Down-Regulation of Nitric Oxide Production by Ibuprofen in Human Volunteers

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ABSTRACT

Ibuprofen has been shown in vitro to modulate production of nitric oxide (NO), a mediator of sepsis-induced hypotension. We sought to determine whether ibuprofen alters NO production and, thereby, vascular tone, in normal and endotoxin-challenged volunteers. Techniques for detecting NO were validated in 17 subjects infused with sodium nitroprusside, a NO donor. Then, endotoxin (4 ng/kg) or saline (vehicle alone) was administered in a single-blinded, crossover design to 12 other subjects randomized to receive either ibuprofen (2400 mg p.o.) or a placebo. Endotoxin decreased mean arterial pressure (MAP; \( P = .002 \)) and increased alveolar NO flow rates (\( P = .04 \)) and urinary excretion of nitrate and nitrate (\( P = .07 \)). In both endotoxemic and normal subjects, ibuprofen blunted the small fall in MAP associated with bed rest (\( P = .005 \)) and decreased alveolar NO flow rates (\( P = .03 \)) and urinary excretion of nitrite and nitrate (\( P = .02 \)). However, ibuprofen had no effect on the decrease in MAP caused by endotoxin, although it blocked NO production to the point of disrupting the normal relationship between increases in exhaled NO flow rate and decreases in MAP (\( P = .002 \)). These are the first in vivo data to demonstrate that ibuprofen down-regulates NO in humans. Ibuprofen impaired the NO response to bed rest, producing a small rise in blood pressure. Although ibuprofen also interfered with the ability of endotoxin to induce NO production, it had no effect on the fall in blood pressure, suggesting that the hemodynamic response to endotoxin is not completely dependent on NO under these conditions.

Nitric oxide (NO) has been proposed as the predominant mediator of sepsis-induced shock (Radomski et al., 1990; Evans et al., 1993). In a healthy state and in early stages of sepsis, NO produced by a constitutive, calcium-dependent NO synthase in endothelial cells (eNOS) regulates vascular tone (Vallance et al., 1989). In a later stage of sepsis, inflammatory mediators induce a calcium-independent isoform of NO synthase (iNOS) that has been closely associated with the hypotension and the catecholamine hyporesponsiveness of septic shock in animals (Radomski et al., 1990; Szabo et al., 1993) and humans (Evans et al., 1993). This hypothesis, linking NO and the pathogenesis of septic shock, has led to an attempt to develop NO synthase inhibitors for treating this syndrome (Petros et al., 1994).

Commonly used cyclooxygenase inhibitors, such as ibuprofen, may represent an alternative approach for controlling the NO pathway in sepsis, as well as in other conditions where NO may play a pathogenic role, such as inflammatory arthritides (Sakurai et al., 1995), neurodegenerative diseases (Smith et al., 1997), and atherosclerosis (Wever et al., 1998). Cyclooxygenase inhibition has been shown in vitro to down-regulate iNOS expression and NO production (Aeberhard et al., 1995; Amin et al., 1995; Farivar et al., 1996; Kepka-Lenhart et al., 1996). However, others have reported iNOS up-regulation (Tetsuka et al., 1994; Xu et al., 1995), and one study with human endothelial cells found eNOS inhibition, but iNOS activation, at clinically achievable concentrations of ibuprofen (Menzel and Kolarz, 1997). Furthermore, ibuprofen has failed to increase blood pressure in septic patients (Bernard et al., 1997) or in volunteers challenged with endotoxin (Martich et al., 1992). These divergent results suggest two possibilities. Either ibuprofen does not decrease NO production in vivo, or it decreases NO production, but other vasoregulatory pathways compensate for this loss and blood pressure is unaffected.

The purpose of this study was to determine whether cyclooxygenase inhibition alters NO production in humans. Blood pressure, NO pathway activity, and prostaglandin synthesis were measured in the presence or absence of ibuprofen in humans.
normal and in endotoxin-challenged volunteers. This model produces a hyperdynamic, vasodilated state that develops over 2 to 3 h and persists for at least 8 h (Suffredini et al., 1989). The accompanying inflammatory response is well characterized, is self-limited (Martich et al., 1991), and spares the pulmonary airways (Boujoukos et al., 1993).

