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Effects of antiglaucoma drugs GLC756, a novel dopamine D_2 agonist and D_1 antagonist, and timolol on endotoxin-induced TNF-alpha release in serum of rats

U.W. LAENGLE[1], R. MARKSTEIN[2], V. SCHNEIDER[1], D. ROMAN[1]

[1]Department of Toxicology/Pathology
[2] Department of Ophthalmics, Novartis Pharma AG, Basel - Switzerland

PURPOSE. *Anti-inflammatory activity of an antiglaucoma drug may be an advantage for long-term treatment of glaucoma since it may reduce the risk of treatment-related inflammatory processes in outer compartments of the eye and probably also prevent or delay progression of glaucomatous retinal neurodegeneration. In this study, the effect of GLC756, a novel mixed dopamine D_2 receptor agonist and dopamine D_1 receptor antagonist, and timolol on endotoxin-induced cytokine tumor necrosis factor-α (TNF-α) release in serum was examined.*
METHODS. *For endotoxin-induced TNF-α release, 8-week-old Lewis rats were intravenously injected with 160 µg lipopolysaccharide (LPS) from* Salmonella typhimurium. *GLC756, timolol, or betamethasone were either systemically (1 mg/kg SC for 5 days) or topically (0.4%, 0.5%, and 0.1%, respectively, 20 µL eye drops given 16 times over 48 hours in left and right eye) administered. TNF-α was measured in serum 2 and 48 hours after LPS induction.*
RESULTS. *A marked TNF-α increase in serum was found 2 hours after LPS induction. Administration of GLC756 and betamethasone, systemically and topically, decreased TNF-α release. However, due to large scattering of mean values only the effect of systemically administered GLC756 was statistically significant. In contrast, timolol increased TNF-α values stronger than LPS alone.*
CONCLUSIONS. *The significant suppression of LPS-induced TNF-α increase by GLC756 suggests an additional anti-inflammatory potential of the dopaminergic compound in the treatment of glaucoma. (Eur J Ophthalmol 2006; 16: 401-6)*

KEY WORDS. *Betamethasone, D_2 agonist, D_1 antagonist, Glaucoma medication, Endotoxin, GLC756, Rat, Serum, Timolol, TNF-α*

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INTRODUCTION

Glaucoma designates a group of chronic eye diseases that are characterized by progressive atrophy of the optic nerve head and visual field loss and, in most cases, by elevation of intraocular pressure (IOP). The etiology of glaucoma is unknown but elevated IOP and impaired perfusion of the optic nerve have been suggested to be important risk factors. Current medical treatment of glaucoma is focused on lowering IOP. Beta-blockers such as timolol have been frequently used to lower IOP (1). Dopaminergic drugs are another class of compounds that lower IOP in experimental animals and in man (2) and in addition have been found to improve perfusion of the optic nerve in experimental animals (3) and therefore may be of particular value for the treatment of glaucoma (4-6).

