Contribution of Nitric Oxide and Tumor Necrosis Factor-α to the Effect of Indomethacin on Endotoxin-Induced Mortality

Bahar TUNÇTAN*, Sedat ALTUĞ**, Orhan ULUDAĞ**, Nurettin ABACIOĞLU**

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Summary
We have previously demonstrated that indomethacin (1, 10 or 100 mg/kg, i.p.) prevented endotoxin (10 mg/kg, i.p.-) induced increase in nitric oxide (NO) and eicosanoid production, whereas it did not affect the mortality rate in an experimental septic shock model in mice. The aim of the present study was to investigate the contribution of NO and tumor necrosis factor (TNF-α) to the effect of indomethacin on endotoxin-induced mortality. Animals were injected with endotoxin (10 mg/kg, i.p.) alone or in combination with indomethacin [cyclooxygenase (COX) inhibitor] (100 mg/kg), aminoguanidine (inducible NO synthase inhibitor) (100 mg/kg), and/or pentoxifylline (TNF-α production and activity inhibitor) (90 mg/kg) at 9:00 a.m. The blood samples were collected 15 h after the drug administration and serum samples were used for the measurement of nitrite concentrations. The endotoxin-induced increase in serum nitrite levels was decreased by indomethacin and aminoguanidine, but not by pentoxifylline. Endotoxin-induced mortality was prevented by aminoguanidine and pentoxifylline, but not by indomethacin. The ineffectiveness of indomethacin on endotoxin-induced mortality was not changed by aminoguanidine and/or pentoxifylline treatment. These results suggest that the overproduction of NO and/or TNF-α may not contribute to the effect of indomethacin on endotoxin-induced mortality.

Key Words : Indomethacin, pentoxifylline, endotoxin, mice, mortality.

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INTRODUCTION

Induction of cyclooxygenase (COX) by endotoxin results in an elevated production of proinflammatory prostaglandins (PGs) correlated with organ dysfunction and mortality\(^1\). Although clinical and experimental studies indicate that COX inhibition by many nonsteroidal antiinflammatory drugs improves hemodynamic abnormalities and reduces mortality in septic shock\(^2,3\), some COX inhibitors, such as

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* Mersin University, Yenişehir Campus, Department of Pharmacology, Faculty of Pharmacy, 33161, Mersin, TURKEY
** Gazi University, Department of Pharmacology, Faculty of Pharmacy, 06330, Ankara, TURKEY
\* Corresponding author e-mail: btunctan@yahoo.com
Tunçtan, Altuğ, Uludağ, Abacıoğlu

indomethacin, have been shown to exert both beneficial and detrimental effects in septic animals\textsuperscript{4,5}. We have previously demonstrated that indomethacin (1, 10 or 100 mg/kg, i.p.) when co-administered with endotoxin at 9:00 a.m. significantly decreased systemic nitric oxide (NO) production dose-dependently, whereas it did not prevent the mortality rate in an experimental septic shock model in mice\textsuperscript{6}. We have also shown that endotoxin injection at 9:00 a.m. caused a peak after 15 h, while 9:00 p.m. injection had two peaks after 9 and 18 h in serum nitrite concentrations\textsuperscript{7}. The peak values obtained from morning and evening injections were significantly decreased by indomethacin and a selective inducible NO synthase (iNOS) inhibitor, aminoguanidine; 6-keto-PGF\textsubscript{1α} (a stable product of prostacyclin) and thromboxane B\textsubscript{2} (TXB\textsubscript{2}) (a stable product of TxA\textsubscript{2}) levels were also decreased by indomethacin when injected with endotoxin at both injection times, but not by aminoguanidine. On the other hand, when mice were injected with endotoxin in the morning or in the evening, it increased the mortality rate within 24 h, which could be abolished by aminoguanidine, but not indomethacin. It has been shown that increased tumor necrosis factor-α (TNF-α) production may be the reason for the detrimental effect of indomethacin on survival\textsuperscript{5,8}. Some in vitro and in vivo studies indicate that co-exposure to indomethacin may strongly enhance TNF-α production in response to endotoxin or other inflammatory stimuli, and the ability of the drug to inhibit PGH synthase I correlates with its potency to induce TNF-α\textsuperscript{5,9,10}. Therefore, we investigated the contribution of NO and TNF-α to the effect of indomethacin on endotoxin-induced mortality.

