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Interaction of inducible nitric oxide synthase and cyclooxygenase-2 inhibitors in formalin-induced nociception in mice

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Abstract

Studies with inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 inhibitor were conducted to assess their synergistic antinociceptive effect and possible therapeutic advantage. The antinociceptive interaction of rofecoxib, a selective cyclooxygenase-2 inhibitor, with aminoguanidine hydrochloride, a selective iNOS inhibitor, was examined in the formalin-induced paw-licking model in mice. Analysis of variance (ANOVA) and the isobolographic method were used to identify the nature of the antinociceptive interaction. Different doses of rofecoxib (1, 3, 10 and 30 mg/kg) and aminoguanidine hydrochloride (10, 30, 100 and 300 mg/kg) alone were administered orally to adult male albino mice (20–30 g). Only high doses of rofecoxib (10 and 30 mg/kg) and aminoguanidine hydrochloride (100 and 300 mg/kg) showed a statistically significant antinociceptive effect. Combination of a subthreshold dose of rofecoxib (1 mg/kg) with increasing doses of aminoguanidine hydrochloride (30, 100 and 300 mg/kg) resulted in potentiated antinociception ($P<0.05$). Combined therapy with a subthreshold dose of aminoguanidine hydrochloride (30 mg/kg) with increasing doses of rofecoxib (1, 3, 10 and 30 mg/kg) also resulted in significant antinociception ($P<0.05$). These results suggest that rofecoxib and aminoguanidine hydrochloride act synergistically in their antinociceptive action in mice. A possible mechanism of interaction is that nitric oxide (NO) stimulates the activity of cyclooxygenase-2 by combining with its heme component. Furthermore, the present results suggest that combination therapy with rofecoxib and aminoguanidine hydrochloride may provide an alternative for the clinical control of pain.
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1. Introduction

Two isoforms of cyclooxygenase, the key enzyme in prostaglandin and thromboxane biosynthesis, referred to as cyclooxygenase-1 and cyclooxygenase-2, have been identified. Cyclooxygenase-1 is expressed constitutively and high levels can be detected in most tissues (O'Neill and Ford-Hutchinson, 1993). In contrast, levels of cyclooxygenase-2 mRNA and protein are usually low or undetectable under basal conditions but are rapidly elevated during inflammation or mitogenic stimulation (Raz et al., 1989; Kujubu et al., 1991). The cyclooxygenase isoforms are the primary target enzymes for nonsteroidal anti-inflammatory drugs (NSAIDs). It was postulated that inhibition of cyclooxygenase-2 mediates the anti-inflammatory and chemopreventive effects of NSAIDs without having a relevant influence on homeostatic reactions (Vane and Botting, 1995).

Nitric oxide (NO) is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS, Palmer et al., 1988). To date, three isoforms of NOS have been identified, viz neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Ogden and Moore, 1995). Both eNOS and nNOS are activated by calcium and are constitutive isoforms, while iNOS is Ca^{2+}-independent and is synthesized de novo following exposure of a wide range of cell types to bacterial endotoxins and/or inflammatory cytokines. In recent years, considerable evidence has accumulated suggesting a role for NO as a mediator of inflammation (Nussler and Billiar, 1993; Lyons, 1995). NO dilates microvascular blood vessels and promotes microvascular perme-

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ability, resulting in edema formation (Hughes et al., 1990). In addition, NO increases the synthesis/release of pro-inflammatory mediators such as cytokines and reactive oxygen species (Marcinkiewicz et al., 1995) and prostanoids (Sautebin et al., 1995).

L-arginine and NO donors such as sodium nitroprusside, nitroglycerine and 3-morpholinosydnonimine (SIN-1) have been demonstrated to produce antinociception in rats with carrageenan-induced hyperalgesia (Kawabata et al., 1992). In contrast, L-NAME suppresses, while L-arginine and NO donors enhance, nociception or inflammatory responses elicited by bradykinin, substance-P, carrageenan and dextran (Hughes et al., 1990). Thus, there exists evidence for both a nociceptive and an antinociceptive role of NO in peripheral tissue.