Materials and Methods

Subjects. This investigation was approved by the Institutional Review Board on Human Experimentation of the National Institute of Allergy and Infectious Diseases. Written, informed consent was obtained from all of the participants. Twenty-nine healthy subjects (22 men, 7 women), ranging in age from 20 to 37 years old (mean, 28 years), were studied. For some of these subjects, data on fever, heart rate variability, and circulating levels of leptin and macrophage inflammatory protein-1 have been published elsewhere (Goden et al., 1996; Bornstein et al., 1998; O’Grady et al., 1999). After an overnight fast, subjects had i.v. and radial artery catheters placed (Arrow International, Reading, PA), and their electrocardiogram and blood pressure were monitored continuously. Heart rate and mean arterial pressure (MAP) were recorded every hour starting before the administration of sodium nitroprusside (SNP) or endotoxin (baseline, time = 0).

Exhaled NO, Exhaled Carbon Dioxide (CO₂), and Airway Flow Measurements. Subjects wore nose clips and breathed NO-free air (Roberts Oxygen, Rockville, MD) through a mouthpiece. A NO standard curve with a sensitivity of 1 part per billion was generated each day of the study. Single-breath vital capacity maneuvers were analyzed for NO (270B Chemiluminescence Analyzer; Sievers Instruments, Inc., Boulder, CO), CO₂ (Capnogard ETCO 2 Monitor; Sievers), and NOx levels, collectively

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Results

SNP Effects (Methods Validation Study). SNP increased heart rate in the high-dose group (Fig. 1A). In both the low- and high-dose groups, MAP was decreased (Fig. 1B)

Dixon (1951). To detect and remove outliers, data were analyzed with a Kruskal-Wallis test. When analyzing 11-dehydro-thromboxane B₂, an outlier was detected and removed with a procedure described by Dixon (1951). To examine the relationship between MAP and the total NO flow rate, a Spearman correlation coefficient was calculated for each subject. These correlations were analyzed with a Kruskal-Wallis test.

SNP Administration (Methods Validation Study). To evaluate the methods used to measure NO, patients were given an infusion of SNP (Elkins-Sinn Inc., Cherry Hill, NJ), a NO donor, diluted in 5% dextrose in water (final concentration, 200 μg/ml). Before the initiation of SNP or placebo (vehicle alone) infusion, all of the subjects received 30 ml/kg of 0.9% saline i.v. over 30 min. A maintenance infusion of 0.9% saline at a rate of 1 ml/kg/h was then administered for the remainder of the study.

Subjects in the high-dose SNP group (n = 8) were administered two drug titrations on the same day, separated by 1 h. An initial rate of 0.5 μg/kg/min was followed by increases of 0.2 μg/kg/min every 3 to 5 min until MAP decreased to 65 mm Hg or heart rate increased to 115 beats/min. The mean maximum infusion rate was 3.9 ± 1.2 μg/kg/min, and the mean total dose was 443 ± 165 μg/kg.

A target infusion rate of 3.0 μg/kg/min was selected for the low-dose SNP group (n = 9) based on data from the high-dose group. Paired, single-blinded randomized experiments of low-dose SNP and placebo (vehicle alone) were separated by 1 week. The infusion rate was titrated upward over 90 min to 3.0 μg/kg/min as tolerated, and then continued for 1 h. The mean maximum infusion rate attained was 2.8 ± 0.2 μg/kg/min, and the mean total dose was 284 ± 21 μg/kg. During the placebo week, each subject was given comparable volumes of 5% dextrose in water.

Endotoxin and Ibuprofen Administration. Twelve subjects were given endotoxin (4 ng/kg i.v.; U.S. Standard Reference Endotoxin, Lot EC-5, Escherichia coli 0113; Bureau of Biologics, U.S. Food and Drug Administration) or 0.9% saline (vehicle) separated by 1 week in a single-blinded, randomized order. Endotoxin in 2 ml of 0.9% saline (vehicle) or vehicle alone was administered over 1 min followed by a 10-ml flush of 0.9% saline. Subjects were randomized further to receive either 800 mg of ibuprofen (Upjohn, Kalamazoo, MI; n = 6) or a lactose placebo (n = 6) p.o. at 1.5 h before, at the time of, and at 3 h after the administration of endotoxin or 0.9% saline.

Statistical Analysis. Data from the SNP infusion study were analyzed by first calculating a summary statistic for each individual (e.g., maximum change). Summary statistics were analyzed for group differences with a nonparametric Kruskal-Wallis test.

