

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number
WO 01/24794 A1

- (51) International Patent Classification⁷: **A61K 31/24**
- (21) International Application Number: PCT/US00/21784
- (22) International Filing Date: 9 August 2000 (09.08.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/147,965 9 August 1999 (09.08.1999) US
- (71) Applicant (*for all designated States except US*):
WEBB-WARING INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; 4200 E. Ninth Avenue,
Denver, CO 80262 (US).
- (72) Inventor; and
- (75) Inventor/Applicant (*for US only*): **REPINE, John, E.**
[US/US]; 2275 Cherry Hills Farm Drive, Englewood, CO
80110 (US).
- (74) Agents: **KOVARIK, Joseph, E. et al.**; Sheridan Ross P.C.,
Suite 1200, 1560 Broadway, Denver, CO 80202-5141 (US).
- (81) Designated States (*national*): AE, AL, AM, AT, AU, AZ,
BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK,
DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,
IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,
RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA,
UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— *With international search report.*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: A METHOD FOR THE TREATMENT OF OCULAR OXIDATIVE STRESS

(57) Abstract: A method for combating the detrimental effects of oxidative stress on the eye involving the use of modifiers (both stimulators and/or inhibitors) of a fundamental nuclear regulatory mechanism in cells that controls both beneficial and detrimental aspects of oxidative stress produced by inflammation and other processes and the responses to these events in the eye. The ability to modulate nuclear wellness, aging and acute and chronic disease processes is achieved through regulation of NFκB controlled events, including the administration of one or more agents.

WO 01/24794 A1

A METHOD FOR THE TREATMENT OF OCULAR OXIDATIVE STRESS

FIELD OF THE INVENTION

The present invention relates to a new method for treating or preventing ocular oxidative stress. More particularly, the invention relates to using strategies to effectively modulate the cascade of effects that are controlled by the central nuclear regulatory factor - NF κ B - in the various cells of the eye, as well as NF κ B controlled events in blood and other cells that impact the structure and/or function of the eye.

BACKGROUND OF THE INVENTION

The eye is a complex, highly vascular, heavily innervated, and environmentally exposed organ that is frequently subjected, during both health and disease, to numerous events that produce oxidative stress. Oxidative stress can rapidly damage key cellular components in the eye including lipids, proteins, RNA, and DNA. In addition, oxidative stress is detrimental to the lens, retina, vitreous and other ocular tissues. Accordingly, approaches that limit oxidative stress to the eye would be valuable in limiting deterioration of eye function.

The sources of oxidative stress in the eye include a number of possibilities. The partial list of endogenous sources encompasses phagocytic cells, most notably, neutrophils, macrophages and eosinophils, which are potent generators of oxidants. Additionally, biochemical reactions involving cyclooxygenase, nitric oxide (NO) synthase and other enzymatic reactions can lead to oxidative stress. Enhanced mitochondrial metabolism of oxygen also produces superoxide anion ($O_2^{\cdot -}$) and other reactive species of oxygen. Exogenous sources of oxidative stress include cigarette smoking, various drugs and photooxidative reactions caused by irradiation to the eye as a consequence of sunlight or laser therapy. Moreover, even without an increased production or exposure to oxidants, the eye might be exposed to increased oxidative stress if any of a number of antioxidants or factors that reduce the toxicity of oxidative stress are decreased or impaired. For example, aging has been reported to decrease levels of the antioxidants and anti-inflammatory agents such as glutathione (GSH) and taurine, respectively. The accumulation of iron (ferritin) that occurs with aging is also believed to increase oxidative stress since uncomplexed iron facilitates the reaction of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) and produces the highly toxic hydroxyl radical (OH).

Furthermore, oxidative stress, cigarette smoke, and other factors mobilize Fe^{++} from ferritin and other complexing proteins.

Oxidative stress occurs in the eye during both health and disease. For example, during normal circumstances, the mitochondrial consumption of O_2 generates oxygen radicals which may not be completely detoxified by antioxidants and consequently, may produce oxidative stress. Another example is apparent in ARMD, the leading cause of blindness in the elderly, a condition in which deposits of an oxidized lipid, lipofuscin, are manifest in the retina. Levels of hydrogen peroxide (H_2O_2) are also increased in the vitreous of the eye and is believed to contribute to cataract, glaucoma and/or other ocular abnormalities. Following surgery, laser therapy, infection, and/or injury, the eye is often subjected to reactions that generate inflammation activating immune and inflammatory cells and other cellular functions that produce oxidative stress.

A number of prior approaches have been proposed to limit oxidative stress in the eye. For example, anti-inflammatory agents have been directed at specific parts of the inflammation process. These agents, however, usually have relatively specific actions and are often not effective for unknown reasons. The lack of effectiveness of these relatively specific inhibitors may be a result of the great redundancy of the inflammatory process. Thus, for example, while a cyclooxygenase inhibitor is advantageous for reducing cyclooxygenase related events, many other processes that produce oxidative stress are not limited by this specific intervention. Another example relates to the recruitment to the eye of neutrophils - cells which are powerful generators of oxidants. Many factors have been identified as chemoattractants for neutrophils. They include interleukin-8 (IL-8), C5a (a complement component), FMLP (a bacterial wall product), and leukotriene B4. There is evidence that selective inhibition of any one chemoattractant (e.g. with IL-8 antibodies) may not reduce neutrophil recruitment in one or another situation because of the operability of alternate chemotaxins.

In sum, there exists a long felt but unsolved need to find a way of inhibiting multiple arms of the redundant inflammatory immune process that would be effective in reducing cell damage due to ocular oxidative and other stress.

SUMMARY OF THE INVENTION

Eyes are frequently subjected to a variety of events that produce or lead to oxidative stress. Oxidative stress is defined as an increase in the production of highly reactive species of oxygen and/or nitrogen ("free radicals") and/or a decrease in antioxidant defense systems. Antioxidant defense systems encompass enzymatic and non-enzymatic detoxifying mechanisms, binders of various cofactors, such as metals that facilitate oxidative reactions, and molecular alterations that make cellular lipids, membranes or DNA less susceptible to damage by oxidants.

