Suppression of Nitric Oxide Generated by Inflammatory Microphages by Calcitonin Gene–Related Peptide in Aqueous Humor

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PURPOSE. Ocular immune privilege is mediated in part by the activity of constitutively produced immunosuppressive cytokines and neuropeptides. Aqueous humor was examined for content of calcitonin gene–related peptide (CGRP), and the potential of CGRP to mediate immunosuppressive activity within aqueous humor was determined.

METHODS. The concentration of CGRP in fresh, normal rabbit aqueous humor was assayed by competitive enzyme-linked immunosorbent assay. The ability of CGRP to suppress interferon (IFN)-γ production by antigen-stimulated, primed lymph node cells was examined by assaying supernatants of stimulated CGRP-treated, primed T-cell cultures for IFN-γ. The anti-inflammatory activity of aqueous humor and CGRP was assayed by treating IFN-γ–lipopolysaccharide (LPS)–activated RAW 264.7 cells (macrophages) with aqueous humor, aqueous humor plus anti-CGRP antibody, or CGRP alone. Culture supernatants of the treated macrophages were examined for nitrite by Griess reagent. The production of inducible nitric oxide synthase (NOS2) protein was examined by immunoblotting cell lysates of treated activated macrophages.

RESULTS. The constitutive level of CGRP in fresh, normal rabbit aqueous humor was 5 ± 1 × 10⁻⁵ M. At its ocular concentration, CGRP did not inhibit IFN-γ production by stimulated effector T cells, but it suppressed nitric oxide generation by activated macrophages. Neutralization of CGRP in normal rabbit aqueous humor prevented the aqueous humor from suppressing nitric oxide generation by macrophages. Neither CGRP nor aqueous humor suppressed NOS2 protein synthesis in activated inflammatory macrophages.

CONCLUSIONS. Calcitonin gene–related peptide is a constitutive neuropeptide in aqueous humor. Through CGRP, aqueous humor suppresses nitric oxide production by activated macrophages. This suppression appears to result from inhibiting NOS2 enzymatic activity, rather than from suppressing NOS2 synthesis. The results imply that the ocular microenvironment has diverse immunoregulatory mechanisms that suppress induction, activation, and mediation of immunogenic inflammation. (Invest Ophthalmol Vis Sci. 1998;39:1372-1378)

The eye is an immune-privileged site that prevents induction of immunogenic inflammation within its microenvironment.1,2 This immunosuppression is considered to be an evolutionary adaptation of several immunosuppressive mechanisms protecting the eye's delicate structure from the damage associated with immunogenic inflammation.3 The immunosuppressive activity of aqueous humor has demonstrated the presence of soluble factors actively suppressing immunogenic inflammatory responses.4 The immunosuppressive activity of aqueous humor is mediated by cytokines, growth factors, and neuropeptides that are constitutively produced and released within the ocular microenvironment.5,7 Currently identified immunosuppressive aqueous humor factors are transforming growth factor-β, and the neuropeptides α-melanocyte stimulating hormone (α-MSH) and vasoactive intestinal peptide.8–12 The activities of α-MSH and vasoactive intestinal peptide in aqueous humor have indicated an important role for neuropeptides in ocular immune privilege.13 Another neuropeptide that is constitutively found in the sensory neurons of the iris and ciliary body is calcitonin gene-related peptide (CGRP).14,15 Calcitonin gene–related peptide, a 37-amino-acid neuropeptide encoded within the calcitonin gene derived from alternate mRNA splicing only within neurons,16 has conflicting neuroimmunomodulating activities. It inhibits the mechanisms of antigen presentation by macrophages and Langerhans' cells in inducing delayed-type hypersensitivity (DTH) and contact hypersensitivity.17,18 In addition, CGRP inhibits production of IFN-γ–induced reactive oxygen intermediates by macrophages.19 This suggests that CGRP suppresses antigen-presenting cell induction of immunogenic inflammation and antagonizes the activity of inflammatory cytokines produced by inflammatory T cells. The anti-inflammatory activity of CGRP is further suggested by the prevention of diabetes through targeted CGRP gene expression in nonobese diabetic mice β cells.20 Therefore, localized site-specific expression of CGRP...
mediates immunosuppression. In addition, CGRP has limited suppressive effects on T cells. It can suppress proliferation of mitogen-stimulated spleen T cells but not of mitogen-stimulated interleukin (IL)-2 production.\textsuperscript{21} Mature peripheral T cells are nonresponsive to CGRP; however, thymocyte proliferation and differentiation are suppressed by CGRP.\textsuperscript{22}