**MATERIALS AND METHODS**

**Drugs**

Endotoxin (Escherichia coli O111:B4 lipopolysaccharide), indomethacin, aminoguanidine and pentoxifylline were obtained from Sigma Chemical Co. (St. Louis, USA). NaHCO\textsubscript{3} was obtained from Merck (Darmstadt, Germany). Indomethacin was dissolved in 5% NaHCO\textsubscript{3} solution. All other drugs were dissolved in saline.

**Animals**

Locally bred male and female albino mice (Refik Saydam Hygiene Center, Ankara, Turkey) weighing 20-40 g were used throughout the experiments according to the proposals of the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed in standard transparent cages (20 per cage) with free access to food and water under environmentally controlled conditions at 24 ± 2°C. They were synchronized by maintenance of controlled environmental conditions for at least two weeks prior to and throughout the duration of the experiments. The circadian rhythmicity of the animals was ensured by a standardized 12 h light/dark cycle (lights on at 9:00 a.m.) with a light intensity of approximately 100 lux. Automatic timer-controlled cool fluorescent bulbs were used to provide lighting. To avoid seasonal variations, all experiments were performed from June to August.

**Treatments**

Endotoxin (10 mg/kg) was administered intraperitoneally alone or in combination with saline, indomethacin (100 mg/kg), pentoxifylline (90 or 180 mg/kg) or aminoguanidine (100 mg/kg) at 9:00 a.m. The blood samples were collected 15 h after the drug administration. After blood samples had clotted at room temperature for 30 min, they were defibrinated and centrifuged at 2000 rpm for 15 min. Serum was aspirated and frozen at -20°C until analysis.

**Nitrite measurement**

In biological systems, conversion of NO in aqueous solution to nitrite and nitrate is thought to favor nitrite production\textsuperscript{11}. It has been reported that nitrite is the only stable end-product of the autooxidation of NO in aqueous solution\textsuperscript{12}; measurement of nitrite concentrations in biological samples is widely accepted as an index for NOS activity\textsuperscript{13}. Therefore, concentrations of nitrite in the sera were measured using the diazotization method based on the Griess reaction, which is an indirect assay for NO
production. Briefly, Griess reagent [1% sulfanylamide (50 µl) and 0.1% N-1-naphthylethylenediamine dihydrochloride (50 ml) in 2.5% orthophosphoric acid] was added to the each well containing the sample (100 ml) in 96-well tissue culture plates. After incubation for 15 min at room temperature, absorbance was measured at 550 nm with a microplate reader (Diagnostic Pasteur, LP 400, Germany). Nitrite concentration was calculated by a standard calibration curve of sodium nitrite solutions.

**Data analysis**

The results are reported as means ± SEM. n refers to the number of animals used. Statistical comparisons were made using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California, U.S.A., http://www.graphpad.com) by one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons or Kruskal-Wallis test followed by Dunn’s test. Student’s t or Mann-Whitney U tests were also used when necessary. % Mortality comparisons were done by Fisher’s exact test. P value of < 0.05 was considered statistically significant.

**RESULTS**

We have previously shown that the injection of endotoxin to mice at 9:00 a.m. or 9:00 p.m. elicited temporal changes in serum nitrite levels which reached a peak by 15 h, or by 9 and 18 h, respectively. In this study, injections were made only at 9:00 a.m. and blood samples were collected 15 h after drug administration. Indomethacin significantly diminished serum nitrite levels (Fig. 1) without any significant improvement on survival (Fig. 2). Aminoguanidine significantly reduced NO production (Fig. 1) and prevented endotoxin-induced mortality (Fig. 2).

