Modulation of Guanylate Cyclase by Lipoxygenase Inhibitors

DANIEL L. CLARK AND JOEL LINDEN

SUMMARY Drugs that inhibit endothelium-dependent relaxation were tested to determine their effect on soluble guanylate cyclase purified from dog aorta. Basal, arachidonic acid (10^-5 M)-stimulated, and nitroprusside (5 x 10^-5 M)-stimulated guanylate cyclase activities were inhibited by methylene blue and the lipoxygenase inhibitors nordihydroguaiaretic acid and eicosatetraynoic acid. The effective inhibitory doses were in the range of those that have been reported to inhibit endothelium-dependent relaxation. Other compounds known to inhibit endothelium-dependent relaxation had little or no effect on guanylate cyclase activity. Basal guanylate cyclase activity was more resistant to inhibition than were activated states of the enzyme. The data suggest that reported inhibition of endothelium-dependent relaxation by some lipoxygenase inhibitors may be the result, at least in part, of their direct effect on guanylate cyclase activity. (Hypertension 8: 947-950, 1986)

KEY WORDS • guanylate cyclase • endothelium-dependent relaxation • nordihydroguaiaretic acid • eicosatetraynoic acid • methylene blue

AC TIVATION of guanylate cyclase and cyclic guanosine 3',5'-monophosphate (GMP) accumulation has an apparent, but still unproven, direct role in mediating smooth muscle relaxation. Nitrovasodilator drugs (e.g., nitroprusside and glyceryl trinitrate) are thought to form a nitric oxide radical that directly activates guanylate cyclase to produce relaxation.1

Another class of vasodilator agents that requires the presence of vascular endothelium to produce relaxation was described in 1980 by Furchgott and Zawadzki.2 These agents, which include acetylcholine, A23187, bradykinin, histamine, and adenosine 5'-triphosphate (ATP), cause the release of a substance (or substances), termed endothelium-derived relaxing factor (EDRF), from endothelial cells that results in vascular relaxation and cyclic GMP accumulation. Nordihydroguaiaretic acid (NDGA), an antioxidant and lipoxygenase inhibitor and eicosatetraynoic acid (ETYA, a lipoxygenase and cyclooxygenase inhibitor) inhibit endothelium-dependent relaxation. Since selective cyclooxygenase inhibitors do not prevent these responses, it has been suggested that EDRF might be a metabolite of arachidonic acid (AA) by way of the lipoxygenase pathway.2,3 Several antioxidants, phospholipase inhibitors, and cytochrome P-450 inhibitors have also been reported to inhibit endothelium-dependent relaxation.4 An alternative hypothesis is that EDRF is a metabolite of AA derived from the cytochrome P-450 pathway.5

Since cyclic GMP accumulation in smooth muscle appears to be a consequence of EDRF release,6,7 it was of interest to us to examine the effect of purported inhibitors of endothelium-dependent relaxation on soluble guanylate cyclase, the enzyme that synthesizes cyclic GMP.

Materials and Methods

Sodium nitroprusside, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), hydroquinone, dithiothreitol, butylated hydroxytoluene, NDGA, and 3-isobutyl-1-methylxanthine were purchased from Sigma Chemical Company (St. Louis, MO, USA). The AA (5,8,11,14-eicosatetraenoic acid) was obtained from Nu Chek Prep (Elyssian, MN, USA), while 5,8,11,14-eicosatetraenoic acid was a gift from Hoffman-LaRoche Labs (Nutley, NJ, USA). The SKF525A and BW 755C were gifts of Smith Kline & French (Philadelphia, PA, USA) and Burroughs Wellcome (Kent,
England), respectively. All other reagents and chemicals were purchased from either Sigma or Fisher Scientific (Dallas, TX, USA).

Partially purified soluble guanylate cyclase was prepared by the method of Kimura et al. Aortas obtained from mongrel dogs of either sex were finely minced, suspended in buffer composed of 50 mM tris(hydroxymethyl)aminomethane (Tris), 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, pH 7.4, and disrupted with a Polytron (Brinkman Instruments, Westbury, NY, USA). The mixture was filtered through gauze and centrifuged at 20,000 g for 10 minutes. The resulting supernatant was centrifuged at 100,000 g for 1 hour. Guanylate cyclase in the 100,000 g supernatant was precipitated by titration to pH 5 with 1 M HCl, and the pellet was centrifuged at 12,000 g for 20 minutes. The pellet was dissolved in 50 mM potassium phosphate (pH 7.8) using 10 strokes in a glass homogenizer fitted with a Teflon pestle. This mixture was centrifuged at 12,000 g for 20 minutes. Aliquots of the final supernatant were stored at −70°C.

Protein was assayed by a modification of the method of Stowell et al. Aliquots of soluble guanylate cyclase or bovine serum albumin standards were dissolved in 0.75 ml of 0.2 N NaOH containing 0.1% (wt/vol) sodium dodecyl sulfate. To these solutions, 0.75 ml of 0.4 N boric acid was added, followed by 1 ml of fluorescamine (1 mg/ml in acetone). Fluorescence was measured at an excitation wavelength of 390 nm and an emission wavelength of 475 nm.