In contrast to its potential immunosuppressive activity, CGRP may have a role in nonimmune mediated inflammation (innate immunity). Tissues with acute and chronic phases of inflammation have a localized increase in CGRP concentration.\textsuperscript{23} Localized tissue increases of CGRP are also associated with induction of neurogenic and endotoxin-induced ocular inflammation.\textsuperscript{24-26} This association has suggested that CGRP could act as a mediator of localized inflammatory activity. These seemingly contradictory activities of CGRP-mediated immunosuppression and inflammation indicate the uncertainty of CGRP activity in host defense mechanisms.

Because the iris and ciliary body are innervated with peptidergic neurons containing CGRP, we assayed normal rabbit aqueous humor for constitutive CGRP. Also, we examined the possible role of CGRP in aqueous humor immunosuppression of IFN-\(\gamma\) production by primed inflammatory T cells and its role in aqueous humor suppression of IFN-\(\gamma\)-induced nitric oxide synthase (NOS) by inflammatory macrophages.

**Materials and Methods**

**Aqueous Humor Collection**

Aqueous humor was obtained from normal New Zealand White rabbits by passive drainage of the anterior chamber.\textsuperscript{11} The rabbits were anesthetized with a mixture of ketamine and xylazine administered intramuscularly and managed according to the guidelines of the U. S. Animal Welfare Act, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The aqueous humor was obtained by limbal paracentesis, using a 27-gauge winged infusion set (Surflo; Fisher Scientific, Pittsburgh, PA). Aqueous humor was passively drained into a siliconized microcentrifuge tube (Fisher). The aqueous humor was used immediately in the CGRP enzyme-linked immunosorbent assay (ELISA) or in the nitrite accumulation experiments.

**Competitive Enzyme-Linked Immunosorbent Assay**

The concentration of CGRP in fresh, normal rabbit aqueous humor was measured, using a competitive ELISA similar to the competitive ELISA we developed for \(\alpha\)-MSH hormone.\textsuperscript{12} The wells of a 96-well flat-bottomed tissue culture plate (Corning, Corning, NY) were coated overnight at 4°C with a 1:500 dilution anti-rabbit IgG (Sigma, St. Louis, MO) in 0.05 M carbonate buffer (pH 9.6). The wells were wash once, resuspended in 100 \(\mu\)l antigen. The cultures were incubated for 24 hours, by hypotonic shock, and the remaining washed spleen cells (1 \(\times\) 10\(^5\)) were plated in a 96-well plate (Corning). The cultures were washed twice with serum-free medium to remove nonadherent cells.