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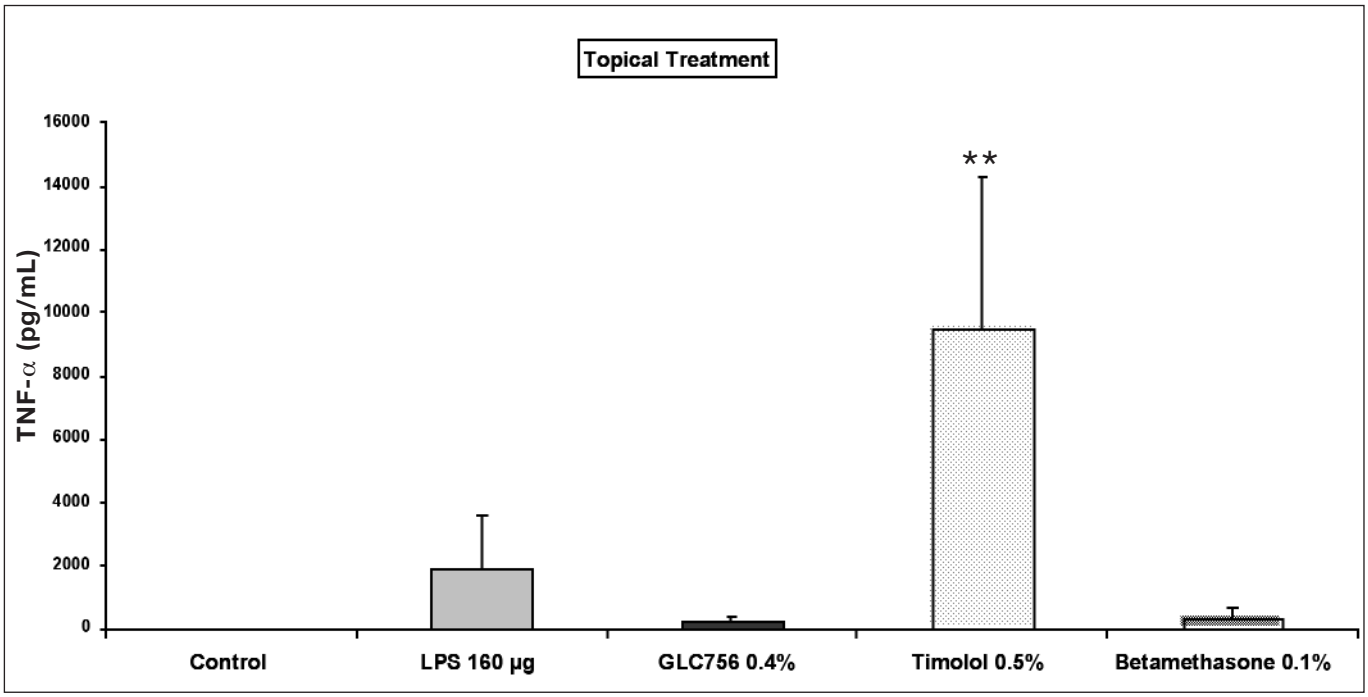

Fig. 1 - *Serum tumor necrosis factor (TNF)-α values 2 hours after lipopolysaccharide (LPS) induction and topical pretreatment with GLC756, timolol, or betamethasone. Each column is a mean of five values and the bars indicate standard deviation. Value was considered significantly different from the LPS group at **p<0.005. Control group: no measurable value.*

Increased numbers of inflammatory cells in outer compartments of the eye were found in humans who have been treated with conventional antiglaucoma drugs (7-9). Mediators of inflammatory response might mediate proliferation of the fibroblasts in the anterior parts of the eye (10). Increased numbers of inflammatory cells and fibroblasts may contribute to the failure of glaucoma filtering surgery (7, 11-14). Therefore, antiglaucoma drugs with an anti-inflammatory property may provide an additional therapeutic benefit.

In glaucomatous optic neuropathy, apoptosis is implicated in the death of retinal ganglion cells (15). Although the relationship of glial activation to neurodegeneration in glaucoma has not been established, increased production of some neurotoxic substances by optic nerve head astrocytes has been found in glaucomatous eyes. For example, increased production of TNF-α has been detected in glaucomatous optic nerve head (16). There is evidence that apoptosis-promoting substances, including TNF-α secreted by activated glial cells after exposure to stress, contribute directly to neuronal cytotoxicity. Therefore, it is suggested that the inhibition of TNF-α release from glial cells may be a novel therapeutic principle for neuroprotection for the treatment of glaucomatous optic neuropathy (17). Bromocriptine, a dopamine D2 receptor agonist, has been reported to reduce TNF-α production in rats (18).

It was therefore of interest to study the effect of the antiglaucoma drugs GLC756, a novel mixed dopamine D_2 receptor agonist and dopamine D_1 receptor antagonist (2), and the beta-blocker timolol on endotoxin-induced TNF-α release in serum of rats. The effects of the two test drugs were compared with that of betamethasone, a drug with proven anti-inflammatory properties.