The present invention is directed to methods and formulations to treat and/or prevent ocular oxidative stress and can be used alone and/or in combination with other approaches to improve eye health and/or to reduce the consequences of ocular disease processes related to aging, age-related macular degeneration (ARMD), cataract, glaucoma, uveitis, iritis and other insults to the eye that involve oxidation stress. The present invention is also beneficial for treating or preventing unwanted consequences in the eye related to infection, trauma, surgery, laser therapy, tumors and/or toxin exposure.

Nuclear factor- κ B (NF κ B) is a heterodimeric transcription factor complex that mediates multiple immune and inflammatory responses. The activated form of NF κ B consists of two proteins, a p65 (aka rel A) subunit and a p50 subunit. Rel, rel BV, v-rel and p52 may also be a part of NF κ B and different forms of activated NF κ B may activate different sets of target genes. Activation of NF κ B in the cytoplasm is associated with a series of events that facilitate the transport of NF κ B to the nucleus when it has its effects on centrally controlled genetic processes (see [Figure 1](#)).

Many stimuli, including cytokines, protein kinase C activators, viruses and oxidants, activate NF κ B (see [Table I](#)).

TABLE I. Stimuli That Activate NF κ B

	Cytokines	
	Tumor necrosis factor α	
	Interleukin -1 β	
5	Interleukin -17	
	Protein kinase C activators	
	Phorbol esters	
	Platelet-activating factor	
	Oxidants	
10	Hydrogen peroxide	
	Ozone	
	Viruses	
	Rhinovirus	
	Influenzavirus	
15	Epstein-Barr virus	
	Cytomegalovirus	
	Adenovirus	
	Immune stimuli	
	Phytohemagglutinin	
20	Anti-CD3 antibodies (by means of T-lymphocyte activation)	
	Antigen	
	Other	
	Lipopolysaccharide	
	Ultraviolet radiation	
25	Agents that inhibit NF κ B activation include pyridoxine dithiocarbamate, NAC, glucocorticoids, acetosalicylic acid, sodium salicylate, glitoxin, interleukin-10 and gold salts. Additional inhibitors of NF κ B include inhibitors of I κ B kinases or certain factors, such as NEMO, that alter kinase related and other factors involved in NF κ B activation. I κ B α or I κ B β superrepressor, especially non-phosphatable forms offer a way of	
30	inhibiting NF κ B. Compounds (e.g. peptides) or strategies that block nuclear localization and/or nuclear uptake of NF κ B can be used to effectively inhibit NF κ B activity. NF κ B can also be inhibited by factors such as P300 creb binding protein that prevent NF κ B from interacting with any basic transcription complex.	
35	As shown in Table II, NF κ B regulates expression of many genes, oftentimes in conjunction with the nuclear factor of interleukin-6 (IL-6) and activator protein (AP-1). NF κ B also acts on genes for granulocyte colony stimulator factor (GCSF), various chemokines (E.G. IL-8), adhesion molecules (e.g. E. Selectin, ICAM), growth factors (e.g. VEGF), inflammation inducing factors (e.g. tumor necrosis factor, IL-1 IL-6) and enzymes (e.g. nitric oxidase (NO) synthase and cyclooxygenase).	

TABLE II. Proteins Regulated by NF κ B

	Proinflammatory cytokines
	Tumor necrosis factor α
	Interleukin-1 β
5	Interleukin-2
	Interleukin-6
	Granulocyte-macrophage colony-stimulating factor
	Macrophage colony-stimulating factor
	Granulocyte colony-stimulating factor
10	Chemokines
	Interleukin-8
	Macrophase inflammatory protein 1 α
	Macrophage chemotatic protein]
	Gro- α , - β , and - γ
15	Eotaxin
	Inflammatory enzymes
	Inducible nitric oxide synthase
	Inducible cyclooxygenase-2
	5-Lipoxygenase
20	Cytosolic phospholipase A ₂
	Adhesion molecules
	Intercellular adhesion molecule]
	Vascular-cell adhesion molecule]
	E-selectin
25	Receptors
	Interleukin-2 receptor (α chain)
	T-cell receptor (β chain)

The comprehensive effects of NF κ B leads to a multidimensional array of events involving many factors which can be modulated at a central level. By way of example, one complex scheme could involve elaboration of neutrophils from the bone marrow, production of chemotaxins, recruitment and activation of neutrophils and the release of enzymes and oxidants from neutrophils. The latter is facilitated by COX2 (prostaglandins, thromboxanes), NO synthase and other molecules which increase blood flow and blood vessel permeability. Thus, the present invention is directed to the ability to modulate the effects of the central coordinating effector NF κ B to significantly reduce all of these events and the many untoward effects including apoptosis, necrosis, and cell differentiation that are unwanted consequences of oxidative stress. This contrasts with the use of a single agent which inhibits only one of these processes and/or the use of multiple inhibitors to accomplish the same goals that could be achieved using the NF κ B tactic. In addition, the ability to inhibit some of these processes is unknown and

therefore appropriate modulation of NF κ B is presently the only way known of controlling these events. By analogy, the present approach involves controlling the events of the army, navy, air force and marines at the central "pentagon level" rather than attempting to alter the effects of a single discrete insult, such as damage from tanks or submarine missiles.

Use of the Invention for Treatment and Prevention

At present, there is no comprehensive way for inhibiting multiple arms of the diverse inflammatory response and the oxidative stress and antioxidant depletion that often ensues and/or complicates oxidative stress. The discovery of a relatively discrete (the "pentagon" approach) way that could simultaneously limit and control inflammation by centrally modulating a variety of factors is needed for the care and maintenance of eye function.