**Interferon-\(\gamma\) Sandwich Enzyme-Linked Immunosorbent Assay**

The wells of a 96-well flexible microtiter plate (Falcon; Oxand, CA) were coated with 50 \(\mu\)l 5 \(\mu\)g/ml monoclonal antibody-detecting anti-IFN-\(\gamma\) (clone R4-6A2; Pharmagen, San Diego, CA), in 0.05 M carbonate-bicarbonate (pH 9.6; Sigma). The plate was incubated for 1 hour at room temperature. The anti-CGRP antibody binds all isoforms of CGRP and has no cross-reactivity to calcitonin, amylin, amylin amide, or substance P. The plate was washed three times with PBS-Tween, and a mixture of biotinylated-CGRP (Peninsula) with aqueous humor sample or known amounts of CGRP protein (Peninsula) was added to the wells. The mixtures were made by combining 1 ng/ml biotinylated CGRP in PBS-BSA with an equal volume of aqueous humor (100 \(\mu\)l total). For a standard curve, known amounts of CGRP protein (0.02-20 ng/ml) in DPBS were mixed with the biotinylated-CGRP. The mixtures were incubated in the wells for 1 hour at room temperature. After three washings with PBS-Tween, 1:1000 streptavidin-\(\beta\)-galactosidase (Gibco, Gaithersburg, MD) was added to the wells. The plate was incubated for 30 minutes at room temperature and washed five times with PBS-Tween. Substrate chlorophenyl-red-\(\beta\)-galactoside (Gibco BRL) was added to the wells, and the color change was read 1 hour later with an electroimmunoassay plate reader (Tecn, Durham, NC). The concentration of CGRP in the aqueous humor was calculated by using the sample's optical density (OD) in an equation fitted to the polynomial regression of CRGP concentration to the OD of wells with known concentrations of CGRP. The competitive ELISA for CGRP was sensitive to a level of 20 pg/ml.
cytokine were added to the wells to form a standard curve. The plate was incubated for 3 hours at room temperature and washed three times with PBS-Tween. One hundred microliters 1 μg/ml biotinylated-monoronal antibody-detecting anti-IFN-γ (clone XMG1.2; Pharmagen), was added. The plate was incubated for 1 hour and washed three times. One hundred microliters streptavidin-β-galactosidase (Gibco) was added to the wells. The plate was incubated for 30 minutes and washed 5 times, after which of chlorophenol red-β-galactopyranose substrate (Gibco) and color was allowed to develop for 30 minutes. The OD of the converted chlorophenol red-β-galactopyranose was read on a standard electroimmunoassay plate reader at a 570-nm wavelength. The cytokine concentration of the standard samples was plotted against their OD to create a standard curve. The concentration of cytokine in the assayed culture supernatant was calculated from the OD of the test well and sample dilution factor, using the standard curve. Sensitivity of the assay was to a level of 5 pg/ml.

**Nitrite Assay**

Accumulation of nitrite in the culture supernatant of IFN-γ-LPS-activated RAW 264.7 cells, a mouse monocytic leukemic cell line (ATCC, Rockville, MD), was assayed as an indicator of nitric oxide production. Twenty-seven RAW cells (6 × 10⁶ cells) in 100 μl phenol red-free RPMI 1640 (BioWittaker) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT) were added to the wells of a flat-bottomed, 96-well culture plate and incubated for 2 hours at 37°C. The medium was removed, and 50 μl of 2 ng/ml LPS and 2 U/ml IFN-γ were added to the wells. An additional 50 μl medium with or without CGRP was added to the wells. The cultures were incubated for 6 hours at 37°C. The supernatant was assayed for nitric oxide by mixing 100 μl supernatant with 100 μl Griess reagent (1% sulfanilamide-0.1% naphthylethylene diamine dihydrochloride/2% H₃PO₄) in a 96-well plate. The plate was incubated 15 minutes at room temperature and read on an electromiunoassay plate reader at 550 nm. The concentration of nitrite in the culture supernatant was determined from a standard curve of known sodium nitrite concentrations (100–0.003 μM).

**Nitric Oxide Synthase-2 Immunoblotting**

Treated RAW cells were washed once in 0.01 M PBS and lysed with lysis buffer (0.01 M Tris-buffered saline, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and the proteins were transferred to a nitrocellulose filter in a minicell and blot module (Xcell II; Novex, Cambridge, MA). The filter was blocked for 30 minutes with 0.01 M Tris-buffered saline plus 1% BSA and treated with a 1:100 dilution blocking buffer of rabbit anti-NOS2 IgG antibody (Santa Cruz Biotechnologies, Santa Cruz CA) overnight. The filter was washed three times with blocking buffer and treated with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma) for 1 hour. The filter was washed three times in blocking buffer and once in distilled water. Bands were revealed by application of the alkaline phosphatase substrate solution (BCIP-NBT; Sigma). The filters were washed with distilled water, digitally photographed, and analyzed using imaging software (version 1.61/ppc; National Institutes of Health, Bethesda, MD).