Soluble guanylate cyclase was assayed by the method of Kimura and Murad. The assay buffer, 50 mM potassium phosphate (pH 7.8), contained 10 mM Mg2+, 5 mM guanosine 5'-triphosphate (GTP), and 0.7 mM 3-isobutyl-1-methylxanthine. Drugs were prepared as concentrated stock solutions and added to the incubation mixture. The sodium salt of AA was prepared by dissolving the free acid in 20 mM NaOH. Stock solutions of NDGA and ETYA were prepared in dimethyl sulfoxide. To these solutions, 0.75 ml of 0.2 N NaOH containing 0.1% (wt/vol) sodium dodecyl sulfate was added, followed by 1 ml of fluorescamine (1 mg/ml in acetone). Fluorescence was measured at an excitation wavelength of 390 nm and an emission wavelength of 475 nm. The sodium salt of AA was prepared by dissolving the free acid in 20 mM NaOH. Stock solutions of NDGA and ETYA were prepared in ethanol. The concentration of ethanol in the final reaction mixture (<1% vol/vol) had no effect on guanylate cyclase activity. All other compounds were dissolved in either buffer or water. Guanylate cyclase assays were conducted for 10 minutes at 37°C. The reaction was terminated by the addition of 0.25 ml of ice-cold 0.1 M ZnSO4 and 0.25 ml of ice-cold 0.1 M Na2CO3. After centrifugation, aliquots of the supernatant were assayed for cyclic GMP by automated radioimmunoassay. Enzyme activity is expressed as picomoles of cyclic GMP formed per minute per milligram of protein.

Results

Accumulation of cyclic GMP was linear with time for up to 20 minutes (data not shown). The sensitivity of guanylate cyclase to inhibitors was assessed by assaying the enzyme under basal conditions or during stimulation by either AA or nitroprusside. To compare inhibition during different states of guanylate cyclase activation, the data have been presented as the percentage of maximal activity for each method of activation (basal, AA, nitroprusside) to normalize for both the variation in activity from preparation to preparation of the enzyme and the variation in enzyme activity resulting from the different methods of stimulation. The AA (10−5 M) stimulated guanylate cyclase approximately 300% in our assay system. At concentrations of 10−4 M or higher, AA inhibited guanylate cyclase activity. Nitroprusside (5 × 10−5 M) activation of the enzyme (800–3000%) varied from preparation to preparation.

The NDGA effectively inhibited soluble guanylate cyclase activity (Figure 1). The NDGA concentrations producing a 50% decrease in basal, AA-stimulated, and nitroprusside-stimulated activities (EC50) are given in Table 1. The NDGA has been reported to inhibit endothelium-dependent relaxation at concentrations ranging from 2.5 to 10 × 10−5 M.12

At concentrations of 3 to 10 × 10−5 M, ETYA produced a 300 to 400% activation of guanylate cyclase activity (Figure 2). Above 10−4 M, ETYA inhibited guanylate cyclase under all conditions of activation studied. Thus, like AA, ETYA had a biphasic effect on guanylate cyclase activity. The ETYA was a slightly less potent inhibitor of guanylate cyclase than NDGA (see Table 1). Eicosatetraynoic acid has been reported to inhibit endothelium-dependent relaxation at a concentration of 10−4 M.12

In contrast to NDGA and ETYA, the selective lipoxygenase inhibitor BW 755C did not markedly inhibit guanylate cyclase activity (see Table 1). Methylene blue, a known inhibitor of guanylate cyclase, was a less potent inhibitor of guanylate cyclase than either NDGA or ETYA (Figure 3; see Table 1). Methylene blue reportedly inhibits endothelium-dependent relaxation at concentrations ranging from 2.5 to 10 × 10−5 M.12

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Effect of nordihydroguaiaretic acid (NDGA) on dog aorta soluble guanylate cyclase activity. The enzyme, prepared as described in Methods, was incubated for 10 minutes at 37°C in 50 mM potassium phosphate buffer containing 10 mM Mg2+, 5 mM GTP, 0.7 mM 3-isobutyl-1-methylxanthine, and additional drugs as indicated. Basal = no drugs; AA = arachidonic acid; NP = nitroprusside. Data represent the mean ± SEM of four to six experiments. Basal enzyme activity was 31.5 ± 5.0 pmol of cyclic GMP formed per mg protein/min.
LIPOXYGENASE INHIBITORS AND GUANYLATE CYCLASE/Clark and Linden

TABLE 1. Effective Concentration (10^{-3} M) for 50% Inhibition of Soluble Guanylate Cyclase Activity

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Basal</th>
<th>Nitroprusside-stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nordihydroguaiaretic acid</td>
<td>≥5</td>
<td>0.126</td>
<td>0.036</td>
</tr>
<tr>
<td>Eicosatetraynoic acid</td>
<td>5</td>
<td>0.219</td>
<td>0.102</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>≥2</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>BW 755C</td>
<td>3</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>≥2</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>SKF 525A</td>
<td>1</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>2</td>
<td>*</td>
<td>0.19</td>
</tr>
<tr>
<td>Phenyl r-butyl nitrene</td>
<td>2</td>
<td>*</td>
<td>54</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>1</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>2</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Assays were performed as described for Figure 1. n = number of experiments performed; EC_{50} = effective concentration, 50%.
*EC_{50} > 1 x 10^{-3} M.
†EC_{50} > 1 x 10^{-1} M.
‡EC_{50} > 3 x 10^{-3} M.

