycerin may be administered by transdermal patch as described in detail in my U.S. patent 5,455,279, referenced above. Alternatively, a long-lasting nitrate formulation, such as isosorbide dinitrate SR tablets which are usually administered every 8-12 hours, are administered more frequently (e.g., every 4 hours) to induce cardiovascular tolerance but preserve their effect on nitrosylation of caspases. It is also useful to administer superoxide dismutase (SOD), catalase, or both, to limit toxicity by decreasing the formation of peroxynitrite from the reaction of NO· with superoxide anion ($O_2^{\cdot -}$).

The compound may be included in a pharmaceutical preparation, using a pharmaceutical carrier (e.g., physiological saline); the exact formulation of the therapeutic mixture depends upon the route of administration. Preferably, the compound is administered orally or intravenously, but it may also be administered sublingually, by nasal spray, by transdermal patch, subcutaneously, intraventricularly, intravitreally, or by

ointment. The preferred compounds, nitroglycerin or their derivatives (including all those preparations commercially available, e.g., those listed in the *Physician's Desk Reference* (1997) under coronary vasodilators or under nitroglycerin or nitroglycerin intravenous and including isosorbide mononitrate, isosorbide dinitrate, nitroglycerin sublingual, Minitran, NT-1, Niotrocor, Nitroderm, Nitrodisc, Nitro-dur, Nitro-Dur II, Nitrofilm, Nitrogard, Nitroglin, Nitropen, Tridil, and 6-chloro-2-pyridylmethyl nitrate) are administered at 0.01 mg - 60 gm/day, in divided doses. Sodium nitroprusside -- $Na_2[Fe(CN)_5NO]-2H_2O$ (from Elkins-Sinn, Inc., Cherry Hill NJ), Nipride (from Roche, Nutley, NJ), or other preparations -- are administered intravenously at 0.5-10μg/min.

Compounds determined to be effective protective agents by the assays described herein are administered as above at a dosage suitable to reduce cellular damage. Generally, such compounds are administered in dosages ranging from 0.01 mg - 60 gm/day, more preferably in dosage of 0.1-5 mg/day.

Those skilled in the art will understand that there are other factors which aid in determining optimum dosage. For example, for NO-conjugated drugs, the dosage used for the unconjugated drug (e.g. tPA a dosage of 0.35-1.08 mg/kg and generally $\leq$ 0.9 mg/kg) is predictive of useful NO-conjugate dosage. Dosages may be divided. It is desirable to maintain levels of NO or related redox species in the brain of 1 nM to 500 μM. Treatment may be repeated as necessary.

Regarding neuronal therapies, polyethylene glycol (PEG) is used to enhance absorption into the central nervous system (CNS) and efficacy of SOD and/or catalase. ~~An~~ SOD mimic, the protein-bound polysaccharide of <u>Coriolus versicolor</u> QUEL, termed "PS-K", may also be

effective by parenteral or oral routes of administration, especially with PEG to enhance CNS absorption, and such mimics may be substituted for SOD in this aspect of the invention. See Kariya et al., Mol. Biother. 4:40-46 (1992); and Liu et al., (1989) Am. J. Physiol. 256:589-593."

Examples

Example 1

We have shown that S-nitrosylation of caspases [e.g., CPP32 (caspase -3, Alnemri et al.) and ICE (caspase-1)] inhibit their ability to cleave the substrate PARP [poly(ADP-ribose)polymerase]. Fluorogenic assays of caspase activity in neuronal and other cellular cultures revealed that S-nitrosylation by either exogenous or endogenous NO species inhibited enzyme activity and therefore prevented apoptosis.

Nitrosylation of the critical cysteine in caspases (which is present in the peptide ICARG) can be verified by the Saville reaction, well known to those skilled the art. Feelish and Stamler, cited above, Ch. 36, p. 527.

