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# Elevated Thromboxane Levels in the Rat during Endotoxic Shock: Protective Effects of Imidazole, 13-azaprostanoic Acid, or Essential Fatty Acid Deficiency

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# Elevated Thromboxane Levels in the Rat during Endotoxic Shock

## PROTECTIVE EFFECTS OF IMIDAZOLE, 13-AZAPROSTANOIC ACID, OR ESSENTIAL FATTY ACID DEFICIENCY

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ABSTRACT The potential deleterious role of the proaggregatory vasoconstrictor, thromboxane $A_2$, in endotoxic shock was investigated in rats. Plasma thromboxane $A_2$ was determined by radioimmunoassay of its stable metabolite thromboxane $B_2$. After intravenous administration of *Salmonella enteritidis* endotoxin (20 mg/kg), plasma thromboxane $B_2$ levels increased from nondetectable levels (<375 pg/ml) in normal control rats to 2,054±524 pg/ml ($n$ = 8), within 30 min to 2,071±429 at 60 min, and decreased to 1,119±319 pg/ml, at 120 min. Plasma levels of prostaglandin E also increased from 146±33 pg/ml in normal controls ($n$ = 5) to 2,161±606 pg/ml 30 min after endotoxin ($n$ = 5).

In contrast to shocked controls, rats pretreated with imidazole, a thromboxane synthetase inhibitor, or essential fatty acid-deficient rats, which are deficient in arachidonate and its metabolites, did not exhibit significant elevations in plasma levels of thromboxane $B_2$. Imidazole did not however inhibit endotoxin-induced elevations in plasma prostaglandin E. Essential fatty acid deficiency significantly reduced mortality to lethal endotoxic shock. This refractoriness could be duplicated in normal rats pretreated with the fatty acid cyclo-oxygenase inhibitor, indomethacin (10 mg/kg), intravenously 30 min before endotoxin injection. Imidazole (30 mg/kg) administered intraperitoneally 1 h before or intravenously 30 min before endotoxin, also significantly ($P < 0.01$) reduced mortality from lethal endotoxin shock to 40% compared to a control mortality of 95% at 24 h. Likewise pretreatment with 13-azaprostanoic acid (30 mg/kg), a thromboxane antagonist, reduced mortality from endotoxic shock at 24 h from 100% in control rats to only 50% ($P < 0.01$). The results suggest that endotoxin induces increased synthesis of thromboxane $A_2$ that may contribute to the pathogenesis of endotoxic shock.

## INTRODUCTION

During endotoxic shock there is an increased synthesis of prostaglandins from arachidonic acid (1–4). Their role in the pathogenesis of endotoxemia however, remains to be clearly elucidated (4–6). Endotoxic shock is also associated with thrombocytopenia, and pulmonary and mesenteric vasoconstriction (7–10). Thromboxane (TX)[1]$A_2$, another arachidonic acid metabolite, is a potent vasoconstrictor and platelet aggregator (11), thus raising the possibility that it may mediate these pathologic sequelae of endotoxemia. This postulate is further supported by previous studies demonstrating that essential fatty acid (EFA)-deficient rats that are depleted of prostaglandin and thromboxane precursors (12, 13) are resistant to endotoxic shock (14).

Fatty acid cyclo-oxygenase inhibitors such as indomethacin block both the formation of prostaglandins and $TXA_2$ from arachidonate. However, the discovery of selective thromboxane synthetase inhibitors such as imidazole (15, 16), and the thromboxane antagonist, 13-azaprostanoic acid (17), provide a means to specifically evaluate the role of $TXA_2$ in endotoxic shock. Thus, this study was undertaken to determine:

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[1] *Abbreviations used in this paper*: EFA, essential fatty acid; PG, prostaglandin; TX, thromboxane.

(*a*) if $TXA_2$ synthesis is increased during endotoxemia; and (*b*) the effects of indomethacin, imidazole, 13-azaprostanoic acid and EFA deficiency on endotoxin-induced mortality in rats.

## METHODS

*Rats.* All rats used in the experiments came from an inbred Long-Evans rat colony maintained by the investigators. The rats employed in these studies were 7–13 wk old.

Control rats were fed the usual colony diet that contained a minimum of 24% protein, 4.5% fiber, and 4% fat (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.). Of the fat content, 36.66% is linoleic acid, 2.45% linolenic acid, and 4.16% arachidonic acid.

