

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^{**}

Contribution of nitric oxide and tumor necrosis factor- α to the effect of indomethacin on endotoxin-induced mortality 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.

Received: 27.07.2005

Revised: 07.11.2005

Accepted: 23.11.2005

Endotoksin ile Oluşan Mortalite Üzerinde İndometazin Etkisine Nitrik Oksit ve Tümör Nekroze Edici Faktör- α 'nın Katkısı Özet

Daha önce farede oluşturulan deneysel septik şok modelinde yaptığımız bir çalışmada, indometazinin (1, 10 veya 100 mg/kg, i.p.) endotoksin (10 mg/kg, i.p.) ile nitrik oksit (NO) ve eikozanoit oluşumundaki artışı önlediği, ancak endotoksin ile artan mortaliteyi etkilemediği gösterilmiştir. Bu çalışmanın amacı, endotoksin ile oluşan mortalite üzerinde indometazinin etkisine NO ve tümör nekroze edici faktör (TNF)- α 'nın katkısı araştırmaktır. Farelere 9:00'da endotoksin (10 mg/kg, i.p.), indometazin (siklooksijenaz (COX) inhibitörü) (100 mg/kg, i.p.), aminoguanidin (indüklenebilir NO sentaz inhibitörü) (100 mg/kg, i.p.) ve/veya pentoksifilin (TNF- α oluşumu ve aktivitesi inhibitörü) (90 mg/kg, i.p.) uygulanmıştır. Madde uygulanmasından 15 saat sonra kan örnekleri alınmış ve serum örnekleri nitrit konsantrasyonları ölçümü için kullanılmıştır.

Endotoksin uygulanmasından sonra artan serum nitrit düzeyleri indometazin ve aminoguanidin ile azalmış, pentoksifilin ile değişmemiştir. Endotoksin ile artan mortalite ise aminoguanidin ve pentoksifilin tamamen önlenirken, indometazin ile değişmemiştir. İndometazinin endotoksin ile oluşan mortalite üzerindeki etkisizliği aminoguanidin ve/veya pentoksifilin ile değişmemiştir. Bu bulgular, endotoksin ile oluşan mortalite üzerinde indometazinin etkisine NO ve/veya TNF- α 'nın katkısının olamayacağını düşündürmektedir.

Anahtar Kelimeler: İndometazin, pentoksifilin, endotoksin, fare, mortalite

INTRODUCTION

Induction of cyclooxygenase (COX) by endotoxin results in an elevated production of proinflammatory prostaglandins (PGs) correlated with organ

dysfunction and mortality¹. 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

* Mersin University, Yeniflehir 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: btuncan@yahoo.com

indomethacin, have been shown to exert both beneficial and detrimental effects in septic animals^{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 mice⁶. 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 concentrations⁷. 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_{1α} (a stable product of prostacyclin) and thromboxane B₂ (TxB₂) (a stable product of TxA₂) 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^{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 PG H synthase I correlates with its potency to induce TNF-α^{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). NaHCO₃ was obtained from Merck (Darmstadt, Germany). Indomethacin was dissolved in 5% NaHCO₃ 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 defibrinized 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 production¹¹. It has been reported that nitrite is the only stable end-product of the autooxidation of NO in aqueous solution¹²; measurement of nitrite concentrations in biological samples is widely accepted as an index for NOS activity¹³. 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

production⁶. Briefly, Griess reagent [1% sulfanylamide (50 μ l) and 0.1% N-1-naphthylethylenediamine dihydrochloride (50 ml) in 2.5% orthophosphoric acid] was added to the each well containing the sample (100 μ l) 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 \pm 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^{6,7}. 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)¹⁴⁻¹⁶ 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