Use of the Invention for Prognosis Purposes

Measurement of various factors or responses of the immune or inflammatory system can be used to predict processes that have pathologic significance. A prime example is the use of measurements of superoxide dismutase (SOD), catalase and/or ferritin to predict the development of the acute respiratory distress syndrome (ARDS) in at-risk subjects. Individuals who sustain trauma, infection, blood transfusions, surgery and other insults become susceptible to the development of ARDS - complicating, highly fatal pulmonary edema. The concern is that ARDS develops variably and in not all of the at-risk subjects. Because the biochemical responses that contribute to ARDS precede the development of lung edema, assessment of these responses ahead of time provides the opportunity to institute therapy earlier. Likewise, the effectiveness of interventions may be assessed by observing changes that occur in these marks or predictors. Using any single factor is not as effective as using a battery of factors since increases or decreases in individual factors may not reflect the concerted response needed to cause ARDS.

By comparison, and with respect to the present invention, when NF κ B is activated, multiple factors are increased. Thus, the concerted response needed to cause ADS or tissue damage is better assessed by measuring the status of NF κ B rather than any

single factor. One aspect of the present invention is directed to the monitoring of the status of NF κ B or NF κ B dependent processes in order to predict the severity of oxidative stress and its response to various interventions in relationship to assessments of eye health and disease.

5 One object of the present invention is to provide a method to modulate NF κ B dependent events that contribute to oxidative stress and other responses that contribute directly or indirectly to vision deterioration and/or eye abnormalities.

 Another object is to provide a method to limit the unwanted consequences of NF κ B activation or inhibition when it is caused by oxidative stress or other processes.

10 A further object is to provide a method that in additive or synergistic fashion to other approaches, maintains or improves vision and/or eye health.

 A still further object is to provide a method that augments other ways or improving vision including, without limitation, mechanical low vision aids, laser therapy, acupuncture, plasmaphoresis, and retinal transplants.

15 An additional objective is to provide a new method for using NF κ B or NF κ B related responses to predict or reflect the development of ocular oxidative stress, to assess individuals in whom oxidative stress is more likely to occur, to quantitate the extent of oxidative stress and/or to monitor the effect of one or more interventions.

 These and other objects of the present invention are achieved by providing a
20 method that modulates NF κ B dependent processes in the eye and cells that are recruited to the eye.

 The present invention is based on the discovery that NF κ B modulation can decrease oxidation stress and that activation of NF κ B by oxidative stress can be decreased by methods that reduce oxidative stress. To this end, the present invention
25 provides a method for maintaining eye health as well as treating eye disorders in which oxidative stress is a cause and/or a contributing factor. Preferably, the agent is a non-toxic, orally active agent such as N=acetylcysteine (NAC) but other agents, including but not limited to agents which affect NF κ B through a variety of genetic and/or pharmacologic mechanisms, may also be used.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing purported reactions involved in the activation of NFκB.

Figure 2 is a figure that depicts a mechanism whereby NFκB dependent processes
5 could contribute to ocular oxidative stress.

Figure 3 indicates the yin-yang responses controlled by NFκB.

DETAILED DESCRIPTION

The following description is provided to enable any person who is skilled in the
10 art to which the present invention pertains, or with which it is most nearly connected, to make and use the same, and sets forth in specific and conceptual terms, the best mode contemplated now by the inventors for the purpose of carrying out their invention.

Pharmacologic, neuropeptide and/or genetic inhibitors of NFκB are identified
by analysis of the effect of interventions on NFκB in resting and simulated cells *in vitro*.
15 Without trying to be limiting in any sense, this is accomplished using cultured cells from various sources. For initial screening, Western blot analysis is conducted on extracts from unstimulated and stimulated cells that have been treated with various activating agents and in some experiments along with various concentrations of inhibitors. These cellular extracts are then analyzed using electrophoretic mobility shift analysis (EMSA)
20 with or without the use of antibodies to identify various NFκB family members. As mentioned, NFκB activation is assessed in cells before and following the application of NFκB activators, such as LS, PMA or IL-1. Inhibitor effectiveness strategies are examined by applying various concentrations of the agent before and following analysis of NFκB activation. In some cases, inhibitors are also identified by testing agents that
25 inhibit the IκB subunits or other parts of the mechanism by which NFκB modulators both in up- or down-regulating ways. To control NFκB and NFκB-dependent response using genetic manipulations of the promoter and/or other systems which regulate NFκB by that mechanism, various cell types are tested to determine NFκB activation which is believed to occur differently in different cell types e.g. the lung is different than the eye and
30 consequently inhibitors are identified that are effective in the eye.

Other agents stimulate NFκB in ways that protect cells against oxidative stress and other insults. For example, pretreatment with IL-1 and/or TNF decreases the

oxidative lung injury that occurs following exposure of the rats to hyperoxia or IL-1 administration intratracheally. The mechanism responsible for the protection appears to depend on NFκB dependent mechanisms. This conclusion is based on studies done *in vitro* in which pretreatment with IL-1 makes rat lung microvascular endothelial cells (RLMEC) resistant to injury caused by stimulated neutrophils. IL-1 pretreatment also increases NFκB activity in RLMEC. However, when NFκB activation is prevented in IL-1 pretreated RLMEC using IκBβ or IκBα transfected RLMEC - a highly specific maneuver which prevents NfκB activation - resistance is decreased. Thus, IL-1 pretreatment by a mechanism which involves NFκB activation has the ability to make cells resistant to the oxidative and other damage usually conferred by stimulated neutrophils. For example, since viruses activate NFκB cultured cells, heat is used to inactivate or modify viruses to activate NFκB in ways that are protective. Based on these findings, the present invention is directed to the administration of IL-1 or other agents as being useful in activating NFκB, and in turn, NFκB dependent processes that protect cells from oxidative and other insults.

NFκB activities in mammalian ocular and other tissues are examined from various subjects that have been subjected to various conditions and/or various interventions. Using the same approaches as described above, organs and/or cells are isolated and cellular extractions are obtained to identify the levels of NFκB. Once the appropriate circumstances are identified, the agents that are postulated to be causing NFκB alterations *in vitro* are retested in simplified cellular systems *in vitro*. This approach identifies agents and approaches that are better for ocular tissues since NFκB activation and inactivation and its consequences vary in different cells.