Data from the endotoxin-challenge experiment were analyzed with a four-way ANOVA, with the main effects for endotoxin, ibuprofen, subject (nested within ibuprofen), and time. In addition to the main effects, all interactions that did not include the subject were present in the statistical model and all interactions that did include the subject were used as the error term. Based on animal data, it was anticipated that it would take 3 h or longer for endotoxin to induce nOS (Szabo et al., 1993; Gardiner et al., 1995). Furthermore, an analysis of the MAP data showed no effects before 3 h after the administration of endotoxin. Thus, for all parameters, the ANOVA was run separately for an early period (0–2 h) and a late period (3–8 h) after the administration of endotoxin. Significance levels were pooled from the early and the late periods with Fisher’s Omnibus test. A separate analysis was conducted in which the endotoxin-ibuprofen interaction was constructed so as to isolate the endotoxin-only treatment group, and the P values were adjusted to control for multiple comparisons.

When analyzing 11-dehydro-thromboxane B₂, an outlier was detected and removed with a procedure described by Dixon (1951). To examine the relationship between MAP and the total NO flow rate, a Spearman correlation coefficient was calculated for each subject. These correlations were analyzed with a Kruskal-Wallis test.

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and the exhaled total NO flow rates were increased (Fig. 1C) compared with the placebo group (see Fig. 1 for P values). Other measures of exhaled NO, including the peak NO concentration and the alveolar NO flow rate, were also increased in the SNP groups (P < .05 for all; data not shown). Urinary NOx excretion increased only in the high-dose group during the poststudy urine collection (Fig. 1D). In contrast, compared with the placebo group, low- or high-dose SNP had no effect on the urinary excretion of cGMP (P ≥ .16 for both; data not shown). Changes from baseline in S-nitrosothiol, considered a more specific measure of endogenous NO production, were decreased by SNP in both the high- and low-dose groups compared with the placebo group (0.3 ± 0.2 and 0.6 ± 0.8 μM, respectively, versus 2.6 ± 1.3 μM; P ≤ .05). Therefore, the exhaled NO flow rates and the urinary excretion of NOx were the most sensitive indicators of systemic NO release.

Next, we examined the relationship between changes in the NO flow rates and changes in MAP. All three of the groups (placebo, low-dose SNP, and high-dose SNP) demonstrated a similar (P = .58) weak, negative correlation between the total NO flow rate changes and MAP changes (r = −0.31, P = .02; all groups combined, data not shown). Thus, increases in the NO flow rates correlated with MAP decreases in normal volunteers, independent of the administration of SNP.

Ibuprofen Effects Independent of Endotoxin. Ibuprofen concentrations were similar at 0, 3, and 6 h, with an overall mean of 46.6 ± 4.3 μg/ml (P = .001). Ibuprofen had no significant effects on heart rate (P ≥ .5; Fig. 2A). In contrast, ibuprofen blunted the small fall in MAP associated with bed rest, both in the early period (<3 h) and the late period (>3 h) after administration of either saline (normal) or endotoxin (P = .005; Fig. 2B). Similarly, ibuprofen decreased temperature (P = .004; Fig. 3A). Furthermore, measures of NO were decreased by ibuprofen in normal and endotoxemic subjects, both in the early and the late period. These measures included the alveolar NO concentration (P = .05; Table 1), the alveolar NO flow rate (P = .03; Table 1), the total NO flow rate (P = .06; Fig. 3B), and the urinary NOx (P = .02; Fig. 3C). The plasma NOx concentrations were decreased by ibuprofen but only in the late period (P = .04; Table 2).

To further understand the effects of ibuprofen on NO production and MAP, correlation coefficients were analyzed and are shown for each individual volunteer (Fig. 4A) and as group means (Fig. 4B). Similar to the SNP part of the study, the total NO flow rate increases correlated with MAP decreases (r = −0.36; P = .009; saline and endotoxin combined) in subjects not treated with ibuprofen (placebo groups), whether they received saline or endotoxin. In contrast, for subjects treated with ibuprofen, the exhaled total NO flow rate increases did not correlate with MAP decreases (r = +0.27; P = N.S.; saline and endotoxin combined). These correlation coefficients comparing placebo- with ibuprofen-treated subjects were significantly different from each other.
TABLE 1
Early and late period effects of ibuprofen and endotoxin (mean changes from baseline ± S.E.M.)