When we consider the NOS and cyclooxygenase pathway simultaneously, it seems that a complex relationship is emerging between both of them. Cyclooxygenase-2 is the inducible form of the enzyme, the synthesis of which is triggered by those cytokines that also induce iNOS (Clancy and Abramson, 1995). The two pathways interact closely and NO can stimulate cyclooxygenase activity (Salvemini et al., 1995). Dual inhibition of cyclooxygenase-2 and iNOS can be produced by glucocorticoids (Newton et al., 1998; Korhonen et al., 2002) but, unfortunately, serious side effects of glucocorticoids limit their clinical use. Perusal of the literature indicated that studies of the interaction of iNOS and cyclooxygenase-2 inhibitors have not been reported. Therefore, it is worthwhile to study the interaction between the iNOS inhibitor, aminoguanidine, with the cyclooxygenase -2 inhibitor, rofecoxib, to know whether combined administration of iNOS and cyclooxygenase-2 inhibitors in different doses offers any therapeutic advantage over the inhibition of either prostaglandin or NO alone. The nature of the positive interaction (i.e. additive or supra-additive) between iNOS inhibitor and cyclooxygenase-2 inhibitor with regard to enhancement of antinociception has not been studied rigorously, thus we are uncertain whether this interaction is truly synergistic or additive. The experiments described here were undertaken to examine the antinociceptive interaction of rofecoxib with aminoguanidine hydrochloride and to analyze the nature of this interaction by isobolographic analysis. Formalin-induced nociception was used because it allows evaluation of the antinociceptive effects of NSAIDs and NOS inhibitors (Morgan et al., 1992). Further, this test does not generate conditional learning and has high prediction of antinociception. The use of formalin as a noxious stimulus in tonic pain research has been strongly supported by the different time course of nociceptive behavior (Wheeler-Aceto et al., 1990).

2. Materials and methods

2.1. Experimental animals

Adult male albino mice (20–30 g) were divided into 20 groups of 6 each. Animals were housed in a temperature-controlled room with a standard light–dark cycle. Food and water were available continuously.

2.2. Drugs and their administration

Rofecoxib, a highly selective cyclooxygenase-2 inhibitor donated by Ranbaxy Laboratories, India, and aminoguanidine hydrochloride, a selective iNOS inhibitor procured from Sigma, USA were used in the study. Both the drugs were administered orally in the form of an aqueous suspension in 1% Tween-80.

2.3. Test of antinociception

The effect of drugs was observed on the pain produced in the hind paw of male albino mice by 20 μl of 2.5% formalin solution (in glass-distilled water) according to the method of Corea and Calixto (1993). One hour before the induction of pain, rofecoxib or aminoguanidine hydrochloride was administered orally at 1, 3, 10, 30 mg/kg or 10, 30, 100 and 300 mg/kg doses, respectively, to mice. The effect of the drugs at different doses was compared with that of vehicle (control). Interaction studies were conducted by co-administering rofecoxib (1 mg/kg) with different doses of aminoguanidine hydrochloride (10, 30, 100 and 300 mg/kg). In a similar way, aminoguanidine hydrochloride (30 mg/kg) was co-administered orally with different doses of rofecoxib (1, 3, 10 and 30 mg/kg). Mice were placed in a glass bell jar to record the time spent licking the injected paw in the late phase (15–30 min) after formalin injection. The results obtained from interaction studies were compared with those for individual drug-treated groups and vehicle-treated controls.

2.4. Data analysis

The data obtained were converted to % maximum possible effect (MPE) by Equation

$$\%\mathrm{MPE} = 100 - \left(\frac{100 \times \text{Time spent licking the formalin} - \text{injected paw by drug} - \text{treated mice}}{\text{Time spent licking the formalin} - \text{injected paw by vehicle} - \text{treated control mice}}\right)$$

The log dose was plotted vs. % MPE, and regression analysis of log dose–response curve was used to calculate ED_{50} and its 95% CL. The interaction between rofecoxib and aminoguanidine hydrochloride was examined by

analysis of variance (ANOVA), and the nature of interaction was further confirmed by isobolographic analysis (Gessner, 1988; Gennings et al., 1990; Nelson and Kursar, 1999).

In the isobolographic analysis, the ED_{50} of rofecoxib was plotted on the ordinate and the ED_{50} of aminoguanidine hydrochloride on the abscissa. A theoretical line of additive interaction was drawn by connecting the ED_{50} for rofecoxib with that for aminoguanidine hydrochloride. The ED_{50} of the combination (rofecoxib + aminoguanidine hydrochloride) (Point 'A' in Fig. 3) was then plotted on the isobolograph. If the confidence intervals surrounding the combination data point (Point 'A' in Fig. 3) did not overlap with the confidence interval surrounding the theoretical line for the additive interaction, then statistically significant synergism or antagonism may be concluded when combination data points come below or above the theoretical line of the additive interaction, respectively. The intensity of the synergistic or antagonistic interaction was represented by the potency ratio (Gessner, 1988; Nelson and Kursar, 1999). The potency ratio is the distance from the origin to the observed ED_{50} for the combination (Point 'A' in Fig. 3) divided by the distance from the origin to the expected ED_{50} coordinates of the theoretical line for the additive interaction (Point 'B' in Fig. 3). The ratio of observed to expected distances represents the potency ratio. A potency ratio of 1 represents additivity. A potency ratio less than 1 reflects synergism and a ratio more than 1 reflects antagonism.