A prime example of a clinically useful agent which is known to modulate NFκB is N-acetylcystein (NAC), a known mucolytic agent. NAC is an N-acetyl derivative ($\text{HS-CH}_2\text{-CH-CHOOH-NH-COCH}_3$) of the naturally occurring amino acid, L-cysteine. NAC can be administered in any convenient manner, e.g. orally, intramuscularly, intravenously, and perhaps intraocularly, transdermally or by aerosolization at doses ranging from 100 to 5000 milligrams daily. NAC is more soluble than cysteine in water and is less easily oxidized than cysteine. Hence, it is preferred to use NAC or an equivalent cysteine derivative rather than cysteine itself, but cysteine or products that deliver cysteine is still effective (although less preferred) in inhibiting NFκB and

consequently, its use can be used in treating or preventing tissue damage such as that observed following oxidative stress.

Use of NAC or its cysteine delivering derivatives to accomplish the intended treatment, i.e., modulation of NF κ B, is believed to occur by one or more means, the possible mechanisms offered by N-acetylcysteine including scavenging (inactivation of)
5 oxygen radicals which activate NF κ B and/or raising intracellular glutathione (GSH) levels, as well as numerous other direct and indirect effects of the agent on NF κ B activation.

NAC is a particularly desirable compound for use according to the present invention as it is currently used clinically to treat patients with chronic disorders, for example, chronic bronchitis, and cancer, as well as acute disorders such as ARDS, AIDS, ALS, and acetaminophen toxicity. NAC has been used safely for many years. For example, an oral dose of 600 mg tid is well tolerated for long periods of time in patients with chronic bronchitis and emphysema. Radiolabelled NAC is rapidly absorbed in
10 humans one hour after administration and then distributed extensively. The mean plasma half-life of NAC is about 1.35 hours and approximately 225 of the dose is excreted in the urine after 24 hours. NAC appears to bind to protein and undergo some metabolism. Adverse effects following NAC treatment are rare but can include nausea, vomiting, pyrosis, dyspepsia, and very rarely, urticaria. These possible side effects can, however,
15 generally be moderated to enable the treatment contemplated herein.

Another aspect of the present invention is that intracellular GSH levels decline with age, and as a consequence, decreased GSH levels is believed to enhance oxidative stress, which contributes to ocular tissue damage. Using the claimed method of treatment, NAC is used to prevent or reverse the ocular dysfunctions typical of elderly
20 patients that would otherwise develop diminished eye function and ocular disease related to oxidative stress.

Advantageously, the NAC is administered in an effective amount, preferably in the range of about 400-600 milligrams daily, more preferably about 250 milligrams. This may be done orally, for example, twice a day, although the dose, frequency and
25 form of administration may be varied depending on other factors.

In a variation of the present invention, other cysteine delivery systems are used to effectively modulate NF κ B either directly or indirectly. L-2-oxothiazolidine-4-

carboxylate (OTC), or procysteine as it is commonly known, is effectively transported into cells where it is converted by the action of 5-oxoprolinase into L-cysteine. By mechanisms similar to those described above with respect to NAC, OTC may increase NFκB levels. OTC has been shown to be effectively transported into mouse and rat
5 brains as evidenced by increased brain cysteine levels in the mouse and rat following OTC administration. The dose of OTC used for present purposes, and the mode of administration are generally similar to those given above for NAC.

In another variation of the invention, mercaptopropionylglycine (MPG) accomplishes the same objective by increasing GSH levels by decreasing oxidative
10 damage both *in vivo* and *in vitro* (i.e., MPG-related myocardial infarct size following ischemia-reperfusion). Free radical scavenging is believed to be the mechanism for the protective effect of MPG. When measured by pulse radiolysis *in vitro*, MPG has been shown to avidly scavenge OH (hydroxy radicals). Experimental pretreatment with MPG increase free sulfhydryl (GSH) content in control animals and maintain normal free
15 sulfhydryl levels in animals subjected to ischemia-reperfusion. Although not bound by theory, the protective mechanism(s) by which MPG functions are believed to include the following. For example, GSH levels in cell tissue are increased or maintained by MPG-induced glutathione synthesis and/or MPH-induced release of protein bound GSH. Alternatively, GSH is spared by MPG. MPG acts as a sulfhydryl radioprotector. In any
20 case, the MPG functions to increase NFκB.

A unique aspect of the present invention is the use of NAC,OTC, MPG alone and/or any other NFκB inhibitor alone, or in a combination of two or more, or in conjunction with one or more antioxidant, anti-inflammatory, or current symptomatic
25 treatment. Neither unacetylated cysteine, nor GSH are believed to thoroughly penetrate cells by themselves. Other antioxidants, for example antioxidant vitamins, such as vitamin A, lutein, zeaxanthin, vitamin E, β-carotene, or vitamin C, are effective because they serve to facilitate desired cell penetration.

Pretreatment with cytokines or cytokine fragments is useful in initiating NFκB activation in ways that confer endogenous protection against oxidative stress. Among
30 other agents interleukin-1 (IL-1) or TNF are believed to be particularly effective. Small non-toxic doses are given alone or in combination to animals or cells. An intrinsic resistance to oxidative stress ensues in approximately 12 to 24 hours. This response

protects the cell or organ from damage induced by oxidative stress following exposure to activated neutrophils, hyperoxia or ischemia-reperfusion.

A further aspect of the present invention is the use of fragments of IL-1, TNF or other molecules that activate NF κ B to produce resistance to oxidative stress. Selected fragments of IL-1 activate certain immune functions that are also activated by native IL-1. In this way, a novel fragment of IL-1 (or other molecule) is selected that activates NF κ B and confers resistance to oxidative stress without inducing all of the other actions that follow administration of native IL-1 or these other similar molecules. The approach regarding stimulation relates in concept to the use of RAS activators, oxidants, stress reaction, UV, viruses, growth factors and toxicological agents either in their native state, or in a specifically modified state, alone and/or in combination.