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Saline/placebo</th>
<th>Saline/ibuprofen</th>
<th>Endotoxin/placebo</th>
<th>Endotoxin/ibuprofen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ Peak NO flow rate (nl · s⁻¹)</td>
<td>0.3 ± 0.3</td>
<td>0.5 ± 0.6</td>
<td>0.8 ± 0.4</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>Δ Alveolar NO flow rate (nl · s⁻¹)</td>
<td>0.6 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.3</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Δ Peak NO concentration (ppb)</td>
<td>35.8 ± 20.3</td>
<td>114.2 ± 59.9</td>
<td>170.4 ± 44.0</td>
<td>46.9 ± 18.2</td>
</tr>
<tr>
<td>Δ Alveolar NO concentration (ppb)</td>
<td>145.8 ± 27.7</td>
<td>21 ± 2.1</td>
<td>62 ± 5.9</td>
<td>1.5 ± 1.8</td>
</tr>
<tr>
<td>Δ Plasma NOx (µM)</td>
<td>-3.7 ± 1.1</td>
<td>-6.2 ± 1.9</td>
<td>-3.3 ± 0.9</td>
<td>-4.7 ± 5.2</td>
</tr>
<tr>
<td>Δ Plasma cGMP (nM)</td>
<td>0.9 ± 0.9</td>
<td>-2.2 ± 1.7</td>
<td>-0.8 ± 1.8</td>
<td>-0.5 ± 1.7</td>
</tr>
<tr>
<td>Δ Plasma S-nitrosothiol (µM)</td>
<td>-1.2 ± 0.4</td>
<td>0.2 ± 0.3</td>
<td>1.5 ± 1.0</td>
<td>-0.3 ± 1.9</td>
</tr>
<tr>
<td>Δ Urine cGMP (nmol · h⁻¹)</td>
<td>1.3 ± 6.7</td>
<td>0.1 ± 7.4</td>
<td>5.2 ± 5.9</td>
<td>1.6 ± 2.5</td>
</tr>
</tbody>
</table>

Early Period Effects

Δ Peak NO flow rate (nl · s⁻¹)
Δ Alveolar NO flow rate (nl · s⁻¹)
Δ Peak NO concentration (ppb)
Δ Alveolar NO concentration (ppb)
Δ Plasma NOx (µM)
Δ Plasma cGMP (nM)
Δ Plasma S-nitrosothiol (µM)
Δ Urine cGMP (nmol · h⁻¹)

Late Period Effects

Δ Peak NO flow rate (nl · s⁻¹)
Δ Alveolar NO flow rate (nl · s⁻¹)
Δ Peak NO concentration (ppb)
Δ Alveolar NO concentration (ppb)
Δ Plasma NOx (µM)
Δ Plasma cGMP (nM)
Δ Plasma S-nitrosothiol (µM)
Δ Urine cGMP (nmol · h⁻¹)

Δ Parts per billion.

b P < .05, ibuprofen-treated groups compared to non-ibuprofen-treated groups (early and late effect combined).

Discussion

This study demonstrates that ibuprofen, a cyclooxygenase inhibitor, reduces NO production and dissociates it from MAP in humans. This observation confirms in vivo a potentially important mechanism of action for ibuprofen and possibly other nonsteroidal anti-inflammatory drugs. Although ibuprofen blocks increases in NO production and blunted bed rest-associated reductions in blood pressure, this agent failed to alter the hemodynamic response to endotoxin, suggesting that vasoregulatory mechanisms other than NO determine vasomotor tone under these conditions.

The in vivo effects of ibuprofen on the NO pathway previously have not been reported in humans and its effects in vitro have been variable. Some in vitro studies have found that cyclooxygenase inhibitors down-regulate NOS expression and function (Aebberhard et al., 1995; Amin et al., 1995; Farivar et al., 1996; Kepka-Lenhart et al., 1996), but others have demonstrated up-regulation (Tetsuka et al., 1994; Xu et
A study with human endothelial cells concluded that the administration of high doses of ibuprofen to humans would likely increase net NO generation (Menzel and Kolarz, 1997). Ibuprofen does have some in vivo effects that might tend to increase rather than decrease NO responses. For example, in human endotoxin-challenged volunteers, ibuprofen increases the release of proinflammatory cytokines (Martinich et al., 1991; O’Grady et al., 1999), an effect which might increase iNOS induction. Although our study demonstrates that ibuprofen down-regulates the NO pathway in vivo, it does not address whether this represents the net effect of several competing mechanisms.