MATERIALS AND METHODS

Animals and study design

Eight-week-old Lewis rats, weighing 140 to 180 g, were obtained from Charles River Company, WIGA, Sulzfeld, Germany. TNF-α release was induced in eight groups of five rats each, by intravenous injection of lipopolysaccharide (LPS) from *Salmonella typhimurium* (Sigma, Switzerland) at 160 µg per rat in a volume of 160 µL. Two of these groups were kept as positive LPS controls. The remaining groups were treated with GLC756, betamethasone, or timolol either topically (0.4%, 0.1%, and 0.5%, respec-

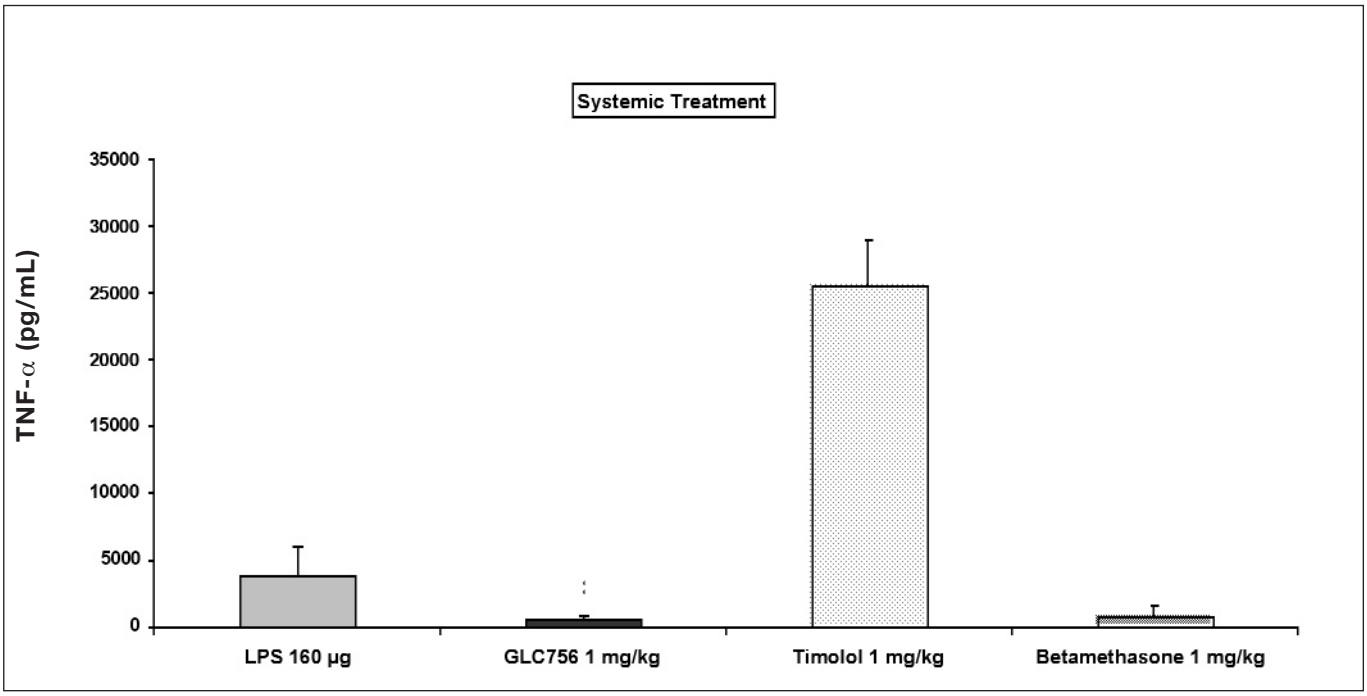

Fig. 2 - *Serum tumor necrosis factor (TNF)-α values 2 hours after lipopolysaccharide (LPS) induction and systemic pretreatment with GLC756, timolol, or betamethasone. Each column is a mean of five values and the bars indicate standard deviation. Values were considered significantly different from the LPS group at *p<0.05 and **p<0.005.*

tively, 20 µL eye drops given 16 times over 48 hours in left and right eye, LPS intravenously about 1 h after the first eye drop) or systemically (1 mg/kg/day subcutaneously for 5 days; LPS intravenously on the third day). An additional group was kept as negative control group treated topically with 0.9% NaCl (20 µL eye drops given 16 times over 48 hours into the right eye.