According to one embodiment of the present invention, an agent which may comprise an amino acid and/or a protein of the present invention (i.e., at least one of NAC, glucocorticoids, acetosalicylic acid, agents that inhibit NF κ B activation, interleukin-1 (IL-1), tumor necrosis factor (TNF), cysteine, N-acetylcysteine (NAC) or procysteine) may be encoded on a nucleic acid, facilitating the delivery of the agent to particular tissues and/or to facilitate gene therapy treatments to ameliorate certain ocular oxidative stress disorders. The homology or percent identity between two or more nucleic acid or amino acid sequences that encode an agent of the present invention is performed using methods known in the art for aligning and/or calculating percentage identity. To compare the homology/percent identity between two or more sequences, for example, a module contained within DNASTAR (DNASTAR, Inc., Madison, Wisconsin) can be used. In particular, to calculate the percent identity between two nucleic acid or amino acid sequences, the Lipman-Pearson method, provided by the MegAlign module within the DNASTAR program, is preferably used, with the following parameters, also referred to herein as the Lipman-Pearson standard default parameters:

- (1) Ktuple = 2;
- (2) Gap penalty = 4;
- (3) Gap length penalty = 12.

According to another embodiment of the present invention, to align two or more nucleic acid or amino acid sequences, for example to generate a consensus sequence or evaluate the similarity at various positions between such sequences, a CLUSTAL

alignment program (e.g., CLUSTAL, CLUSTAL V, CLUSTAL W), also available as a module within the DNASTAR program, can be used using the following parameters, also referred to herein as the CLUSTAL standard default parameters:

Multiple Alignment Parameters (i.e., for more than 2 sequences):

- 5 (1) Gap penalty = 10;
 (2) Gap length penalty = 10;

Pairwise Alignment Parameters (i.e., for two sequences):

- (1) Ktuple = 1;
 (2) Gap penalty = 3;
10 (3) Window = 5;
 (4) Diagonals saved = 5.

As used herein, stringent hybridization conditions refer to standard hybridization conditions under which nucleic acid molecules are used to identify similar nucleic acid molecules. Such standard conditions are disclosed, for example, in Sambrook et al.,
15 *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, 1989. Sambrook et al., *ibid.*, is incorporated by reference herein in its entirety (see specifically, pages 9.31-9.62). In addition, formulae to calculate the appropriate hybridization and wash conditions to achieve hybridization permitting varying degrees of mismatch of nucleotides are disclosed, for example, in Meinkoth et al., 1984, *Anal. Biochem.* 138,
20 267-284; Meinkoth et al., *ibid.*, is incorporated by reference herein in its entirety.

More particularly, stringent hybridization and washing conditions, as referred to herein, refer to conditions which permit isolation of nucleic acid molecules having at least about 70% nucleic acid sequence identity with the nucleic acid molecule being used to probe in the hybridization reaction, more particularly at least about 75%, and most
25 particularly at least about 80%. Such conditions will vary, depending on whether DNA:RNA or DNA:DNA hybrids are being formed. Calculated melting temperatures for DNA:DNA hybrids are 10°C less than for DNA:RNA hybrids. In particular embodiments, stringent hybridization conditions for DNA:DNA hybrids include hybridization at an ionic strength of 6X SSC (0.9 M Na⁺) at a temperature of between
30 about 20°C and about 35°C, more preferably, between about 28°C and about 40°C, and even more preferably, between about 35°C and about 45°C. In particular embodiments, stringent hybridization conditions for DNA:RNA hybrids include hybridization at an

ionic strength of 6X SSC (0.9 M Na⁺) at a temperature of between about 30°C and about 45°C, more preferably, between about 38°C and about 50°C, and even more preferably, between about 45°C and about 55°C. These values are based on calculations of a melting temperature for molecules larger than about 100 nucleotides, 0% formamide and
5 a G + C content of about 40%. Alternatively, T_m can be calculated empirically as set forth in Sambrook et al., *supra*, pages 9.31 TO 9.62.

In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (i.e., that has been subject to human manipulation). As such, "isolated" does not reflect the extent to which
10 the nucleic acid molecule has been purified. An isolated nucleic acid molecule can include DNA, RNA, or derivatives of either DNA or RNA. There is no limit, other than a practical limit, on the maximal size of a nucleic acid molecule in that the nucleic acid molecule can include a portion of a gene, an entire gene, or multiple genes, or portions thereof.

15 An isolated nucleic acid molecule of the present invention can be obtained from its natural source either as an entire (i.e., complete) gene or a portion thereof capable of forming a stable hybrid with that gene. An isolated nucleic acid molecule can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Isolated nucleic acid molecules include
20 natural nucleic acid molecules and homologues thereof, including, but not limited to, natural allelic variants and modified nucleic acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications provide the desired effect. An isolated nucleic acid molecule of the present invention can include degeneracies. As used herein, nucleotide degeneracies refers to
25 the phenomenon that one amino acid can be encoded by different nucleotide codons. Thus, the nucleic acid sequence of a nucleic acid molecule that encodes a protein of the present invention can vary due to degeneracies.

A nucleic acid molecule homologue can be produced using a number of methods known to those skilled in the art (see, for example, Sambrook et al., *ibid.*). For example,
30 nucleic acid molecules can be modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant DNA techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce

mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, PCR amplification and/or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules and combinations thereof. Nucleic acid molecule homologues can be selected from a mixture of modified nucleic acids by screening for the function of the protein encoded by the nucleic acid and/or by hybridization with a wild-type gene.

The present invention includes a recombinant vector, which includes at least one isolated nucleic acid molecule of the present invention, inserted into any vector capable of delivering the nucleic acid molecule to a desired tissue. Such a vector can contain nucleic acid sequences that are not naturally found adjacent to the isolated nucleic acid molecules to be inserted into the vector. The vector can be either RNA or DNA and typically is a plasmid. One type of recombinant vector, referred to herein as a recombinant molecule and described in more detail below, can be used in the expression of nucleic acid molecules.

As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified nucleic acid molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention.

In particular, expression vectors of the present invention contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules of the present invention. Recombinant molecules of the present invention may also include transcription control sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences.