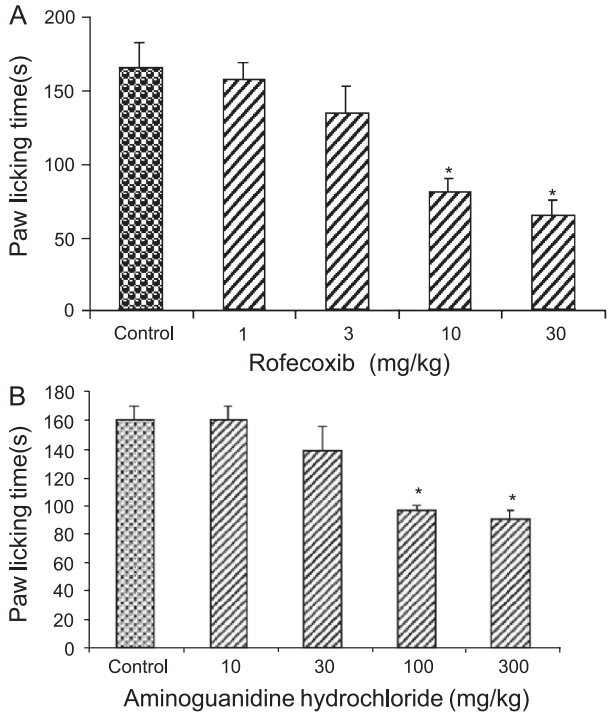

Fig. 1. Antinociceptive effect of rofecoxib (A) and aminoguanidine hydrochloride (B) in the formalin-induced paw-licking model. Rofecoxib (A) and aminoguanidine hydrochloride (B) were administered orally 1 h prior to subplantar injection of formalin. Control animals were injected with an appropriate volume of vehicle. Results show means ± S.E.M. (vertical bars; paw-licking time (s) in the late phase, 15–30 min). $n=6$. *$P<0.05$ (ANOVA).

3. Results

3.1. Antinociceptive effect of rofecoxib and aminoguanidine hydrochloride alone and in combination

Rofecoxib and aminoguanidine hydrochloride produced a statistically significant reduction in paw-licking time (antinociception) in the late phase of formalin-induced nociception (Fig. 1A and B). Rofecoxib at higher doses (10 and 30 mg/kg) produced a statistically significant antinociceptive effect, whereas lower doses of rofecoxib (1 and 3 mg/kg) did not produce a significant reduction in the late phase of formalin-induced nociception (Fig. 1A). Similarly, lower doses of aminoguanidine hydrochloride (10 and 30 mg/kg) did not produce a statistically significant antinociceptive effect, whereas higher doses of aminoguanidine hydrochloride (100 and 300 mg/kg) showed a statistically significant antinociceptive effect (Fig. 1B). ED_{50} values were 13.63 and 306.19 mg/kg for rofecoxib and aminoguanidine hydrochloride, respectively, by regression analysis. On the basis of the dose–response relationship of rofecoxib and aminoguanidine hydrochloride, administered alone, the subthreshold doses of rofecoxib (1 mg/kg) were combined with increasing doses of aminoguanidine hydrochloride (10–300 mg/kg), and aminoguanidine hydrochloride (30 mg/kg) was combined with rofecoxib (1–30 mg/kg). Combination treatment with rofecoxib and aminoguanidine hydrochloride augmented antinociception in the late phase (Fig. 2A and B). Combined treatment with a fixed dose of rofecoxib (1 mg/kg) with variable doses of aminoguanidine hydrochloride (30–300 mg/kg) produced a statistically significant reduction ($P<0.05$) in formalin-induced late phase paw licking (Fig. 2A). In a similar way, combined treatment with a fixed dose of aminoguanidine hydrochloride (30 mg/kg) with different doses of rofecoxib (1–30 mg/kg) showed a statistically significant antinociceptive effect ($P<0.05$) in the late phase of nociception (Fig. 2B).

