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Morphine-induced nitric oxide production in isolated, iris-ciliary bodies

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Abstract

Considerable evidence suggests that the nitric oxide (NO)/cGMP signaling pathway plays an integral role in opioid receptor-mediated responses in the cardiovascular and immune systems. Previous studies in our laboratory and others have shown that nitric oxide (NO) plays a role in morphine-induced reduction of intraocular pressure (IOP) and pupil diameter (PD) in the New Zealand white (NZW) rabbit. The present study is designed to determine the effect of morphine on NO production in the isolated, iris-ciliary body (ICB), site of aqueous humor production, as this effect could be associated with morphine-stimulated changes in aqueous humor dynamics and iris function. ICBs obtained from normal NZW rabbits were utilized in these experiments. In some experiments, ICB samples were treated with morphine (1, 10 and 100 μ M) for 1 hour and later examined for changes in NO levels using a NO detection kit. In other experiments, tissue samples were pretreated with naloxone (non-selective opioid receptor antagonist), L-NAME (non-selective NO synthase inhibitor) or GSH (sulfhydryl reagent) for 30 minutes, followed by treatment with morphine (10 μ M). Morphine caused a concentration-dependent increase in the release of NO from ICBs. Levels of NO detected in the incubation medium of ICB samples increased from 1.49 ± 0.19 (control) to 8.81 ± 2.20 μ M/mg protein (morphine treated; 100 μ M). Morphine-stimulated release of NO was significantly inhibited in tissues pretreated with 10 μ M naloxone, L-NAME, or GSH. Results obtained from this study suggest that morphine stimulates NO release from the ICB through a mechanism that involves activation of NO-releasing opioid receptors. These results support the *in vivo* effects of morphine demonstrated in previous studies.

Keywords

Opioid receptors; morphine; nitric oxide; iris-ciliary body

1. Introduction

Considerable evidence suggests that the nitric oxide (NO)/cGMP signaling pathway plays an integral role in opioid receptor-mediated responses in the immune (Kowalski, 1998; Coussons-Read and Giese, 2001) and cardiovascular (Rebrova et al., 2001; Guo et al., 2005; Sun et al., 2006) systems. Although morphine-induced reduction of intraocular pressure (IOP) and pupil diameter (PD) has been previously reported, the exact mechanism(s) involved in these actions has not been completely elucidated. Initial studies by Myashita (1913) demonstrated that

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morphine raised IOP, but subsequent reports revealed morphine's ability to lower IOP (Leopold and Comroe, 1948; Fanciullacci et al., 1980; Drago et al., 1985; Dortch-Carnes and Russell, 2006; Bonfiglio et al., 2006). The ocular effects of morphine (particularly the miosis), are generally thought to occur primarily through centrally-mediated signaling mechanisms (Lee and Wang, 1975; Murray, et al., 1983). To date, however, a direct intraocular component has not been ruled out.

Aqueous humor is produced by the ciliary epithelial cells located in the ciliary body (Krupin et al., 1986). IOP is determined primarily by the dynamic equilibrium between the production of aqueous humor in the ciliary body and its efflux mainly through the trabecular meshwork and Schlemm's canal (Wiederholt et al., 1991). In addition to the pressure-dependent trabecular meshwork and Schlemm's canal pathways, the pressure-independent uveoscleral pathway provides an additional outflow mechanism that may play a role in morphine-induced reduction of IOP.

Since recent studies in our laboratory have generated evidence of a role of NO in morphine-mediated reduction of IOP and PD (Dortch-Carnes and Russell, 2006), the present study is designed to determine the effect of morphine on NO formation in the ICB, because the ciliary body may be a site that contributes to the NO production generated following acute morphine treatment. With the use of an inhibitor of NO synthesis, and a sulfhydryl compound (GSH) that inactivates opioid (Gionnini et al., 1989; Gioannini et al., 1990; Liu and Quirion, 1992) and other membrane bound receptors containing disulfide bonds (Gilbert, 1982), additional evidence that morphine-mediated changes in NO production in the ICB is linked to activation of opioid receptors capable of stimulating NO release has also been established.

2. Materials and Methods

2.1. Preparation of Iris-Ciliary Bodies and Drug Treatments

In this study, the iris-ciliary body (ICB) was used for two reasons: (1) previously, morphine was shown to reduce the intraocular pressure (IOP) and pupil diameter of New Zealand white (NZW) rabbits in part by NO related signaling; (2) morphine increased the levels of NO in aqueous humor samples from NZW rabbits. This study was done to determine if the ICB (site of aqueous humor production) plays a role in morphine-stimulated NO production.