In cell toxicity experiments we demonstrate inhibition of caspase-induced apoptosis by endogenous NO in HEK-293-nNOS cells. HEK-293 cells [Bredt et al., *Nature* 351:714-719 (199)} overexpressing nNOS were transiently transfected with mICE-lacZ (containing the caspase-1 construct [Miura et al., *Cell* 75:653-660 (1993)] or control placZ using the calcium phosphate precipitation method. Following transfection, cells were incubated in absence (0μM) or presence of 6μM 4-Br-A23187 for 48 h. Cells were then permeabilized, fixed, and stained with propidium iodide. Apoptotic nuclei were counted in $\geq$12 fields and results expressed as a percentage of total nuclei. The results are shown in Fig. 1. Values are the mean $\pm$ SEM for $n \geq 3$ from at least

two experiments. A Fisher's protected least significance difference post-hoc test indicated a highly significant decrease in apoptosis of HEK-293-nNOS cells after caspase-1 transfection and 4-Br-A23187 exposure to increase Ca^{2+} and thus activate the nNOS to produce NO ($P \leq 0.007$).

Example 2

Fig. 2 depicts the results of one specific experiment in which the pseudo-caspase enzyme IQACRG ("VICE") demonstrably decreases the apoptosis induced by the excitotoxin N-methyl-D-aspartate (NMDA) plus glycine (an NMDA receptor co-agonist.) Note that VICE's entry into cells is facilitated by coupling the antennapedia peptide (a signal sequence allowing translocation across cell membranes). Note also that the NMDA receptor is a subtype of glutamate receptor, which, when overexcited, causes neuronal damage. The reduction in NMDA-induced (300 μM NMDA/5 μM glycine) neuronal apoptosis effected by 200 nM VICE is significant.

These findings support my conclusion that S-nitrosylation of caspase inhibits apoptosis. The pseudo-enzyme IQACRG containing the caspase active site also prevents apoptosis. The combination of the two is synergistic.

What is claimed is:

1. A method of ameliorating apoptotic non-neuronal and non-ophthalmological cell death or injury in a mammal by administering an effective amount of an S-nitrosylating compound to the mammal.

2. The method of claim 1 in which the S-nitrosylating compound reduces enzymatic activity of a caspase.

3. The method of claim 1 in which the compound is selected from the group consisting of:

nitroglycerin;
sodium nitroprusside;
isosorbide dinitrate (isordil);
S-nitroso captopril (SNOCAP);
Serum albumin coupled to nitric oxide ("SA-NO");
Cathepsin coupled to nitric oxide (cathepsin-NO);
tissue plasminogen activator coupled to NO (tPA-NO);
SIN-1 (or molsidomine) cation-nitrosyl complexes [including Fe^{2+}-nitrosyl complexes];
Nicorandil;
S-nitrosoglutathione;
NO coupled to an adamantane derivative such as memantine;
S-nitrosothiols including S-nitrosocysteine;
quinones (including pyrroloquinoline quinone (PQQ), ester derivatives of PQQ, or ubiquinone);
sydnonimines, such as SIN-1.

4. The method of claim 1 in which the compound is a NONOate having the formula:

$$X\text{-}[N(O)NO]^-$$

where X is any nucleophile including an amine; and agents which generate an oxidizing cascade similar to that generated by NO including: α-lipoic acid (thioctic acid and its enantiomers); dihydrolipoate; glutathione; ascorbate; and vitamin E.

5. The method of claim 1 in which the compound is a nitroxyl (NO^-) generator.

6. The method of claim 5 in which the compound is Piloty's acid, Angeli's salt (Oxi-NO), or sulfi-NO.

7. The method of claim 1 in which the compound is an NO-generating compound listed in Chapter 7 of Feelisch and Stamler, Methods in Nitric Oxide Research, Wiley and Sons, Chichester, UK, (1996), pp 71-115, which is hereby incorporated by reference.