Notably, ibuprofen prevented endotoxin from augmenting NO production, but the fall in blood pressure was unaffected. However, ibuprofen did have vascular effects, as it decreased the fall in blood pressure that occurred with the institution of bed rest. The inability of ibuprofen to alter inflammation-associated abnormalities of vascular tone has been reported before in septic patients (Bernard et al., 1997) and in volunteers challenged with endotoxin (Martinich et al., 1992). In contrast, cyclooxygenase inhibitors have been shown to raise blood pressure in septic pigs (Weitzberg et al., 1995), dogs (Jacobs et al., 1982), and baboons (Fletcher et al., 1976). NO responses vary across species and do not always correlate well with changes in vascular tone (Evans et al., 1993; Jacob et al., 1993; van den Berg et al., 1994; Cobb et al., 1995). Therefore, the administration of low doses of endotoxin to humans may provide insights into blood pressure regulation that are not identical with those obtained from animal models (Suffredini et al., 1989; Martinich et al., 1992).

In our subjects, mechanisms other than NO probably contribute to endotoxin-induced vasodilatory responses. In addition to preventing increases in NO production, ibuprofen inhibited synthesis of prostacyclin and thromboxane A2. Blockade of both vasodilatory and vasoconstrictive prostanoids could cause either increases or decreases in blood pressure, depending on their net effect on vascular tone. Furthermore, in endotoxin-challenged volunteers, ibuprofen decreases the release of vasoconstrictive catecholamines (Revhaug et al., 1988), an effect that favors vasodilation. Alternatively, a fall in blood pressure in the absence of increased NO production could be caused by increased NO effectiveness. Nonsteroidal anti-inflammatory drugs block production of superoxide by cyclooxygenases (Wolin, 1996). Because superoxide inactivates NO (Gaboury et al., 1993), a decrease in superoxide could increase NO bioavailability.

Regardless of the mechanism, our results demonstrate that in the presence of ibuprofen, endotoxin-induced vasodilation occurs without a measurable increase in NO production, possibly because of reciprocal changes in other vasoregulatory pathways.

Ibuprofen was found to reduce NO production in both normal and endotoxin-challenged volunteers, although this effect was more pronounced after endotoxin. This suggests that ibuprofen may also interfere with homeostatic NO production, which primarily arises from constitutive isoforms of NOS. An in vitro study with human endothelial cells (Menzel and Kolarz, 1997) and an in vivo study conducted in swine (Dahm et al., 1997) both support the possibility that cyclooxygenase inhibitors decrease the activity of eNOS, a constitutive isoform. In our study, ibuprofen may have suppressed NO production in normal volunteers by blunting eNOS responses, by decreasing low basal levels of iNOS expression, or both.

Among methodologies used as markers for NO production, we found that the exhaled NO flow rates and the urinary NOx excretion were the most sensitive. Exhaled NO has a number of sources, but increases caused by SNP and endotoxin in this study likely arose from NO release within the pulmonary vasculature (Husain et al., 1994; Persson et al., 1994; Stitt et al., 1997). Although airway inflammation increases exhaled NO (Khatriyov et al., 1994), the administration of low doses of endotoxin to humans produces a self-limited inflammatory response that spares the airways (Boujoukos et al., 1993). Importantly, increases in exhaled NO paralleled increases in urinary NOx excretion, a measure that reflects global changes in NO production. Other tests used in this study, plasma NOx, S-nitrosothiol, and cGMP or urinary cGMP, were relatively insensitive to changes in NO production. Notably, plasma S-nitrosothiol, a more specific measure of endogenous NO production than NOx (Stamler et al., 1992), decreased in response to SNP.

In summary, the administration of ibuprofen to humans blocked NO production but did not prevent an endotoxin-induced fall in blood pressure. These results suggest that mediators other than NO may contribute to alterations in vascular tone during endotoxemia, particularly in the presence of cyclooxygenase inhibition. In support of this concept, iNOS-deficient “knockout” mice have been shown to have NO-independent mechanisms of endotoxin-induced hypotension and death (Macmicking et al., 1995). Thus, the vasodilatory response to endotoxin is complex and may be regulated by a network of overlapping mediators. The ability of ibuprofen to decrease NO production is a mechanism of action that has possible experimental and therapeutic applications.

References


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