ICBs were dissected from the eyes of New Zealand white rabbits and cut into 2 equal pieces. Segments were placed in individual microfuge tubes and equilibrated in a humidified atmosphere (95% O₂, 5% CO₂) at 37 °C for 30 minutes in 200 µl of Earl's Balanced Salt Solution (EBSS) supplemented with L-arginine, bovine serum albumin (BSA) and a protease inhibitor cocktail. L-arginine was included in the media of control and treated tissues to stimulate NO production *in vitro*. Following equilibration, some tissue segments were treated with morphine (1, 10, or 100 µM) for 1 hour. Others were pretreated with either naloxone (non-selective opioid receptor antagonist), L-NAME (non-selective NO synthase inhibitor) or GSH (non-selective inactivator of membrane bound receptors) for 30 minutes followed by treatment with morphine (10 µM) for 1 hour. Following all treatment regimens, tissue samples were removed from the tubes, snap frozen in liquid nitrogen and later analyzed for protein using the Bio-Rad protein assay.

2.2. Nitric Oxide Determination

NO is a very unstable molecule in solution with a half-life of only a few seconds. Therefore, in these studies NO was measured as its stable metabolites, nitrate and nitrite. In the cell, NO undergoes a series of reactions with several molecules present in biological fluids and is eventually metabolized to nitrite (NO₂⁻) and nitrate (NO₃⁻). The incubation medium

surrounding the tissue samples was assayed for NO (nitrates + nitrites) levels using a microplate assay from Active Motif, Carlsbad, California. The principle of the NO quantitation kit is that nitrate in the sample is converted to nitrite in the presence of nitrate reductase and cofactors. Then, nitrate and nitrite levels are assayed using Griess Reagent.

2.3. Statistics

Data were analyzed for differences using one-way analysis of variance followed by the Holm-Sidak method for multiple comparisons. Results are expressed as mean values $\pm$ SEM and were considered significant when $P < 0.05$.

3. Results

Morphine (1, 10 and 100 μ M), caused a concentration-dependent increase in the release of NO from ICBs (figure 1). The levels of NO detected in the incubation medium surrounding ICB samples increased from 1.49 ± 0.19 (control) to 8.81 ± 2.20 μ M/mg protein (100 μ M, morphine-treated). Morphine-stimulated (10 μ M), NO release was inhibited when ICB samples were pretreated for 30 minutes with the non-selective opioid receptor antagonist, naloxone (10 μ M), indicating that the response is opioid receptor-mediated (figure 2). When administered alone, naloxone did not cause any significant change in the levels of NO detected in the incubation medium as compared to control.

To provide initial evidence that morphine-induced release of NO from the ICB is associated with activation of receptors linked to NO release, experiments using the nonselective NOS inhibitor, L-NAME, were performed. When administered alone, L-NAME did not cause a significant change in NO release from ICB samples as compared to control samples (figure 3). Pretreatment with the NOS inhibitor, however, resulted in almost complete inhibition of morphine-induced NO release (figure 3).

The sensitivity of the morphine response to reduced GSH was determined because mu opioid receptors are thought to be more sensitive to inactivation by reduced glutathione than other opioid receptors (Makman et al., 1996). Experiments were conducted examining the effect of this sulfhydryl compound on morphine-stimulated NO production in the ICB preparation (figure 4). In the presence of GSH, disulfide bonds present in opioid receptors are broken which reduces receptor activity. As with the opioid receptor antagonist (naloxone) and the NOS inhibitor L-NAME, GSH (10 μ M) did not produce a significant change in the levels of NO detected in the incubation medium when it was administered alone. Pretreatment with the agent, however, completely inhibited morphine-induced NO release.

4. Discussion

As part of the future directions of previous studies in our laboratory (Dortch-Carnes and Russell, 2006) and others (Bonfiglio et al., 2006), that have demonstrated a role of NO in the ocular hypotensive and miotic effects of morphine, as well as the ability of morphine to stimulate the release of NO into the aqueous humor of NZW rabbits (Dortch-Carnes and Russell, 2007), the present study was designed to determine if the iris-ciliary body (ICB) could be a site of morphine-stimulated NO production.

The synthesis of NO is widely distributed in the eye (Geyer et al., 1997). NO is synthesized by three NO synthases (endothelial, neuronal and inducible NOS), via conversion of the amino acid L-arginine to L-citrulline (Moncada et al., 1989; Moncada and Higgs, 1993; Ignarro et al., 1999; Alderton et al., 2001). NO diffuses out of the cell where it is formed, and diffuses into target cells where it binds to the heme group of soluble guanylate cyclase (sGC) and induces enzyme activation, resulting in increased formation of cGMP (Ignarro, 1990). The L-

arginine/NO/cGMP pathway is a widespread transduction mechanism underlying a number of different functions which include mu-3 receptor mediated regulation of immune (Welters et al., 2000) and cardiovascular responses (Stefano et al., 2002).