8. The method of claim 1 in which the mammal is a human patient characterized by at least one medical indication selected from the group consisting of:

non-neuronal or non ophthalmological oxidative stress, autoimmune diseases, including diseases of lymphocytes, systemic lupus erythematous (SLE), rheumatoid arthritis (RA), fibroblasts (scleroderma), defective hematopoiesis, atherosclerosis, gastrointestinal diseases associated with cell death including hepatobiliary disease, cell-mediated cytotoxicity, drug and chemical toxicity, carcinogenesis, viral disease, T-cell depletion associated with AIDS, glomerulonephritis, cystic renal disease, renal tubular injury, atherosclerosis, myocardial ischemia or infarction, diabetic nephropathies, Chagas' disease, polycystic kidney disease, glomerulonephritis, hypocellular end-stage kidney disease, kidney disease associated with diabetes mellitus, Sjögren's syndrome, fulminant hepatitis (hepatitis B and C), red cell pathology; polycythemia, thalassemia, deficiency in folate, vitamin B12, iron, glucose-6-phosphate dehydrogenase abnormalities, bone marrow failure, myelodysplasia, chronic inflammatory disease, and trauma leading to non-neurological and non-ophthalmological hypoxia and ischemia.

9. A method treating a medical indication characterized by apoptosis, the method comprising administering to a therapeutic composition comprising a pseudocaspase enzyme to the patient.

10. The method of claim 9 in which the medical indication is characterized by injury of central nervous system neurons, mediated by excitotoxicity, e.g., excessive stimulation of glutamate receptors including the NMDA and non-NMDA receptor complexes.

11. The method of claim 9 in which the pseudocaspase enzyme comprises the sequence QACRG.

12. The method of claim 11 in which the pseudocaspase enzyme comprises the sequence IQACRG.

13. The method of claim 12 in which the pseudocaspase enzyme is IQACRG.

14. The method of any of claims 9-13 in which the pseudocaspase enzyme is included in a therapeutic composition which comprises a transfer component enhancing permeation of the blood-brain barrier and/or cell membranes.

15. The method of claim 14 in which the transfer component is an antennapedia protein and the antennapedia protein is conjugated to said pseudocaspase enzyme.

16. The method of any of claims 9-13 in which the therapeutic composition comprises liposomes to enhance permeability.

17. The method of any of claim 9 in which the medical indication is Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis, autoimmune inflammation of the nervous system, multiple sclerosis, demyelinating diseases, autoimmune encephalomyelitis, status epilepticus and other seizure disorders, neurological mechanical trauma, neuronal hypoxia, hypoglycemia, or ischemia, Huntington's disease, AIDS dementia, stroke, neuropathic pain, metabolic disorders (including homocyst(e)inemia), Tourette's syndrome, and drug addiction, tolerance, withdrawal, or dependency.

18. The method of claim 9 in which the medical indication is non-neuronal or non ophthalmological oxidative stress, autoimmune diseases, including diseases of lymphocytes, systemic lupus erythematous (SLE), rheumatoid arthritis (RA), fibroblasts (scleroderma), defective hematopoiesis, atherosclerosis, gastrointestinal diseases associated with cell death including hepatobiliary disease, cell-mediated cytotoxicity, drug and chemical toxicity, carcinogenesis, viral disease, T-cell depletion associated with AIDS, glomerulonephritis, cystic renal disease, renal tubular injury, atherosclerosis, myocardial infarction, diabetic nephropathies, Chagas' disease, polycystic kidney disease, glomerulonephritis, hypocellular end-stage kidney disease, kidney disease associated with diabetes mellitus, Sjögren's syndrome, fulminant hepatitis (hepatitis B and C), red cell pathology; polycythemia, thalassemia, deficiency in folate, vitamin B12, iron, glucose-6-phosphate dehydrogenase abnormalities, bone marrow failure, myelodysplasia, chronic inflammatory disease, and trauma leading to non-neurological and non-ophthalmological hypoxia and ischemia..

19. A method of treating a patient characterized by an ophthalmological condition, the method comprising intravitreal administration of a composition comprising a pseudocaspase enzyme.

20. The method of claim 17 or claim 19 in which the therapeutic composition comprises liposomes.

21. The method of claim 17 or claim 19 in which the therapeutic composition comprises an antennapedia protein conjugated to the pseudocaspase enzyme.

22. The method of claim 21 in which the ophthalmological condition is glaucoma.

23. The method of claim 19 in which the ophthalmological indication is an optic neuropathy.

24. The method of claim 21 in which the therapeutic composition is administered in a liposome.

25. The method of claim 9 further comprising administering to the patient an S-nitrosylating compound.

26. A method treating drug withdrawal, drug tolerance, or drug addiction in a patient, by administering an effective amount of an S-nitrosylating compound to the patient.