3.2. Isobolographic analysis

The data for the combination of rofecoxib and aminoguanidine hydrochloride are presented as an isobologram in Fig. 3. In the graph presented, the doses of rofecoxib are on the ordinate and those of aminoguanidine hydrochloride are on the abscissa. The ED_{50} value for rofecoxib and that of aminoguanidine hydrochloride were plotted on the ordinate and the abscissa of isobolograph,

respectively. Then these points were connected by a solid theoretical line of additivity. The 95% confidence limits for a theoretical additive interaction were 1.49 and 2.82 mg/kg for rofecoxib and aminoguanidine hydrochloride, respectively. The ED_{50} (66.37 mg/kg) for the combination i.e. fixed dose of rofecoxib (1 mg/kg) with different doses of aminoguanidine hydrochloride (10, 30, 100 and 300 mg/kg), and the ED_{50} (1.16 mg/kg) for the other combination, i.e. fixed dose of aminoguanidine hydrochloride (30 mg/kg) with different doses of rofecoxib (1, 3, 10 and 30 mg/kg) were then plotted on the isobolograph (Point 'A' in Fig. 3). The expected ED_{50} for the combination (Point 'B' in Fig. 3) was then plotted and the potency ratio (0.30) was calculated as described in Materials and Methods to know the intensity of the interaction. The combination data point (Point 'A' in Fig. 3) came under the theoretical line of additive interaction, and the 95% confidence interval surrounding the combination data point did not overlap with that of the theoretical line for an additive interaction. Further, the potency ratio (obtained from the isobolograph) was less than 1, thus indicating a synergistic interaction.

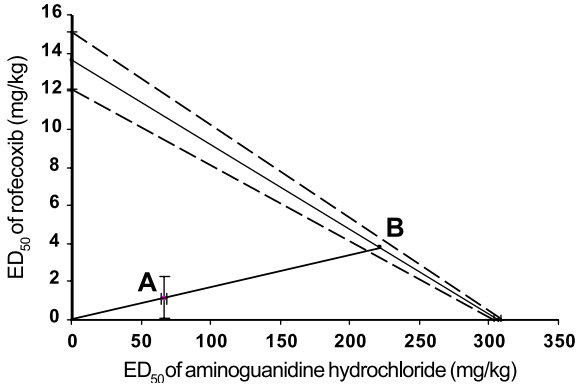

Fig. 3. Isobolographic analysis of an experiment indicating synergism between rofecoxib and aminoguanidine hydrochloride. The solid diagonal line is the zero interaction isobole constructed from experiments with each compound alone. Point 'A' below the zero interaction isobole is the concentration of the combination that produced 50% antinociceptive effect along with the 95% confidence intervals. The ratio of the line originating from the origin to Point 'A' and from origin to Point 'B' is a measure of synergism. The 95% confidence intervals for the additive points (ED_{50} values of rofecoxib and aminoguanidine hydrochloride) are also presented in the figure (dotted line).

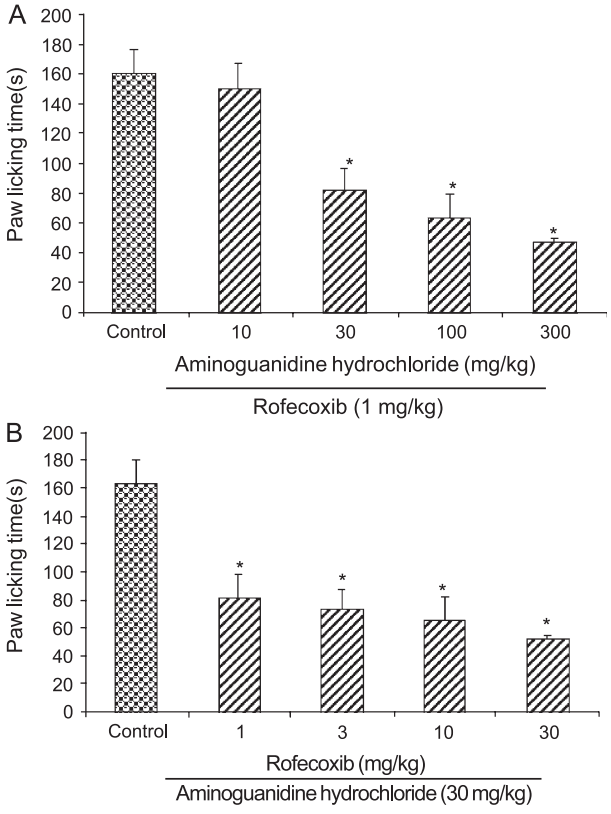

Fig. 2. Antinociceptive effect of rofecoxib (1 mg/kg) combined with increasing doses of aminoguanidine hydrochloride (A) and aminoguanidine hydrochloride (30 mg/kg) combined with increasing doses of rofecoxib (B) in the formalin-induced paw-licking model. Drugs were administered orally 1 h prior to subplantar injection of formalin. Control animals were injected with an appropriate volume of vehicle. Results show means ± S.E.M. (vertical bars; paw-licking time (s) in late phase, 15–30 min). $n=6$. $^*P<0.05$ (ANOVA).