Morphine is known to mediate its effects through activation of mu opioid receptors (Matthes et al., 1996). Of the three currently recognized mu opioid receptor subtypes (mu-1, mu-2 and mu-3), the mu-3 subtype is the only one which is known to be opiate alkaloid selective and coupled to NO synthase derived NO release (Stefano, 1999). In addition, the mu-3 receptor appears to be much more sensitive to inactivation by reduced glutathione (GSH) than the other mu, delta or kappa opioid receptors (Makman, et al., 1996).

In the present study, morphine caused a concentration-dependent increase in the release of NO from isolated ICBs. The bilayered epithelium of the ciliary body is known to be the site of aqueous humor production. Aqueous humor is secreted into the posterior chamber of the eye by the ciliary epithelium of the ciliary body. To date, cAMP has been considered to be the most important regulator of aqueous humor formation, but cGMP has been found to be an inhibitory modulator of aqueous humor production (Korenfeld and Becker, 1989; Becker, 1990; Millar et al., 1997). The flow of aqueous humor has been shown to be regulated by cGMP (Wiederholt et al., 1994). Activation of anterior segment guanylate cyclase elevates cGMP levels, leading to decreased aqueous humor production and thus to reduced IOP (Nathanson, 1987; Korenfeld and Becker, 1989).

Results from the current study have also shown that the morphine-stimulated increase in NO release from the isolated ICB is a naloxone sensitive response, indicating that this effect is opioid receptor mediated. Having established the opioid receptor activity of morphine, experiments examining the effect of L-NAME (nonselective NO-synthase inhibitor) or GSH (non-selective membrane bound receptor inactivator) on morphine-stimulated NO release were performed. Pretreatment of tissue samples with either of the agents almost completely inhibited (L-NAME) or completely abolished (GSH), morphine-induced NO release, thereby suggesting that the response is mediated by activation of a NO-releasing receptor that is inactivated in the presence of GSH. Because morphine can interact with mu, delta and kappa opioid receptors at the concentration used in this study, morphine-stimulated NO release from the ICB cannot be attributed to a specific opioid receptor subtype.

This is one of very few reports documenting a link between opioid receptors and the NO signaling cascade in the eye. The proposed signaling cascade (figure 5) that we surmise to be operating at the level of the ciliary body starts with activation of opioid receptors, followed by increased NO production and release. Once released, NO stimulates an increase in the activity of guanylate cyclase resulting in the formation of cGMP, which ultimately lowers IOP by reducing the formation of aqueous humor.

Aqueous humor produced in the ciliary body leaves the eye mainly via the trabecular meshwork and Schlemm's canal. IOP is maintained as a result of a balance between the secretion of aqueous humor by the ciliary processes and its outflow through the trabecular and uveoscleral outflow pathways. Because NO readily diffuses out of, and into cells, an alternate mechanism of morphine-induced reduction of IOP may involve activation of the cGMP pathway in the trabecular meshwork by NO, released in response to morphine, from the ciliary body and possibly other sites including the trabecular meshwork cells themselves. The trabecular meshwork is thought to be a smooth muscle-like tissue with contractile properties (Barany, 1962; Lepple-Wienhues et al., 1991). It has been shown that smooth muscle relaxing substances, including NO, decrease trabecular tone (Wiederholt et al., 1994; Wiederholt et al., 1995; Wiederholt et al., 1997; Brubaker, 2003; Llobet et al., 2003), leading to an increase in intertrabecular spaces through which outflow can occur. The NO-induced relaxation of the

trabecular meshwork that leads to increased aqueous humor outflow and decreased IOP is mediated by the second messenger, cGMP (figure 5).

Another possible scenario involves the outflow of aqueous humor through the uveoscleral outflow pathway (figure 5). The ciliary muscle plays a significant role in the regulation of aqueous humor dynamics. Contraction of the muscle facilitates aqueous humor outflow via the trabecular pathway by loosening the trabecular meshwork, while the uveoscleral outflow is inhibited, presumably by narrowing of the spaces between the muscle fiber bundles. In contrast, ciliary muscle relaxation facilitates uveoscleral outflow and inhibits outflow through the trabecular meshwork. There is evidence that NO regulates IOP by inducing relaxation of ciliary muscle and trabecular meshwork (Wiederholt et al., 1994; Ding and Abdel-Latif, 1997), thereby decreasing the resistance of the uveoscleral outflow pathway which results in increased aqueous humor outflow via this route and reduced IOP. With this information in mind, it is reasonable to propose that NO produced in response to morphine can reduce IOP by increasing uveoscleral outflow.