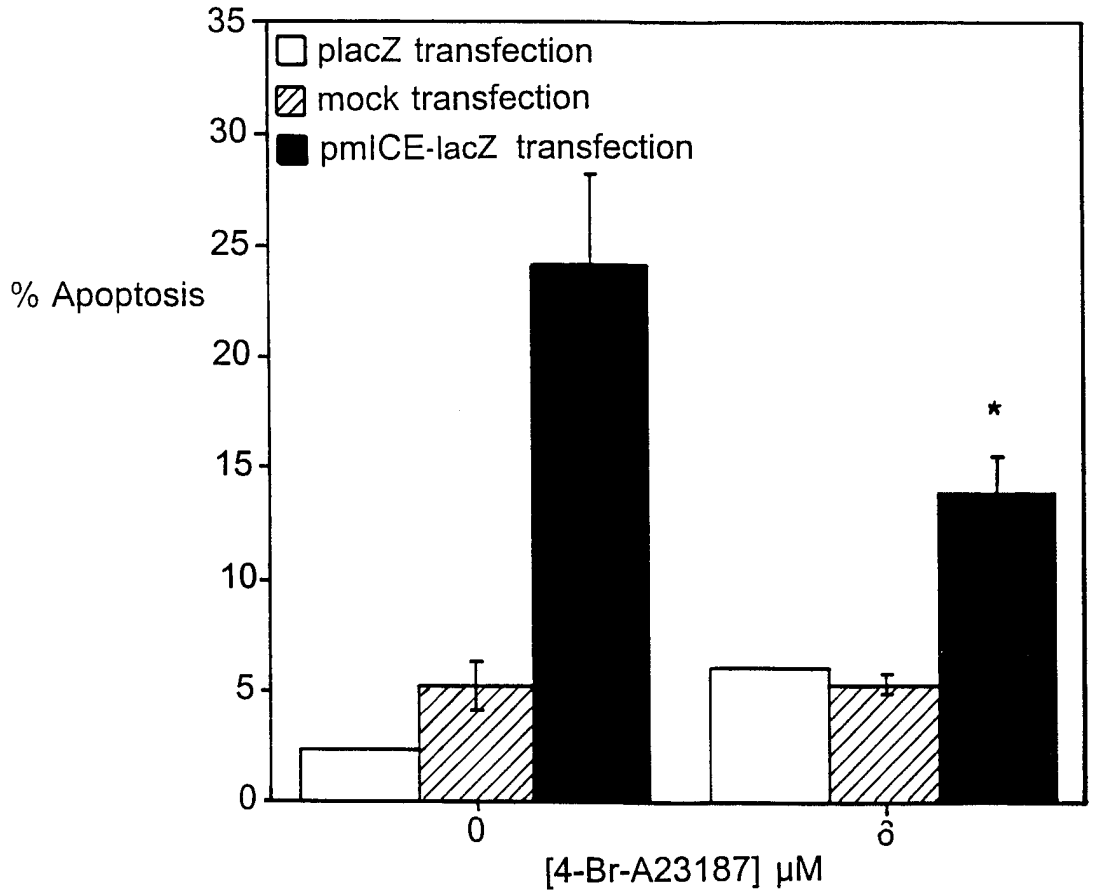
35
30
25
20
15
10
5
0
% Apoptosis
placZ transfection
mock transfection
pmICE-lacZ transfection
*
0
ô
[4-Br-A23187] µM