Drug preparation

GLC756 was received as a powder and as 0.4% eye drops from Novartis Pharma AG, Switzerland. The GLC756 powder was reported to be 100% pure by certificate of analysis. The powder was dissolved in a 2.5% glycerol 1.26 (99.1% pure, from Novartis Pharma AG) solution. Timolol was available commercially as a powder (100% pure) from Sigma, Switzerland, and as 0.5% eye drops from Ursapharm, Germany. The powder was dissolved in a 2.5% glycerol solution. Betamethasone was available commercially as a powder (99.7% pure) from Sigma, Switzerland, and as 0.1% eye drops from Dr. Winzer Pharma GmbH, Germany. The powder was dissolved in a 40% polyethyleneglycol 300 (PEG300, Fluka Chemie AG, Switzerland) solution.

TNF-α assay

TNF-α was determined in serum 2 and 48 hours after LPS induction using rat TNF-α, enzyme-linked immunosorbent assay from Endogen Inc., MA (sensitivity: <10 pg/mL, assay range: 31 to 2500 pg/mL).

Statistical analysis

For all groups mean ± standard deviation were obtained. These values were subjected to an analysis of variance one-way Dunnett's test. Values were considered significantly different from corresponding control at $p<0.05$.

RESULTS

In control animals no TNF-α could be detected in any serum sample (Fig. 1).

In the LPS group a clear increase of TNF-α in the serum occurred 2 hours after intravenous LPS induction (Figs. 1 and 2). The increase was no longer seen 48 hours after LPS induction.

Systemic pretreatment with GLC756 suppressed statistically significantly the LPS-induced TNF-α increase in serum 2 hours after LPS induction (Fig. 1). Topical pretreatment also clearly decreased TNF-α in comparison to that of the LPS group, however, without reaching statistical significance (Fig. 2). No TNF-α could be detected 48 hours after LPS induction.

Timolol, both after systemic and topical pretreatment, produced statistically significant increases of TNF-α values in comparison to the LPS group (Figs. 1 and 2). There, as well, no TNF-α could be detected in serum 48 hours after LPS induction.

Treatment with betamethasone resulted in a marked decrease of serum TNF-α values when compared to the LPS group, however, without statistical significance after systemic or topical administration (Figs. 1 and 2). No TNF-α could be detected after 48 hours of LPS induction.

DISCUSSION

To address anti-inflammatory properties of antiglaucoma drugs, an *in vivo* study in LPS-induced Lewis rats (EIU) was performed. EIU in the Lewis rat is a model for severe uveitis in humans (19). TNF-α as one of the major proinflammatory cytokines, beside IL1 and IL6, was selected as parameter for inflammation in the serum. TNF-α, like IL1, IL6, IL11, and IL17, belongs to the cytokines that are involved in acute and chronic inflammatory responses. TNF-α is produced acutely in large amounts, as in the case of bacterial sepsis, or chronically in lesser amounts, as in the case of chronic infections. During sepsis with Gram-negative organisms, LPS (endotoxin) released from bacteria trigger the widespread production of TNF-α (and subsequently IL1 and IL6) by macrophages (20). Since the presence of macrophages is primarily characteristic for chronic inflammation (21), one can suggest that drugs reducing the inflammation mediator TNF-α significantly in a severe acute uveitis model should also be able to prevent a slight inflammatory reaction caused by chronic eye drop administration and possibly to possess a neuroprotection in the treatment of glaucomatous optic neuropathy.

In this study, timolol and GLC756, a novel mixed dopamine D_2 receptor agonist and dopamine D_1 receptor antagonist, and betamethasone were assessed for their effect on TNF-α release in serum.

TNF-α has been detected in serum of rats after systemic injection of endotoxin, an LPS from the outer membrane of Gram-negative bacteria (22-24). TNF-α, one of the first cytokines released in the cascade of inflammation (25), is an important mediator of inflammation (26) and is produced mainly by activated macrophages and monocytes. The Lewis rat seems to be a good model for production of inflammatory cytokines after stimulation with LPS (22). In the present study, serum TNF-α level was clearly increased 2 hours after intravenous LPS induction compared to control animals where no TNF-α could be detected in serum. An increase of TNF-α was no longer evident 48 hours after LPS induction.