Recombinant molecules of the present invention may also (a) contain secretory signals (i.e., signal segment nucleic acid sequences) to enable an expressed protein of the present invention to be secreted from the cell that produces the protein and/or (b) contain

fusion sequences which lead to the expression of nucleic acid molecules of the present invention as fusion proteins. Examples of suitable signal segments include any signal segment capable of directing the secretion of a protein of the present invention. Preferred signal segments include, but are not limited to, tissue plasminogen activator (t-PA), interferon, interleukin, growth hormone and histocompatibility and viral envelope glycoprotein signal segments. Suitable fusion segments encoded by fusion segment nucleic acids are disclosed herein. Eukaryotic recombinant molecules may include intervening and/or untranslated sequences surrounding and/or within the nucleic acid sequences of nucleic acid molecules of the present invention.

Another embodiment of the present invention includes a recombinant cell comprising a host cell transformed with one or more recombinant molecules of the present invention. Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. Transformed nucleic acid molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

Still other embodiments of the present invention are directed to antibodies directed against proteins of the present invention (as referred to above). Also included in the present invention is the use of such proteins, nucleic acid molecules, antibodies and other inhibitors, as well as therapeutic compositions, to treat ocular oxidative stress disorders and/or abnormalities.

It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, a protein refers to one or more proteins, or to at least one protein. As such, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably. An isolated agent and/or protein of the present invention can, for example, be obtained from its natural source, be produced using recombinant DNA technology, or be synthesized chemically. As used herein, an isolated protein of the present invention can be a full-length protein or any homologue

of such a protein, such as a protein in which amino acids have been deleted (e.g., a truncated version of the protein, such as a peptide), inserted, inverted, substituted and/or derivatized (e.g., by glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitation, amidation and/or addition of glycosylphosphatidyl inositol).

5 A homologue of a protein of the present invention is a protein having an amino acid sequence that is sufficiently similar to a natural protein amino acid sequence of the present invention that a nucleic acid sequence encoding the homologue is capable of hybridizing under stringent conditions to (i.e., with) a nucleic acid molecule encoding the natural protein of the present invention (i.e., to the complement of the nucleic acid
10 strand encoding the natural protein amino acid sequence). A nucleic acid sequence complement of any nucleic acid sequence of the present invention refers to the nucleic acid sequence of the nucleic acid strand that is complementary to (i.e., can form a complete double helix with) the strand for which the sequence is cited.

There is no limit, other than a practical limit, on the maximal size of a nucleic
15 acid molecule in that the nucleic acid molecule can include a portion of a gene, an entire gene, or multiple genes, or portions thereof. Similarly, the minimal size of a protein homologue of the present invention is from about 4 to about 6 amino acids in length, with preferred sizes depending on whether a full-length, multivalent (i.e., fusion protein having more than one domain each of which has a function), or functional portions of
20 such proteins are desired.

As used herein, a mimetope of a protein of the present invention refers to any compound that is able to mimic the activity of such a protein, often because the mimetope has a structure that mimics the protein. Mimetopes can be, but are not limited to: peptides that have been modified to decrease their susceptibility to degradation; anti-
25 idiotypic and/or catalytic antibodies, or fragments thereof; non-proteinaceous immunogenic portions of an isolated protein (e.g., carbohydrate structures); and synthetic or natural organic molecules, including nucleic acids. Such mimetopes can be designed using computer-generated structures of proteins of the present invention. Mimetopes can also be obtained by generating random samples of molecules, such as oligonucleotides,
30 peptides or other organic or inorganic molecules, and screening such samples by affinity chromatography techniques using the corresponding binding partner.

One embodiment of the present invention is a fusion protein that includes an protein-containing domain attached to one or more fusion segments. Suitable fusion segments for use with the present invention include, but are not limited to, segments that can: enhance a protein's stability; act as an immunopotentiator to enhance an immune
5 response against a protein of the present invention; and/or assist purification of a protein (e.g., by affinity chromatography). A suitable fusion segment can be a domain of any size that has the desired function (e.g., imparts increased stability, imparts increased immunogenicity to a protein, and/or simplifies purification of a protein). Fusion segments can be joined to amino and/or carboxyl termini of the domain of the protein of
10 the present invention and can be susceptible to cleavage in order to enable straightforward recovery of a protein of the present invention.

In another embodiment, a protein of the present invention also includes at least one additional protein segment and/or compound and/or treatment that is capable of protecting an animal from one or more diseases, in addition to those related to ocular
15 oxidative stress. For example, in addition to the administration of an agent of the present invention, a method of treatment may further include the use of mechanical low vision aids, acupuncture, laser radiation lasmaphoresis, pharmaceuticals and/or neutraceuticals.

Targeting carriers are herein referred to as "delivery vehicles." Delivery vehicles of the present invention are capable of delivering a therapeutic composition of the
20 present invention to a target site in an animal. A "target site" refers to a site in an animal to which one desires to deliver a therapeutic composition. Examples of delivery vehicles include, but are not limited to, artificial and natural lipid-containing delivery vehicles. Natural lipid-containing delivery vehicles include cells and cellular membranes. Artificial lipid-containing delivery vehicles include liposomes and micelles. A delivery
25 vehicle of the present invention can be modified to target to a particular site in an animal, thereby targeting and making use of a nucleic acid molecule of the present invention at that site. Suitable modifications include manipulating the chemical formula of the lipid portion of the delivery vehicle and/or introducing into the vehicle a compound capable of specifically targeting a delivery vehicle to a preferred site, for example, a preferred
30 cell type. Specifically targeting refers to causing a delivery vehicle to bind to a particular cell by the interaction of the compound in the vehicle to a molecule on the surface of the cell. Suitable targeting compounds include ligands capable of selectively (i.e.,

specifically) binding another molecule at a particular site. Examples of such ligands include antibodies, antigens, receptors and receptor ligands.

Another preferred delivery vehicle comprises a recombinant virus particle vaccine. A recombinant virus particle vaccine of the present invention includes a
5 therapeutic composition of the present invention, in which the recombinant molecules contained in the composition are packaged in a viral coat that allows entrance of DNA into a cell so that the DNA is expressed in the cell. A number of recombinant virus particles can be used, including, but not limited to, those based on alphaviruses, poxviruses, adenoviruses, herpes viruses, arena virus and retroviruses.