EFA-deficient rats were fed a fat-free diet obtained from ICN Nutritional Biochemical (Cleveland, Ohio), consisting of 21.10% vitamin-free casein, 16.45% alphacel, 58.45% sucrose, 4.00% salt mixture, and vitamin supplements. The protocol for induction of EFA-deficiency has been described in Cook et al. (14).

*Radioimmunoassay of $TXB_2$ and prostaglandin (PG)E.* Blood, obtained via cardiac puncture, for $TXB_2$ and PGE determinations was collected into plastic syringes that contained 0.1 ml indomethacin (100 μg/ml) dissolved in 0.1 M sodium phosphate buffer, pH 8.0, and three drops of heparin (1,000 U/ml). The blood was centrifuged (400 *g*) at 4°C in a refrigerated centrifuge and the plasma was collected and frozen at −20°C until extraction. To 1 ml of plasma, [$^3$H]$TXB_2$ or [$^3$H]$PGE_2$ (1,500 cpm) was added to correct for recovery losses. The plasma was acidified to pH 3.5 with formic acid and extracted twice with 3 vol of ethyl acetate. The ethyl acetate layer was removed and dried under $N_2$. The dried extract was reconstituted and applied to a silicic acid column (0.5 g) and the PGE and $TXB_2$ fraction was collected and dried under nitrogen (18). The dried extract was reconstituted in 1 ml gelatin phosphate-buffered saline (18). $TXB_2$ was determined using a previously described radioimmunoassay. After incubation for 4 h at 37°C, free [$^3$H]$TXB_2$ was separated from bound using charcoal-dextran solution (18). The antibody was provided by Dr. J. B. Smith, Cardeza Foundation, Philadelphia, Pa. The minimum detectable amount in 1 ml of plasma was ~375 pg/ml. The antibody does not significantly cross-react (≤0.04%) with other prostaglandins and the interassay variability is 17% ($n$ = 10) (18). PGE was determined using a previously described radioimmunoassay after conversion to PGB (19).

*Materials.* The following were purchased from commercial sources: lipopolysaccharide, Boivin preparation of *Salmonella enteritidis* Difco. Laboratories, Detroit, Mich., control 690467; [5,6,8,9,11,12,14,15-$^3$H]$TXB_2$ (60–70 Ci/mM) and [5,6,8,11,12,14,15-$^3$H]$PGE_2$ (117 Ci/mM), New England Nuclear, Boston, Mass.; organic solvents, Burdick & Jackson Laboratories, Muskegon, Mich.; silicic acid, Biosil A 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.; charcoal and dextran, Schwarz/Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.; imidazole, Aldrich Chemical Co., Milwaukee, Wis. The following were gifts: $TXB_2$ and $PGE_2$ standards, Dr. John Pike, Upjohn Company, Kalamazoo, Mich.; 13-azaprostanoic acid, Dr. Guy Lebreton and Dr. Duane Venton, University of Illinois, Chicago, Ill., and Dr. Peter Anzeveno and Dr. Robert Broersma, Dow Pharmaceuticals, Indianapolis, Ind.

Indomethacin and imidazole were dissolved in phosphate-buffered saline, and 13-azaprostanoic acid was prepared by dissolving 60 mg in 0.07 ml of ethanol, 0.25 ml of 1 N NaOH and 3.68 ml of saline.

*Statistical analysis.* Statistical analysis of differences in $TXB_2$ levels was based upon the Student's $t$ test (20). The chi-square test (20) was employed for mortality studies. All data is expressed as mean±SE of the mean.

## RESULTS

*Plasma $TXB_2$ time-course.* The time-course of appearance of $TXB_2$ in plasma after intravenous endotoxin (20 mg/kg) was determined at 30, 60, and 120 min ($n$ = 8 per period) postinjection (Fig. 1). Within 30 min after endotoxin injection, plasma $TXB_2$ levels increased from nondetectable levels (<375 pg/ml) in normal control rats ($n$ = 10) to 2,054±524 pg/ml ($P < 0.01$), peaked at 60 min, 2,071±429 pg/ml, and started to decline by 120 min to 1,119±319 pg/ml (Fig. 1). Plasma PGE levels also increased from 146±33 pg/ml ($n$ = 5) in nonshocked controls to 2,161±606 pg/ml ($n$ = 5) 30 min after endotoxin administration. Plasma $TXB_2$ levels failed to rise significantly at either 30 or 60 min after endotoxin in control rats pretreated 60 min before injection with imidazole (30 mg/kg i.p.) or in EFA-deficient (7–8 wk old) rats (Fig. 1). Plasma PGE levels, however, remained elevated (1,669±475 pg/ml) in the imidazole pretreated group ($n$ = 5) and did not vary significantly from shocked controls.