To investigate the effect of indomethacin on the TNF-α production in response to endotoxin, pentoxifylline (inhibitor of TNF-α production and activity) at 90 or 180 mg/kg was co-injected with endotoxin. Pentoxifylline did not significantly change serum nitrite levels using either dose (Fig. 1). However, co-administration of pentoxifylline at 180 mg/kg dose with indomethacin significantly decreased nitrite levels induced by endotoxin alone or endotoxin plus pentoxifylline. Pentoxifylline at 90 mg/kg dose alone or in combination with aminoguanidine significantly increased nitrite levels induced by indomethacin. Endotoxin-induced mortality rate was prevented by 90 mg/kg dose of pentoxifylline, but not by 180 mg/kg dose (Fig. 2). Indomethacin-induced mortality was not changed by aminoguanidine and/or pentoxifylline at either dose.
DISCUSSION

Together with our previous findings\(^6\),\(^7\), the results of this study indicate that indomethacin prevented increased eicosanoid and NO production, but not mortality, induced by endotoxin. Indomethacin-induced inhibition on eicosanoid levels was similar at both injection times, whereas morning or evening injection of indomethacin did not significantly reduce endotoxin-induced mortality\(^7\). These results suggest that the overproduction of NO and eicosanoids may be responsible for endotoxin-induced mortality and that exposure to endotoxin at different times of the day may change the effects of the enzyme inhibitors in the experimental septic shock model in mice. On the other hand, the effect of indomethacin on mortality induced by endotoxin does not seem to be dependent on injection time. The findings confirm the previous results that PGs at high concentrations (> 50 ng/ml) inhibit TNF-α production in murine macrophage cell culture\(^17\) and that endotoxin-induced TNF-α production in septic mice is up-regulated by indomethacin\(^8\), suggesting that endogenous PGs downregulate TNF-α production. Therefore, increased TNF-α production may be responsible for indomethacin-induced mortality. TNF-α was shown to induce all symptoms of endotoxic shock in animals, and inhibitors of TNF-α production, such as pentoxifylline at 3-100 mg/kg doses, were shown to have beneficial effects on survival\(^14\)-\(^16\),\(^18\)-\(^20\). However, since inhibition of TNF-α production by pentoxifylline did not prevent the indomethacin-induced mortality, we suggest that the effect of indomethacin on endotoxin-induced mortality may not depend on the enhancement of TNF-α, in contrast to the results of Campanile et al\(^5\). Therefore, we may conclude that NO or eicosanoids are not responsible for the detrimental effects of indomethacin. On the other hand, the mediators of endotoxin-induced mortality may be NO and/or TNF-α. Indeed, aminoguanidine reduced the serum nitrite levels and prevented the mortality induced by endotoxin, as reported by the previous studies which showed that aminoguanidine protects the animals from the lethal effects of endotoxin, inhibiting NO and cytokine production\(^6\). Furthermore, pentoxifylline at 90 mg/kg dose prevented the endotoxin-induced mortality, as previously shown at 80 or 100 mg/kg doses of pentoxifylline, by inhibiting TNF-α and interleukin-6 production\(^19\),\(^20\). The beneficial effect of pentoxifylline on mortality may not be attributed to the inhibition of NO production as previously shown by Wu et al.\(^16\), since pentoxifylline at 90 mg/kg dose did not decrease endotoxin-induced serum nitrite levels. On the other hand, our results with 180 mg/kg pentoxifylline did not completely confirm the data of Jilg et al.\(^21\) and Badger et al.\(^22\) who showed that similar doses of pentoxifylline enhance survival. Our results support the findings of Hadjiminas et al.\(^23\) who reported that the beneficial (at low dose) or detrimental (at high dose) effects of pentoxifylline on survival appear dose-dependent.

In summary, although indomethacin decreases the production of eicosanoids and NO, it does not improve survival in the endotoxin-induced septic shock model in mice. The effect of indomethacin on endotoxin-induced mortality may not be associated with the increased production of NO and/or TNF-α. The mechanism underlying the effect of indomethacin on endotoxin-induced mortality may be due to the overproduction of endogenous mediators with the exception of eicosanoids, NO, or TNF-α.

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