Unpublished data from our laboratory indicate the presence of kappa opioid receptors in the ICB of rabbits. However, as yet, there is no direct evidence of opioid receptors in the trabecular meshwork. Functional data generated in our laboratory (Russell et al., 2000; Moore and Potter, 2001; Russell and Potter, 2002) and others (Leopold and Comroe, 1946; Drago et al., 1985) do indicate that all three subtypes may be present in those tissues, thereby, suggesting that morphine could activate the receptors to release NO. Since morphine-stimulated NO release was reduced by a NOS inhibitor and a sulfhydryl compound, it is possible that the response is mediated by the mu-3 receptor, which has consistently been shown to be a NO-releasing receptor (Nieto-Fernandez et al., 1999; Welters et al., 2000; Rialas et al., 2000; Yahyavi-Firouz-Abadi et al., 2007).

In conclusion, morphine-induced NO release from isolated, iris-ciliary bodies is a naloxone, L-NAME and GSH sensitive response mediated by activation of opioid receptors that stimulate NO release. Results from this study have confirmed the ICB as a site of morphine-stimulated NO production in the eye. In addition, these results support previous *in vivo* studies from our laboratory (Dortch-Carnes and Russell, 2006) and others (Bonfiglio et al., 2006) that demonstrated a role of NO in the ocular hypotensive and miotic effect of morphine. Whether the IOP lowering effect of morphine is generated by reducing aqueous humor production, augmenting its egress or a combination of the two mechanisms, remains to be determined. Since the ciliary body preparation used in this study contained a small portion of the iris, due to the fact that they are fused in the rabbit, no conclusions regarding whether NO was produced by the iris or within the ciliary body can be drawn. Future studies are, however, aimed at using isolated ciliary epithelial and trabecular meshwork cells to further elucidate the mechanism of morphine-induced NO production in the anterior segment of the eye.

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Fig. 1.

Concentration response of morphine on NO release from isolated, iris-ciliary bodies. ICB samples were treated with the indicated concentrations of morphine for 1 h. At the end of the 1-h incubation period, the tissue samples were snap frozen and later analyzed for protein. NO in the incubation medium was analyzed as [nitrates + nitrites] using a microplate assay kit. Data are mean $\pm$ SEM of 8 ICB segments from 4 rabbits. * $P < 0.05$ compared to control.

Fig. 2.

Effect of Naloxone on morphine-induced NO release from isolated, iris-ciliary bodies. ICB segments were pretreated with naloxone (10 μM) for 30 min, followed by treatment with morphine (10 μM) for 1 h. At the end of drug treatments, the tissue samples were snap frozen and later analyzed for protein. NO in the incubation medium was measured as [nitrates + nitrites] using a microplate assay kit. Data are mean ± SEM of 8 ICB pieces from 4 rabbits.

* $P < 0.05$, compared to control; + $P < 0.05$, compared to morphine.

Fig. 3.

Effect of L-NAME on morphine-induced NO release from isolated, iris-ciliary bodies. ICB pieces were pretreated with L-NAME (10 μM) for 30 min, and subsequently incubated with morphine (10 μM) for 1 h. At the end of drug treatments, the ICB samples were snap frozen and later analyzed for protein. NO in the incubation medium was measured as [nitrates + nitrites] with a microplate assay kit. Data are mean $\pm$ SEM of 8 ICB pieces from 4 rabbits.

* $P < 0.05$, compared to control; + $P < 0.05$, compared to morphine.

Fig. 4.

GSH sensitivity of morphine-stimulated NO release from isolated, iris-ciliary bodies. ICB samples were pretreated with GSH (10 μ M) for 30 min, and subsequently incubated with morphine (10 μ M) for 1 h. At the end of drug treatments, the ICB samples were snap frozen and later analyzed for protein. NO in the incubation medium was measured as [nitrates + nitrites] with a microplate assay kit. Data are mean $\pm$ SEM of 8 ICB pieces from 4 rabbits.

* $P < 0.05$, compared to control; + $P < 0.05$, compared to morphine.

Fig. 5.

Schematic of proposed NO related signaling events associated with morphine-induced reduction of IOP. The proposed signaling cascade begins with activation of opioid receptors (μ -3), and subsequent rise in NO production and release. NO stimulates an increase in the activity of sGC, which increases the formation of the second messenger, cGMP. cGMP either increases the outflow of aqueous humor via the trabecular meshwork or uveoscleral routes or decreases the production of aqueous in the ciliary body. The end result of either increased outflow, decreased formation, or a combination of the two processes is decreased IOP.