Discussion

While the precise role of guanylate cyclase and cyclic GMP in smooth muscle relaxation has not been definitively established, several studies, including some studies of endothelium-dependent relaxation, have shown a close correlation between cyclic GMP levels and smooth muscle relaxation. One recent report suggests that EDRF can directly activate guanylate cyclase.

The biochemical pathway (or pathways) from which EDRF arises and its chemical nature have been inferred from the known chemical and biochemical activities of the various drugs that have been shown to inhibit endothelium-dependent relaxation. Thus, EDRF has been postulated to be a short-lived, possibly free radical, metabolite of AA derived from either the lipoxygenase pathway or the cytochrome P-450 pathway. Although AA is thought to be released from the cell membrane through activation of a phospholipase, individual lipoxygenase inhibitors and phospholipase inhibitors, which would be expected to modulate AA metabolism, do not inhibit endothelium-dependent relaxation in all blood vessels studied, nor do they inhibit relaxation elicited by all endothelium-dependent agents.

Other investigators have shown that high concentrations of AA do not relax smooth muscle or increase cyclic GMP after removal of the endothelium. In addition, treatment with melittin or phospholipase to release AA from membranes does not relax arteries without endothelium. A possible explanation for this is our observation that high concentrations of AA inhibit guanylate cyclase. In our assay system, 10^{-5} M AA was a maximally stimulatory concentration. At 10^{-4} M AA, guanylate cyclase was virtually completely inhibited.

Interestingly, ETYA, like AA, had a biphasic effect on guanylate cyclase activity. Low concentrations activated the enzyme, while high concentrations were inhibitory. Higher concentrations of ETYA than of AA were required to obtain the observed stimulatory and inhibitory effects on guanylate cyclase activity. The similarity of the chemical structures of AA and ETYA.

Figure 2. Effect of eicosatetraynoic acid (ETYA) on dog aorta guanylate cyclase activity. The enzyme was assayed as described for Figure 1. Data represent the mean ± SEM of five experiments. Basal enzyme activity was 42.7 ± 4.8 pmol of cyclic GMP formed per mg protein/min.

Figure 3. Effect of methylene blue on dog aorta soluble guanylate cyclase activity. The enzyme was assayed as described for Figure 1. Data represent the mean ± SEM of three experiments. Basal enzyme activity was 38.3 ± 8.4 pmol of cyclic GMP formed per mg protein/min.
may explain their similar effects on guanylate cyclase activity.

Spies et al.20 have previously shown that ETYA and NDGA can inhibit cyclic GMP accumulation in rat ductus deferens induced by Ca²⁺, acetylcholine, nor-epinephrine, or nitroprusside. The ETYA had no effect on the induced contractile response, while NDGA inhibited contraction of the ductus deferens. They suggested that a lipoxygenase metabolite of AA may be involved in the regulation of cyclic GMP formation.

The present study showed that NDGA and ETYA can directly inhibit soluble guanylate cyclase of dog aorta at drug concentrations higher than those that inhibit lipoxygenase (EC₅₀ ≈ 1.6 × 10⁻³ M),21,22 but similar to those that inhibit endothelium-dependent relaxation.12 Inhibition of guanylate cyclase by NDGA and ETYA may indicate that possible lipoxygenase contamination of the partially purified enzyme preparation may produce metabolites that activate guanylate cyclase. However, the lack of effect of BW 755C, a specific lipoxygenase inhibitor, on guanylate cyclase activity suggests that NDGA and ETYA may directly inhibit guanylate cyclase.

In general, AA-stimulated and nitroprusside-stimulated guanylate cyclase activities are more sensitive to inhibition by several drugs than is the basal enzyme activity. Thus, while the doses of drugs used in many reports to inhibit the endothelium-dependent relaxation caused by EDRF may not greatly affect basal guanylate cyclase activity, they may markedly inhibit EDRF-induced activation of guanylate cyclase and thereby prevent relaxation. From the current data, inferring a role for lipoxygenase in the synthesis of EDRF based on the ability of NDGA and ETYA to inhibit endothelium-dependent relaxation may not be appropriate. In aorta ring preparations, methylene blue effectively inhibits endothelium-dependent relaxation in the same dose range13 that inhibited AA-stimulated and nitroprusside-stimulated guanylate cyclase activity in our system, 1 to 5 × 10⁻⁵ M. The definitive determination of the biosynthetic pathway for EDRF, as well as its chemical nature, will require careful investigation of the mechanism by which drugs, such as NDGA and ETYA, inhibit endothelial-dependent relaxation before the involvement of specific pathways can be pinpointed.

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References

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