**RESULTS**

### Expression of Calcitonin Gene–Related Peptide in Normal Rabbit Aqueous Humor

Aqueous humor from humans, rabbits, mice, and other mammals have similar immunosuppressive activities and contain constitutive levels of immunosuppressive cytokines and neuropeptides that are evolutionarily conserved in structure, function, and concentration in biologic fluids. Because of this evolutionary conservation we could use rabbit aqueous humor as a model system to examine the mechanisms of immunosuppression and immune privilege in mammalian eyes. We have developed a competitive ELISA that is specific for CGRP, because we have found that aqueous humor inhibits immunoprecipitation of radioimmune assays, which results in artificially high values of assayed antigens. Using the CGRP-specific competitive ELISA, we assayed normal rabbit aqueous humor for immunoreactive CGRP. We found 1.98 ± 0.45 ng/ml (5 ± 1 × 10⁻⁷ M) CGRP in seven samples of normal rabbit aqueous. The concentration of CGRP in aqueous humor falls within the range of its reported immunomodulating activities.

### Effect on Interferon-γ Production by Effector T Cells

Aqueous humor suppression of DTH has been linked to suppression of IFN-γ production by stimulated primed T cells. This immunosuppression is mediated by cytokines and neuropeptides present in normal aqueous humor. Because the concentration of CGRP in rabbit aqueous humor fell within its reported range of known bioactivity, we examined whether CGRP at its aqueous humor concentration could suppress IFN-γ production by stimulated primed T cells.

Primed T cells were stimulated with anti-T-cell receptor antibody to produce IFN-γ (Fig. 1). When the T cells were stimulated in the presence of CGRP at 2 ng/ml, the level of IFN-γ remained unchanged. In contrast, α-MSH, a known aqueous humor immunosuppressive neuropeptide, suppressed IFN-γ production as expected (Fig. 1). The inability of CGRP to suppress effector T-cell production of IFN-γ suggests that CGRP is not a mediator of T-cell–directed immunosuppression found in mammalian aqueous humor.

### Effect of Anti–Calcitonin Gene–Related Peptide Antibody on Aqueous Humor Suppression of Nitric Oxide Generated by Inflammatory Macrophages

Regarding DTH, one of most important IFN-γ-mediated inflammatory activities is the activation of inflammatory macrophages, and one of the most damaging factors generated by inflammatory macrophages is nitric oxide. Nitric oxide generation within macrophages is dependent on and mediated by IFN-γ and LPS. The macrophage cell line, RAW 264.7, was treated with a mixture of 1 U/ml IFN-γ and 1 ng/ml LPS for 6 hours, after which the supernatant was colorimetrically assayed indirectly for nitric oxide, using Griess reagent to quantitate the concentration of nitrate, a relatively stable end product of nitric oxide and water. Interferon-γ-LPS-activated RAW cells produced 1.6 ± 0.25 μM nitric oxide in vitro; however, nitric oxide production by IFN-γ-LPS-activated RAW cells was suppressed to background levels when the cells were activated in the presence of...
Figure 1. Effects of calcitonin gene–related peptide (CGRP) on interferon (IFN)-γ production by antigen-stimulated, primed T cells. The 48-hour culture supernatant of primed T cells stimulated with antigen-pulsed adherent spleen cells (ASC) was assayed for IFN-γ by sandwich enzyme-linked immunosorbent assay. Primed T cells were stimulated by: (A) antigen-pulsed ASC; (B) ASC only (no antigen); (C) antigen-pulsed ASC in the presence of α-melanocyte-stimulating hormone (30 pg/ml); or (D) antigen-pulsed ASC in the presence of CGRP (2 ng/ml). The data are presented as the mean ± SEM of four experiments.