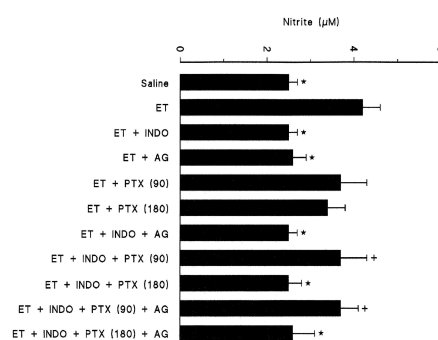


Figure 1. Effects of indomethacin (INDO, 100 mg/kg, i.p.), aminoguanidine (AG, 100 mg/kg, i.p.) and/or pentoxifylline (PTX, 90 or 180 mg/kg, i.p.) alone or in combination with each other on endotoxin (10 mg/kg, i.p.)-induced increase in the serum nitrite levels in mice. Data were expressed as the means \pm S.E.M., n = 6-17. * P < 0.05 vs. endotoxin-treated group determined using Student's t or Mann-Whitney U tests. ⁺P < 0.05 vs. endotoxin + INDO-treated group determined using one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons or Kruskal-Wallis test followed by Dunn's test.

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.

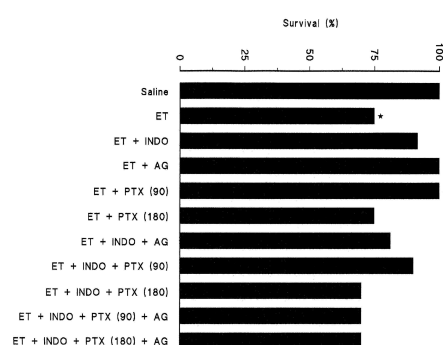


Figure 2. Effects of indomethacin (INDO, 100 mg/kg, i.p.), aminoguanidine (AG, 100 mg/kg, i.p.) and/or pentoxifylline (PTX, 90 or 180 mg/kg, i.p.) alone or in combination with each other on endotoxin (10 mg/kg, i.p.)-induced mortality in mice. Data were expressed as the percentage of surviving mice in each group, n = 6-17. * P < 0.05 vs. saline-treated group determined using Fisher's exact test. ⁺P < 0.05 vs. endotoxin-treated group determined using Fisher's exact test.

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 mortality⁷. 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 culture¹⁷ and that endotoxin-induced TNF- α production in septic mice is up-regulated by indomethacin⁸, 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 al⁵. 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 production⁶. 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 Hadjiminis 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 .

Acknowledgements

This study was supported by the Research Foundation of Gazi University (Project Code No: EF.02/98-06). Results of this paper were presented at the XVth National Congress of Pharmacology, Manavgat, Antalya, Turkey, 1-5 November 1999.