Systemic pretreatment with GLC756 suppressed in a statistically significant manner the LPS-induced TNF-α increase in serum 2 hours after LPS induction. Topical pretreatment also resulted in clearly decreased TNF-α values, which, however, were not statistically significantly different from TNF-α values obtained in the LPS group, which might have been related to a relatively small number of animals and high standard deviations. Influence of topically administered GLC756 on serum TNF-α levels might be partly related to a high systemic exposure due to a drug effect on conjunctival vessels. A drug-induced increase in perfusion of the conjunctival vessels can be assumed, since GLC756, a mixed dopamine D_2 receptor agonist and dopamine D_1 receptor antagonist, is known to improve perfusion of the optic nerve (3). On the other hand, a high topical dose was applied, which is common in preclinical topical toxicity studies in order to obtain the maximal tolerated effect of the drug. The mechanism of GLC756 on TNF-α release seems to be related to its stimulatory effect on dopamine D_2 receptors. Dopamine D_2 receptors stimulation has been reported to suppress LPS-induced TNF-α production (26). It was also found that dopamine D_2 but not dopamine D_1 receptors are involved in LPS-induced modulation of TNF-α production *in vivo* (27). Furthermore, GLC756 has, in addition, affinity to several other receptors such as alpha- and beta-adrenergic and serotonin receptors, which could also have an effect on the TNF-α production. In literature, inhibition of TNF-α has been reported for α_2-adrenoceptor blockade (28), and after stimulation of β_2-adrenergic (29) and of 5-HT_{2c} receptors (30). A receptor-related mechanism of the TNF-α reducing effect by GLC756 needs to be evaluated in a separate *in vivo* study. In the present study, the decreasing effect of GLC756 on TNF-α levels in serum was similar to or even stronger than the one obtained with the corticosteroid betamethasone, which neither systemically nor topically produced a statistically significant decrease of

serum TNF-α levels. The inhibition of an important inflammation mediator suggests a beneficial anti-inflammatory property of GLC756. At least the suppression of TNF-α seemed to be consistent with reduced intraocular inflammation since GLC756 produced a statistically significant reduction in inflammatory cell infiltration in the retina after topical and in the ciliary/vitreous body, and retina after systemic pretreatment in the same animal model as shown in a separate examination (31). Therefore, GLC756 might also be of value in the treatment of glaucoma that results from long-term steroid treatment in patients with chronic inflammatory eye disease such as chronic uveitis (32). With evidence that apoptosis-promoting substances, including TNF-α secreted by activated glial cells after exposure to stress, contribute directly to neuronal cytotoxicity (17), it cannot be excluded that the inhibitory effect of GLC756 on TNF-α production protects ganglionic cells from certain deleterious effects involved in the sequence of events leading to glaucomatous damage of the optic nerve. The effects of GLC756 on other cytotoxic cytokines such as IL1-beta and IL6, which could lead to neurodegeneration when elevated (33), or, e.g., on the anti-inflammatory cytokine IL13, which could induce death of activated microglia and therefore prevent neurodegeneration (34), were not examined in this study due to limited quantity of available serum.

For timolol, a mixed beta(1)/beta(2)-adrenergic receptor antagonist, both systemic and topical pretreatment resulted in statistically significantly increased TNF-α levels in serum compared to the LPS-group, 2 hours after induction. The increasing effect of timolol on TNF-α levels in this study might be related to its affinity to beta adrenergic receptors. The antagonist effect on beta-(2)-adrenergic receptor has been reported to inhibit almost completely the decrease of LPS-induced TNF-α release after agonistic activation of beta-(2)-adrenergic receptor (35, 36). However, in this study, the effect of timolol on TNF-α was always related to a previous LPS induction. So far, it is not known whether timolol increases TNF-α serum levels in a situation where no pretreatment with LPS takes place. In contrast, timolol decreased TNF-α levels in aqueous humor after laser iridotomy in rabbits (37).

In summary, the ability of GLC756 to significantly suppress LPS-induced TNF-alpha release suggests additional anti-inflammatory properties of this dopaminergic compound in the treatment of glaucoma.

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Reprint requests to:
Ulrich.W. Laengle, PhD
Department of Toxicology/Pathology
MUT-2881.133
CH-4002 Basel, Switzerland
Ulrich.Laengle@novartis.com

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