4. Discussion

Rofecoxib and aminoguanidine hydrochloride produced a significant reduction in paw-licking time, suggesting antinociception against formalin-induced pain. This is probably the first study where an iNOS inhibitor (aminoguanidine hydrochloride) and cyclooxygenase-2 inhibitor (rofecoxib) showed a potent antinociceptive effect in the late phase of formalin-induced nociception. Rofecoxib and aminoguanidine hydrochloride have been found to possess antinociception in other models of pain (Chan et al., 1999; Jose et al., 2002).

Formalin-induced pain is biphasic, with an early neurogenic component followed by a late tissue-mediated response (Wheeler-Aceto and Cowan, 1991). The first-phase response is believed to represent a direct effect of formalin on sensory C fibers, while the late-phase response is secondary to the development of an inflammatory response and the release of algesic mediators (Hunskaar and Hole, 1987). Shibata et al. (1989) reported that substance P and bradykinin participate in the manifestation of first-phase responses, and histamine, serotonin, prostaglandin and bradykinin are involved in second-phase responses, indicating that the formalin test is a useful method for examining nociception and its modulation by pharmacological or other means. Previously, it has also been documented that nitric oxide is responsible for the nociceptive behavior in the second phase of the response induced by intraplantar injection of formalin in mice (Moore et al., 1993).

This is probably the first study to analyze the effects of the combination of rofecoxib and aminoguanidine hydrochloride. One of our goals was to identify the possible interaction between both drugs. Treatment of mice with a combination of aminoguanidine hydrochloride and rofecoxib resulted in a significantly greater antinociceptive effect in the late phase (15–30 min) of the formalin-induced paw-licking response than did administration of either drug alone. The degree of potentiation observed was too great to be accounted for by a simple additive effect, thereby suggesting a synergistic interaction between the two drugs. A similar synergistic antinociceptive effect has been noticed following co-administration of L-NG-nitro-arginine methyl ester (L-NAME), an inhibitor of NO synthase, with NSAIDs like flurbiprofen (Morgan et al., 1992), or 7-nitroindazole, a selective neuronal NO synthase inhibitor, co-administered with flurbiprofen (Gaffen et al., 1994). Our study suggests that the interaction is not confined to nonselective cyclooxygenase and NOS inhibitors, and an nNOS inhibitor, as observed by these workers, but also includes inducible NOS inhibitors and cyclooxygenase-2 inhibitors, as well. These interactions can be exploited in inflammatory conditions where iNOS and cyclooxygenase-2 are upregulated.

For studying the drug interaction, the variable dose-ratio method was used in which a particular dose is held constant and varying amounts of the second drug are given in combination to yield ED_{50} values and confidence intervals. Examples of isobolograms depicting the results of variable dose-ratio experiments are not uncommon (Masuda et al., 1981; Foltin et al., 1983; Carter et al., 1998). The isobolographic analysis of the present study showed that the interaction represented a highly significant synergism and fulfilled all the three important conditions (mentioned in results) required for synergism (Gessner, 1988; Nelson and Kursar, 1999).

The mechanism of interaction between aminoguanidine hydrochloride and rofecoxib is clearly of interest but cannot be established from the present data. It was previously reported that the NOS and cyclooxygenase pathways interact closely and that NO can stimulate cyclooxygenase activity, possibly via a reaction with heme component which binds to the active site of cyclooxygenase enzyme (Mitchell et al., 1993; Salvemini et al., 1993). Prostaglandin, an important mediator, is released during the second phase of formalin-induced nociception (Shibata et al., 1989). The released prostaglandin is responsible for nociception and produces hyperalgesia through a cAMP-dependent pathway. NO also facilitates the cAMP-dependent hyperalgesia produced by prostaglandin (Aley et al., 1998). Inhibition of iNOS by aminoguanidine hydrochloride suppresses the NO formation which is required to stimulate cyclooxygenase activity and the cyclic AMP pathway, thus causing a synergistic effect. This effect is in addition to the direct inhibition of cyclooxygenase-2 by rofecoxib and iNOS by aminoguanidine hydrochloride.

In conclusion, aminoguanidine hydrochloride (a selective iNOS inhibitor) administered orally with rofecoxib (a selective cyclooxygenase-2 inhibitor) produced a synergistic antinociceptive effect against formalin-induced nociception in mice. The clinical application of combination therapy in nociception needs further investigation.

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