Figure 2. Effects of aqueous humor on nitric oxide generation by inflammatory macrophages. Culture supernatants (incubated for 6 hours) of interferon (IFN)-γ–lipopolysaccharide (LPS)–treated RAW 264.7 cells (6 × 10⁶ cells/well) were assayed for nitrite (NO₂⁻) by Greiss reagent. RAW cells were treated with: (A) IFN-γ–LPS only; (B) no IFN-γ–LPS; (C) IFN-γ–LPS and fresh rabbit aqueous humor (diluted 50%); and (D) IFN-γ–LPS and aqueous humor with anti-calcitonin gene–related peptide. The data are presented as the mean ± SEM of nine experiments.
Figure 3. Effects of calcitonin gene–related peptide (CGRP) on nitric oxide production by inflammatory macrophages. Culture supernatant (incubated for 6 hours) of RAW 264.7 cells treated with interferon (IFN)-γ-lipopolysaccharide (LPS) in the presence of calcitonin gene–related peptide (CGRP) at various concentrations was assayed for nitrite using Griess reagent. The data represent the mean percentage inhibition ± SEM in four experiments of detected nitrite, compared with the amount of nitrite in the supernatant of RAW cells treated with only IFN-γ-LPS, minus the amount in supernatants of untreated RAW cells.

polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with anti–NOS2-specific antibody.

Immunoblots of untreated RAW cell lysates showed no NOS2 protein, whereas lysates of IFN-γ-LPS-activated RAW cells showed a distinct band at 130 kDa that corresponds to a single protein chain of NOS2 (Fig. 4). Nitric oxide synthase-2 protein was also present in the lysates of RAW cells activated in the presence of aqueous humor or CGRP (Fig. 4). The addition of anti-CGRP antibody to the aqueous humor used in treating activated RAW cells had no affect on the level of NOS2 protein in the cell lysates (Fig. 4). Therefore, the CGRP in aqueous humor did not suppress IFN-γ-LPS induction of NOS2 protein synthesis in macrophages; rather, it is probable that CGRP inhibited nitric oxide synthase enzymatic activity.

Discussion

Aqueous humor suppression of DTH has mostly been described by its immunosuppressive effects on effector T-cell activity.8,11,12 We have now demonstrated the ability for aqueous humor to suppress DTH further by inhibiting IFN-γ-induced inflammatory activity of macrophages. This anti-inflammatory activity is mediated by CGRP, independent of the neuropeptides and growth factors mediating T-cell-directed immunosuppression. The results demonstrate that aqueous humor is anti-inflammatory, suggesting that the immune privileged ocular microenvironment not only suppresses the T cells that trigger DTH, but also IFN-γ-dependent inflammation associated with DTH.

Appreciating the ability of aqueous humor to suppress induction of IFN-γ-mediated macrophage inflammatory activity extends our understanding of the mechanisms of immunosuppression within the eye. It has been proposed that the central function of ocular immune privilege is to prevent immunogenic inflammation from damaging the eye’s delicate structures and threatening vision.3 Our results suggest that the inflammatory activities of T cells and macrophages are regulated in the ocular microenvironment. This implies that suppression of macrophage activity would also prevent inflammation triggered by insults beyond activated T cells. Therefore, the ocular microenvironment appears to have adopted mechanisms to suppress inflammation at multiple levels (T-cell activation, IFN-γ production, and macrophage activation).

Our demonstration that CGRP is ineffective in suppressing IFN-γ production by activated, primed T cells corresponds to the report that mature peripheral T cells lack receptivity to CGRP.22 In contrast, macrophages and antigen-presenting cells are receptive and sensitive to CGRP.17,18,51 Although the focus of this study was not antigen-presenting activity of macrophages, we know that CGRP suppresses antigen presentation by Langerhans’ cells and macrophages, thereby inhibiting DTH and contact hypersensitivity.