10 An effective administration protocol (i.e., administering a therapeutic composition (e.g., a compound, agent, protein, formulation, etc. capable of treating ocular oxidative stress disorders) in an effective manner) comprises suitable dose parameters and modes of administration that result in treatment of a disease. Effective dose parameters and modes of administration can be determined using methods standard
15 in the art for a particular disease. Such methods include, for example, determination of side effects (i.e., toxicity) and progression or regression of disease.

In accordance with the present invention, a suitable single dose size is a dose that is capable of treating an animal with disease when administered one or more times over a suitable time period. Doses can vary depending upon the disease being treated. Doses
20 of a therapeutic composition of the present invention suitable for use with direct injection techniques can be used by one of skill in the art to determine appropriate single dose sizes for systemic administration based on the size of an animal. The number of doses administered to an animal is dependent upon the extent of the disease and the response of an individual patient to the treatment.

25 Preferred methods of systemic administration of therapeutic compositions include intravenous injection, aerosol, oral and percutaneous (topical) delivery. Intravenous injections can be performed using methods standard in the art. Aerosol delivery can also be performed using methods standard in the art (see, for example, Stribling et al., *Proc. Natl. Acad. Sci. USA* 189:11277-11281, 1992, which is incorporated herein by reference
30 in its entirety). Oral delivery can be performed by complexing a therapeutic composition of the present invention to a carrier capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers, include plastic capsules or

tablets, such as those known in the art. Eye drops can be used and/or topical delivery can be performed by mixing a therapeutic composition of the present invention with a lipophilic reagent (e.g., DMSO) that is capable of passing into the skin.

Also included in the present invention is a method to transfer a nucleic acid
5 molecule into a given cell type. The method can be accomplished *in vivo*, *ex vivo*, or *in vitro* and can, in one embodiment, effect gene therapy. That is, the nucleic acid molecule is capable of correcting a genetic defect. A composition that is able to effect gene therapy includes a delivery vehicle that is genetically engineered to effect stable gene therapy in the targeted cell type by, for example, being able to effect integration of the
10 gene into the host genome, maintaining the fused cell as a heterokaryon, or using other mechanisms to stably maintain the gene in the treated cell type. Such a composition is administered to a mammal *in vivo*, or *ex vivo* using techniques such as those developed for other gene delivery vehicles.

While various embodiments of the present invention have been described in
15 detail, it is apparent that further modifications and adaptations of the invention will occur to those skilled in the art. However, it is to be expressly understood that such modifications and adaptations are within the spirit and scope of the present invention.

What is claimed is:

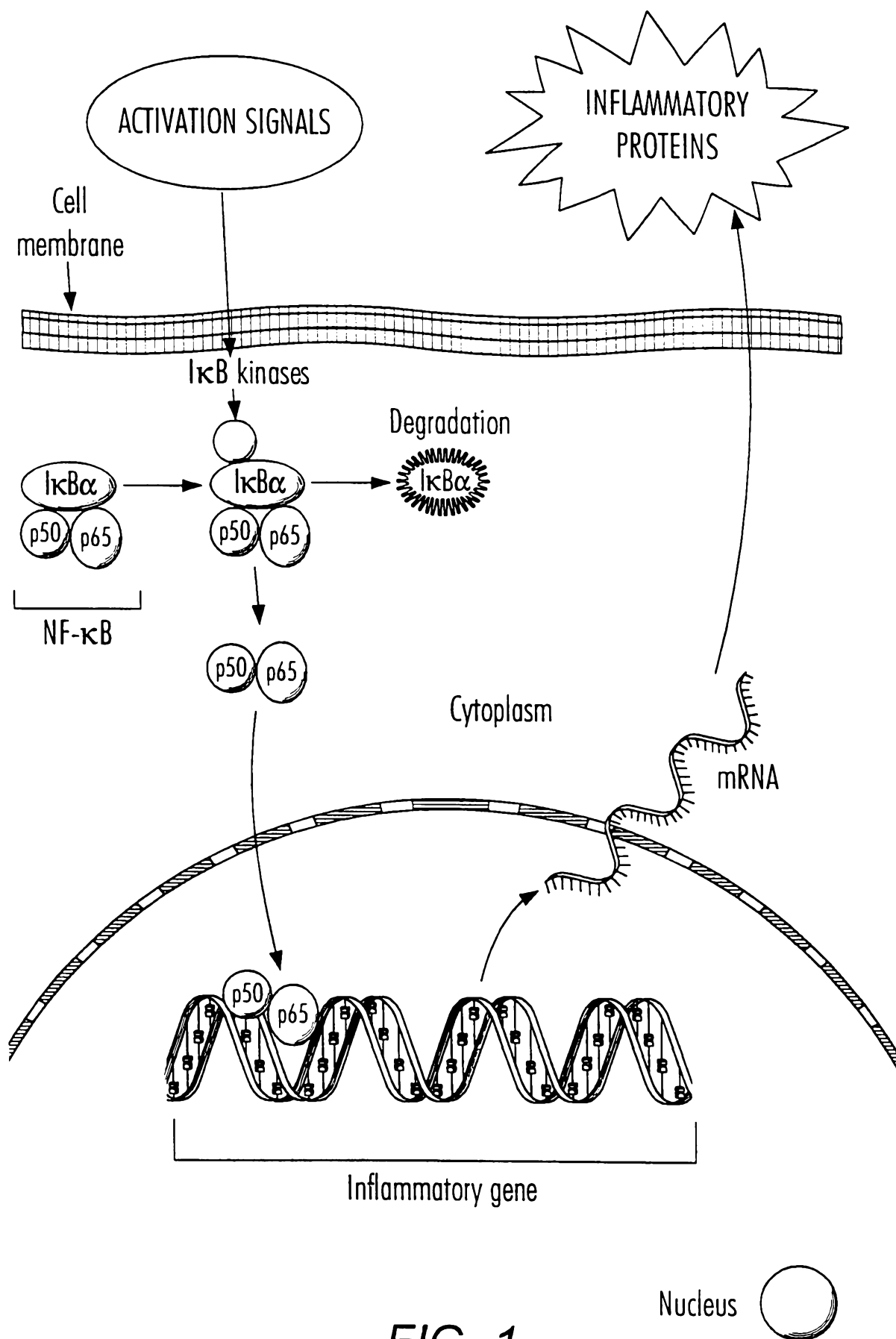
1. A method of treating ocular oxidative stress in mammals in need of such treatment, comprising administering to a mammal an effective amount of an agent that modulates NFκB and NFκB dependent processes.
- 5 2. The method of Claim 1, wherein said agent comprises at least one of NAC, glucocorticoids, acetosalicylic acid, agents that inhibit NFκB activation, interleukin-1 (IL-1), and tumor necrosis factor (TNF).
3. A method of treatment according to Claim 1 wherein said ocular oxidative stress is associated with age-related macular degeneration (ARMD), cataract, iritis,
10 uveitis or glaucoma.
4. A method of treatment as described in Claim 1, further comprising using mechanical low vision aids, acupuncture, laser radiation lasmaphoresis, pharmaceuticals and/or neutraceuticals.
5. A method of treatment as described in Claim 1, wherein the effective
15 agent is administered intraocularly, topically or by injection.
6. A method of treatment as described in Claim 1, wherein the effective agent comprises cysteine, N-acetylcysteine (NAC) or procysteine.
7. A method to diagnose oxidative stress comprising evaluating alterations in NFκB or NFκB dependent events.
- 20 8. A method of assessing the responsiveness to interventions which alter NFκB or NFκB dependent processes in the eye, comprising:
evaluating alterations in NFκB dependent events by assessing secondary or intermediate alterations in genetic factors, metals, proteins or lipids.
9. The method as set forth in Claim 8, wherein said genetic factors consist
25 essentially of STAT3, said metals consist essentially of Fe⁺⁺, said proteins consist essentially of interleukin-6 and said lipids consist essentially of oxidized lipid hydroperoxides.
10. The method as set forth in Claim 1, wherein said agent comprises inhibitors of IκBα or IκBβ superrepressors, peptides capable of blocking nuclear
30 localization or nuclear uptake of NFκB or that prevent or decrease NFκB interacting with basic transcription complexes.