*Mortality studies.* Having established that imidazole and EFA-deficiency markedly reduced plasma $TXB_2$ levels after endotoxin, we sought to determine if drugs that alter prostaglandin or $TXA_2$ formation and EFA deficiency would improve survival from endotoxic shock. Within 5 h after endotoxin injection, mortality in shocked control rats was 50% (Table I) as compared to no mortality in EFA-deficient rats in

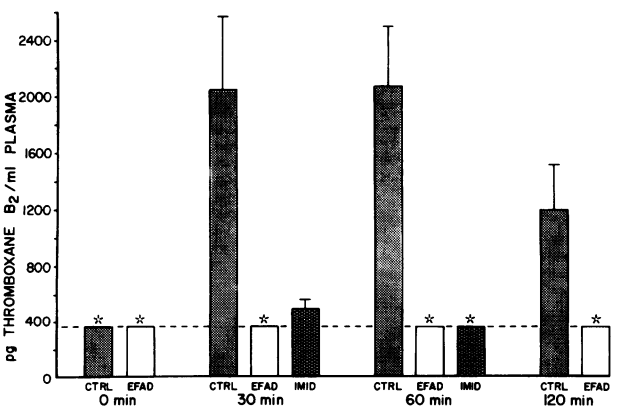


FIGURE 1 Plasma $TXB_2$ levels in control (CTRL), imidazole (IMID) treated, and EFA-deficient (EFAD) rats at 30, 60, 120 min after intravenous *S. enteritidis* endotoxin (20 mg/kg). Imidazole was administered intraperitoneally (30 mg/kg) 60 min before endotoxin. Data expressed as mean±SEM, $n$ = 8–10 rats per group. Both imidazole-treated and EFA-deficient rats varied significantly from control ($P < 0.01$). (*) Minimum detectable plasma $TXB_2$ was 375 pg/ml plasma.

TABLE I
*Protective Effect of EFA Deficiency, Indomethacin, or Imidazole in Endotoxic Shock*

| Group | Time after endotoxin, h | | | |
|---|---|---|---|---|
| | 5 | 12 | 24 | 48 |
| | *Dead/Total* | | | |
| Control | 14/20 | 19/20 | 19/20 | 19/20 |
| EFAD (7–8 wk old) | 0/12 | 1/12 | 7/12 | 8/12 |
| EFAD (12–13 wk old) | 0/14 | 0/14 | 0/14 | 0/14 |
| Indomethacin i.v. | 0/8 | 0/8 | 1/8 | 1/8 |
| Imidazole i.v. | 1/10 | 2/10 | 4/10 | 4/10 |
| Imidazole i.p. | 1/11 | 4/11 | 4/11 | 4/11 |

Imidazole (30 mg/kg) was administered intravenously 30 min or intraperitonealy 60 min before endotoxin. Indomethacin (10 mg/kg was administered intravenously 30 min before intravenous *S. enteritidis* endotoxin (20 mg/kg).

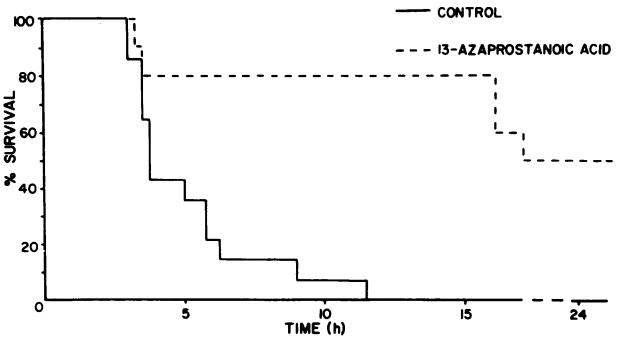


FIGURE 2 Survival time of control ($n$ = 14) and 13-azaprostanoic acid treated rats ($n$ = 10). The antagonist was injected intravenously (30 mg/kg) conjointly with *S. enteritidis* endotoxin (20 mg/kg). Controls received an isovolumetric dose of the injection vehicle. Survival at 24 h was significantly ($P < 0.01$) different between treated and control rats.