FIG. 1

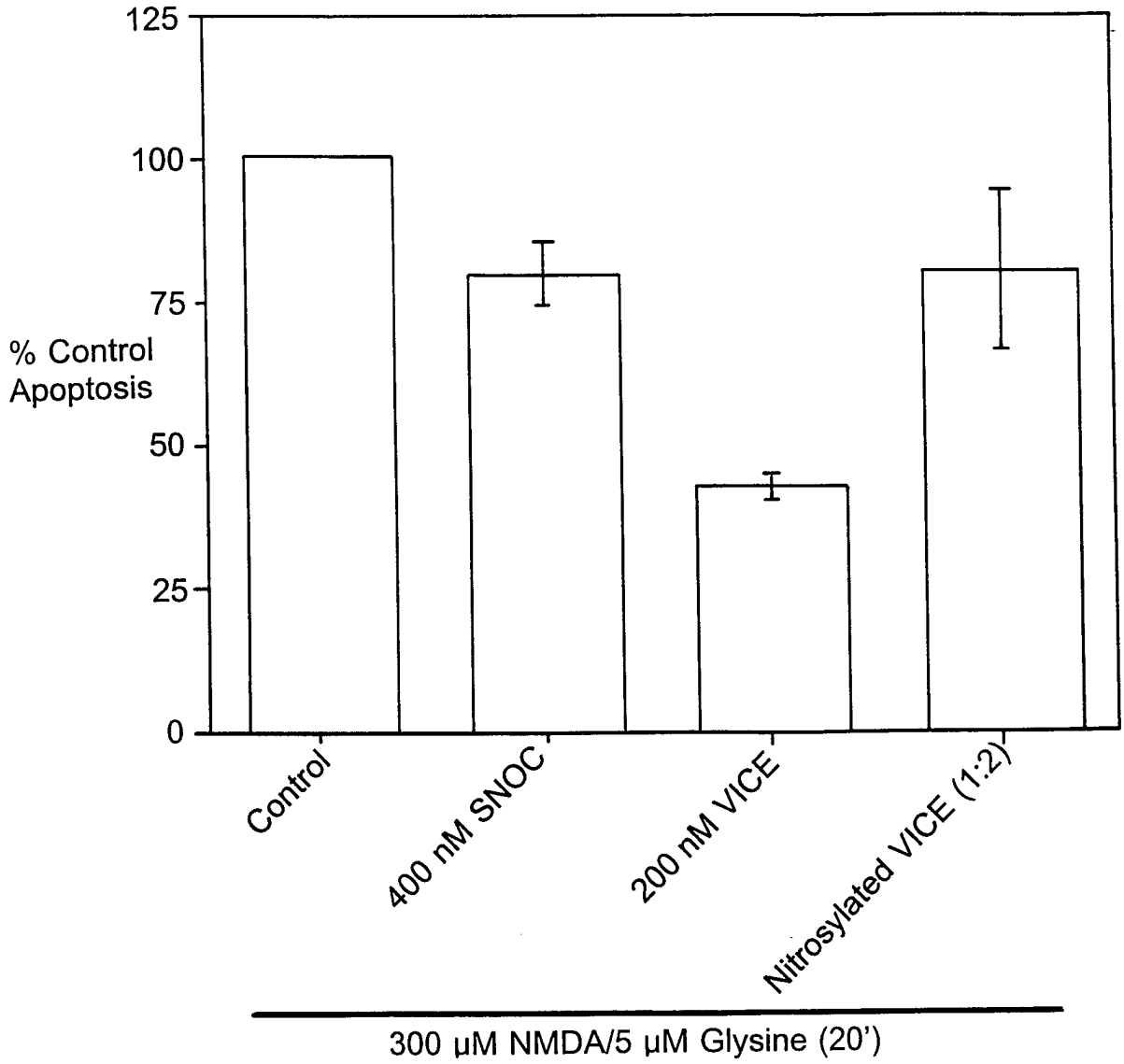
125
100
75
50
25
0
% Control Apoptosis
Control
400 nM SNOC
200 nM VICE
Nitrosylated VICE (1:2)
300 µM NMDA/5 µM Glysine (20')

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06287

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/04, 38/43

US CL :424/94.1, 94.3, 514/502, 611, 558, 579, 589, 657

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.1, 94.3, 514/502, 611, 558, 579, 589, 657

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, APS, BIOSIS, MEDLINE, WPIDS
search terms: nitro? and apopt? and caspase?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TROY, C.M. et al. The Contrasting Roles of ICE Family Proteases and Interleukin-1β in Apoptosis Induced by Trophic Factor Withdrawal and by Copper/Zinc Superoxide Dismutase Down-regulation. Proceedings of the National Academy of Science, USA. May 1996, Vol. 93, pages 5635-5640, see the entire document.	1-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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