In addition to its anti-inflammatory activity, CGRP is also a potent vasodilator.24,25,32,34 It is associated with, and may mediate activation of, nitric oxide production by endothelial cells. In this case the nitric oxide–generating enzyme is endothelial nitric oxide synthase (eNOS, or NOS3), which is a different protein encoded by a different gene from inducible NOS (NOS2) under different regulatory mechanisms and does not generate tissue-damaging levels of nitric oxide.30,35,36 Linkage of CGRP with endotoxin-induced uveitis has also been made; however, unlike nitric oxide generation by NOS2 in immunogenic inflammation, CGRP-mediated inflammation may be through vasodilation induced by nitric oxide generated by...
FIGURE 4. Immunoblot of nitric oxide synthase (NOS)-2 protein in activated macrophage cellular lysates. RAW 264.7 cells were treated as previously described, except that they were lysed 6 hours after treatment. Equal amounts of protein lysate per lane were electrophoresed and transferred to nitrocellulose filters. The filters were blotted with anti-NOS2 antibody. Raw cells were treated with (lane 1) interferon (IFN)-γ-lipopolysaccharide (LPS) and aqueous humor (50% dilution); (lane 2) IFN-γ-LPS and aqueous humor with anti-calcitonin gene-related peptide (CGRP); (lane 3) IFN-γ-LPS and CGRP (2 ng/ml); (lane 4) IFN-γ-LPS only; and (lane 5) no IFN-γ-LPS. The figure represents results of three experiments.

NOS3, the constitutive nitric oxide synthase of endothelial cells. However, the level of CGRP needed to induce this inflammatory response is in the range of 10 to 1000 nanomoles, at least 20 times higher than the concentration we found constitutively in aqueous humor. Our dose-response curve reveals that increasing amounts of CGRP have a diminishing ability to suppress nitric oxide generation in activated macrophages. Therefore, it is possible that after neural stimulation or response to endotoxin, increasing concentrations of CGRP in aqueous humor would actually diminish the anti-inflammatory benefits of CGRP. Rising levels of CGRP could mediate vasodilation through induction of NOS3 activity, which, paradoxically, would amplify the inflammatory response.

The mechanism by which CGRP in aqueous humor suppresses nitric oxide production by IFN-γ-LPS-activated macrophages appears to be through a posttranslational regulation of NOS2. Nitric oxide synthase-2 is a 130 kDa protein that is sequestered into cytoplasmic vesicles. For NOS2 to be active, it must dimerize. The dimerization is mediated by the binding of iron protoporphyrin IX (heme) to the oxygenase domain of NOS2. Because nitric oxide binding to heme renders it insoluble, the iron released from the oxygenase domain prevents dimerization of NOS2 proteins and uncouples already formed NOS2 dimers. Thus nitric oxide may regulate its own production. L-Arginine and tetrahydrobiopterin (H4B) also bind through the oxidative domain. In addition, there is a reductase domain in NOS2 that binds flavin adenine dinucleotide, flavin mononucleotide, and calmodulin. Binding of calmodulin is also required for NOS2 activity. However, this is Ca2+-dependent, unlike constitutive NOSs (NOS1 and NOS3). How CGRP prevents activation of NOS2 is unknown, mainly because the intercellular signaling pathways of CGRP in macrophages is uncertain. It is possible that CGRP receptor ligation may mediate intracellular events that regulate NOS2 dimerization and iron binding. However, binding of calmodulin to NOS2 is Ca2+-independent, unlike constitutive NOSs (NOS1 and NOS3). How CGRP prevents activation of NOS2 is uncertain, mainly because the intercellular signaling pathways of CGRP in macrophages is uncertain. It is possible that CGRP receptor ligation may mediate intracellular events that regulate NOS2 dimerization and ion binding. However, binding of calmodulin to NOS2 is Ca2+-independent, unlike constitutive NOSs (NOS1 and NOS3). How CGRP prevents activation of NOS2 is unknown, mainly because the intercellular signaling pathways of CGRP in macrophages are uncertain. It is possible that CGRP receptor ligation may mediate intracellular events that regulate NOS2 dimerization and ion binding. However, binding of calmodulin to NOS2 is Ca2+-independent, unlike constitutive NOSs (NOS1 and NOS3).
References