both the 7- to 8-wk-old and 12- to 13-wk-old group ($P < 0.01$). Within 24 h, control groups exhibited an approximate 95% mortality relative to a 60% mortality in the younger EFA-deficient rats ($P < 0.05$). The older EFA-deficient rats, however, were totally refractory to the shock and exhibited a 100% survival ($P < 0.001$). Indomethacin, like EFA deficiency, markedly improved survival as denoted by only a 12.5% mortality within 24 h ($P < 0.01$). The mortality in the imidazole-treated groups was 9–10% at 5 h postendotoxin injection and 36–40% within 24 h ($P < 0.01$). Subsequent mortality patterns did not change over an observed 48-h period with the exception of a 67% mortality in EFA-deficient rats.

To further test the hypothesis that $TXA_2$ is a significant pathogenic factor in endotoxic shock, we evaluated the effect of a $TXA_2$ antagonist, 13-azaprostanoic acid. Rats received 13-azaprostanoic acid (30 mg/kg) intravenously, or its vehicle simultaneously with the injection of endotoxin. Within 4 h 50% of the shocked control rats ($n$ = 14) were dead as opposed to only a 20% mortality in the 13-azaprostanoic acid-treated group. By 24 h no rats survived in the control group, whereas 50% of the rats ($P < 0.01$) pretreated with 13-azaprostanoic acid survived (Fig. 2). Indeed 40% of these rats survived over an observed 72 h period.

## DISCUSSION

The therapeutic efficacy of fatty acid cyclo-oxygenase inhibitors in improving endotoxic shock has not been reported to be uniformly beneficial (4–6). Some of these variable results may reflect differences in the route and time of administration of the drugs as well as species variation. Our results however clearly demonstrate that intravenous administration of indomethacin (10 mg/kg) before induction of endotoxic shock in the rat markedly improves survival. Indomethacin has other modes of action in addition to blocking fatty acid cyclo-oxygenase that may be salutory to its protective effect (4, 21). However, EFA-deficient rats, which are deficient in arachidonate and its metabolites (i.e., prostaglandins and $TXA_2$), also are remarkably refractory to highly lethal endotoxic shock. The combined results of these two separate experimental approaches, therefore suggest a role for prostaglandins and/or $TXA_2$ in the pathogenesis of endotoxic shock.

Subsequent studies were undertaken to discern which of these arachidonate metabolites are potentially deleterious in shock. Our results demonstrated that $TXA_2$, a potent platelet aggregator and vasoconstrictor (11), increases early and markedly during endotoxic shock in rats. Imidazole significantly suppressed plasma $TXB_2$ levels and reduced mortality from endotoxic shock, however, it did not significantly modify endotoxin induced elevations of PGE. These observations are consistent with the concept that imidazole exerts its protective action via inhibition of thromboxane synthetase and/or shunting the metabolism of arachidonic acid to prostacyclin, an anti-aggregatory, vasodilator prostaglandin (22, 23). Imidazole like indomethacin has numerous other effects (24), therefore, the lower levels of plasma $TXB_2$ may have been the result of a protective action of imidazole not mediated through inhibition of thromboxane synthetase. However, the postulate that $TXA_2$ is a pathogenic factor in shock, is further supported by the observation that 13-azaprostanoic acid, a $TXA_2$ antagonist (17) which does not alter arachidonic acid metabolism, nor effect the actions of prostacyclin, or $PGE_1$, was effective in decreasing lethality caused by endotoxic shock. Furthermore, EFA-deficient rats that are refractory to the lethal endotoxic shock did not

exhibit elevated plasma $TXB_2$ levels. Because of the altered hemodynamic and thrombotic events observed during endotoxic shock (7–10), and the above observations, $TXA_2$ may well be a primary factor eliciting the early cascade of pathogenic events ultimately progressing to irreversibility.

Our composite observations therefore suggest a potential benefit of three therapeutic modalities in endotoxic shock (i.e., fatty acid cyclo-oxygenase inhibitors and selective $TXA_2$ synthetase inhibitors or antagonists). Because optimum doses and regimens of treatment are yet to be established, it is possible that selective inhibition of $TXA_2$ formation, coupled with diversion of arachidonic acid metabolism toward increased prostacyclin synthesis (21, 22, 25), or $TXA_2$ antagonism, may prove to be more appropriate therapeutic approaches. The subsequent delineation of the tissue sources and potential pathogenic role of $TXA_2$ in endotoxic shock clearly merits further investigation.

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