REFERENCES

1. Basu S, Eriksson M. Oxidative injury and survival during endotoxemia, *FEBS Lett.* 438, 159-160, 1998.
2. Bernard GR, Wheeler AP, Russell JA, Schein R, Summer WR, Steinberg KP, Fulkerson WJ, Wright PE, Christman BW, Dupont WD, Higgins SB, Swindell BB. The effects of ibuprofen on the physiology and survival of patients with sepsis. The Ibuprofen in Sepsis Study Group, *N. Engl. J. Med.* 336, 912-918, 1997.
3. Mansilla-Rosello A, Ferron-Orihuela JA, Ruiz-Cabello F, Garrote-Lara D, Fernandez-Mondejar E, Delgado-Carrasco ML. Differential effects of IL-1 beta and ibuprofen after endotoxic challenge in mice, *J. Surg. Res.* 67, 199-204, 1997.
4. Ashorobi RB, Williams PA. Indomethacin and alpha-tocopherol enhanced survival in endotoxic rats, *Cent. Afr. J. Med.* 41, 216-219, 1995.
5. Campanile F, Giampietri A, Grohmann U, Belladonna ML, Fioretti MC, Puccetti P. Evidence for tumor necrosis factor as a mediator of the toxicity of a cyclooxygenase inhibitor in Gram-negative sepsis, *Eur. J. Pharmacol.* 307, 191-199, 1996.
6. Tunçtan B, Uludağ O, Altuğ S, Abacıoğlu N. Effects of nitric oxide synthase inhibition in lipopolysaccharide-induced sepsis in mice, *Pharmacol Res.* 38, 405-411, 1998.
7. Tunçtan B, Altuğ S, Uludağ O, Abacıoğlu N. Time-dependent variations in serum nitrite, 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 levels induced by lipopolysaccharide in mice, *Biol. Rhythm. Res.* 31, 499-514, 2000.
8. Pettipher ER, Wimberly DJ. Cyclooxygenase inhibitors enhance tumor necrosis factor production and mortality in murine endotoxic shock, *Cytokine*, 6, 500-503, 1994.
9. Sironi M, Gadina M, Kankova M, Riganti F, Mantovani A, Zandalasini M, Ghezzi P. Differential sensitivity of in vivo TNF and IL-6 production to modulation by anti-inflammatory drugs in mice, *Int. J. Immunopharmacol.* 14, 1045-1050, 1992.
10. Griswold DE, Hillegass LM, Breton JJ, Esser KM, Adams JL. Differentiation in vivo of classical non-steroidal antiinflammatory drugs from cytokine suppressive antiinflammatory drugs and other pharmacological classes using mouse tumour necrosis factor alpha production, *Drugs Exp. Clin. Res.* 19, 243-248, 1993.
11. Butler AR, Flitney FW, Williams DLH. NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective, *Trends Pharmacol. Sci.* 16, 18-22, 1995.
12. Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine, *Proc. Natl. Acad. Sci. USA*, 90, 8103-8107, 1993.
13. Prakash H, Ali A, Bala M, Goel HC. Anti-inflammatory effects of Podophyllum hexandrum (RP-1) against lipopolysaccharides induced inflammation in mice, *J. Pharm. Pharm. Sci.* 8, 107-114, 2005.
14. Netea MG, Blok WL, Kullberg BJ, Bemelmans M, Vogels MT, Buurman WA, van der Meer JW. Pharmacologic inhibitors of tumor necrosis factor production exert differential effects in lethal endotoxemia and in infection with live microorganisms in mice, *J. Infect. Dis.* 171, 393-399, 1995.
15. Schade UF. Pentoxifylline increases survival in murine endotoxin shock and decreases formation of tumor necrosis factor, *Circ. Shock*, 31, 171-181, 1990.
16. Wu C-C, Liao M-H, Chen S-J, Yen M-H. Pentoxifylline improves circulatory failure and survival in murine models of endotoxemia, *Eur. J. Pharmacol.* 373, 41-49, 1999.
17. Milano S, Arcoleo F, Dieli M, D'Agostino R, D'Agostino PD, De Nucci G, Cillari E. Prostaglandin E_2 regulates inducible nitric oxide synthase in the murine macrophage cell line J774, *Prostaglandins*, 49, 105-115, 1995.
18. Louie A, Baltch AL, Franke MA, Ritz WJ, Smith RP, Singh JK, Gordon MA. Effect of pentoxifylline on the course of systemic *Candida albicans* infection in mice, *J. Antimicrob Chemother.* 37, 943-954, 1996.

19. Noel P, Nelson S, Bokulic R, Bagby G, Lippton H, Lipscomb G, Summer W. Pentoxifylline inhibits lipopolysaccharide-induced serum tumor necrosis factor and mortality, *Life Sci.* 47, 1023-1029, 1990.
20. Lundblad R, Ekstrom P, Giercksky KE. Pentoxifylline improves survival and reduces tumor necrosis factor, interleukin-6, and endothelin-1 in fulminant intra-abdominal sepsis in rats, *Shock*, 3, 210-215, 1995.
21. Jilg S, Barsig J, Leist M, Küsters S, Volk H-D, Wendel A. Enhanced release of interleukin-10 and soluble tumor necrosis factor receptors as novel principles of methylxantine action in murine models of endotoxic shock, *J. Pharmacol. Exp. Ther.* 278, 421-431, 1996.
22. Badger AM, Olivera DL, Esser KM. Beneficial effects of phosphodiesterase inhibitors BRL-61063, pentoxifylline, and rolipram in a murine model of endotoxin shock, *Circ. Shock.* 44, 188-195, 1994.
23. Hadjiminis DJ, McMasters KM, Robertson SE, Cheadle WG. Enhanced survival from cecal ligation and puncture with pentoxifylline is associated with altered neutrophil trafficking and reduced interleukin-1 β expression but not inhibition of tumor necrosis factor synthesis, *Surgery*, 116, 348-355, 1994.