11. The method as set forth in Claim 1, wherein said agent comprises P300 CREB binding protein.

12. The method as set forth in Claim 1, wherein said agent comprises an antioxidant or anti-inflammatory agent that alters cell redox status or that alters
5 apoptosis-related mechanisms capable of modulating NFκB activity.

13. A method as set forth in Claim 1, wherein said ocular oxidative stress is associated with age-related macular degeneration, cataract, iritis, uveitis or glaucoma.

1/2

**FIG. 1**

2/2

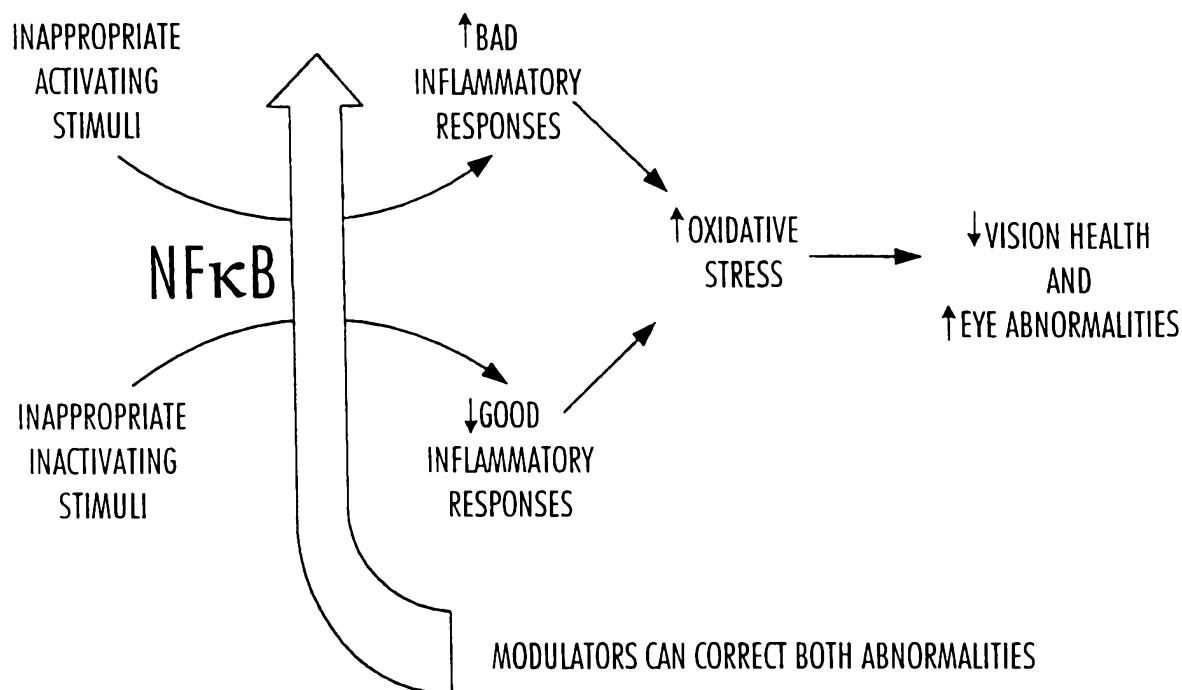
Dual Role of NF κ B

FIG. 2

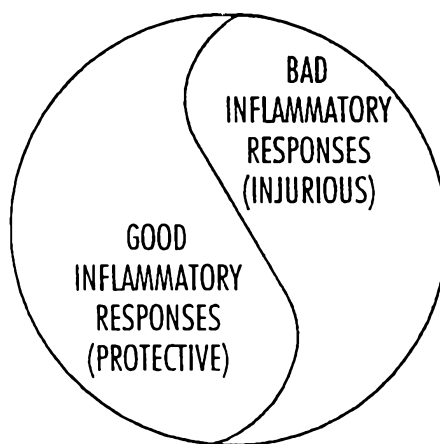
NF κ B Yin-Yang Responses

FIG. 3