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Selective Thromboxane Synthetase Inhibition by Picotamide and Effects on Endotoxin-Induced Lethality (42637)

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Abstract. The efficacy of *N,N'*-bis-(3-picolyl)-methoxyisophthalamide (picotamide) as an *in vitro* thromboxane synthetase inhibitor and its effect on endotoxin (LPS)-induced lethality in rats were assessed. Picotamide at 0.5 and 1.0 m*M* concentrations significantly ($P < 0.05$) inhibited basal and LPS-stimulated synthesis of TxA_2 measured by its stable immunoreactive (i) metabolite TxB_2 in rat peritoneal macrophages. This compound did not inhibit synthesis of i6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, and produced significant shunting to i6-keto-$PGF_{1\alpha}$. For lethality studies rats were pretreated, by gavage with picotamide, at either 75, 150, 300, or 600 mg/kg 2 hr prior to iv *S. enteritidis* (LPS, 20 mg/kg). Both 150 and 300 mg/kg doses of picotamide significantly ($P < 0.05$) improved survival in endotoxin shock at 48 hr. These studies demonstrate that picotamide is a selective thromboxane synthetase inhibitor, and that it may be useful during disease states characterized by increased TxA_2 synthesis.

Bacterial endotoxins, lipopolysaccharides (LPS), are known to elicit a broad spectrum of biological responses including lethal shock in laboratory animals (1, 2). Among the variety of endogenous substances stimulated by LPS, the arachidonic acid metabolites have been implicated as significant pathogenic mediators (3, 4). Administration of LPS in several species of laboratory animals has been shown to enhance synthesis of the fatty acid cyclooxygenase products thromboxane A_2 (TxA_2) and prostacyclin (PGI_2) (5–8). TxA_2, a potent vasoconstrictor, bronchoconstrictor, and platelet aggregator, has been implicated as a possible mediator of certain pathogenic sequelae of endotoxic shock (3, 4). Several specific thromboxane synthetase inhibitors including imidazole (9), 7-IHA (10), Dazoxiben (11), pyridine derivatives (12), and OKY-1581 (13) have been tested in experimental endotoxic shock in the rat. These compounds have been shown to ameliorate many of the deleterious effects of endotoxemia such as reductions in systemic coagulopathies (4), reduced lysosomal labialization (9, 13), improved cardiac output and tissue perfusion (14), and enhanced survival (9, 10, 13).

Previous studies with *N,N'*-bis-(3-picolyl)-methoxyisophthalamide (G137, or picotamide) have shown that this compound has a chemical structure similarity and pharmacologic actions suggestive of a thromboxane synthetase inhibitor and receptor antagonist (15, 16). Picotamide has been shown to be well tolerated during prolonged oral administration in patients and to have effects on coagulation, fibrinolysis, and platelet aggregation (17, 18). Peritoneal macrophages have been shown to synthesize TxA_2 and PGI_2 in response to endotoxin stimulation *in vitro* and to provide a useful approach to test the pharmacologic selectivity of putative drugs that alter arachidonic acid metabolism (3, 19). These observations prompted us to assess the potential thromboxane synthetase inhibitory action of picotamide. Specifically, the effect of this compound on *in vitro* synthesis of immunoreactive (i) TxB_2, the stable metabolite of TxA_2, and i6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, by rat peritoneal macrophages stimulated with endotoxin was examined. Since TxA_2 has been implicated as a pathogenic mediator of endotoxemia, we further tested the effect of picotamide pretreatment in rats on LPS-induced lethality.

Materials and Methods. For *in vitro* experiments, resident peritoneal cells were removed from male, Long–Evans rats by peritoneal lavage with RPMI 1640 medium with

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L-glutamine (GIBCO, Grand Island, NY), containing penicillin (50 units/ml), streptomycin (50 μg/ml), and sodium heparin (10 units/ml), accordingly with a previously reported procedure (19). The harvested cells were diluted to 1×10^6 cells/ml, plated on plastic petri dishes (4 ml/dish), and allowed to adhere for 2 hr at 37°C in 95% air/5% CO_2. Nonadherent cells were removed by washing the plates 3 times with 5% dextrose. For *in vitro* studies, picotamide was dissolved in a mixture of sterile saline:dimethylsulfoxide (10:1; picotamide vehicle). Such a vehicle, with or without the proper amount of picotamide, was subsequently diluted 20 times in RPMI 1640 medium, adjusted to pH 7.4, and added to the cell cultures. Cells were incubated with fresh RPMI containing only the picotamide vehicle (group 1); picotamide (1 m*M*; group 2), *S. enteritidis* LPS (Difco Lab., Detroit, MI) plus picotamide (0.5 m*M*, group 3), LPS plus picotamide (1 m*M*), LPS picotamide (1 m*M*, group 4), or LPS plus picotamide vehicle (group 5). The dose of LPS used *in vitro* was 50 μg/ml. After a 4-hr incubation, the media samples were collected and stored at −20°C for later analysis of $iTxB_2$ and i6-keto-$PGF_{1\alpha}$.

The radioimmunoassay of $iTxB_2$ and i6-keto-$PGF_{1\alpha}$ was carried out as previously described (20). 14,15-^{3}H(N)-TxB_2 and 14,15-^{3}H(N)- i6-keto-$PGF_{1\alpha}$ were purchased from New England Nuclear (Boston, MA).

Male Sprague–Dawley rats (250–320 g/body wt), fed on a standard diet and with *ad libitum* tap water, were used for mortality experiments. Endotoxic shock was induced by a single intravenous (iv) injection of 20 mg/kg (LD_{90}) of *S. enteritidis* LPS. Picotamide, dissolved in carboxymethyl-cellulose, was given by gavage to the rats at doses of 75, 150, 300, or 600 mg/kg, 2 hr before endotoxin injection. Control rats received only picotamide vehicle 2 hr before endotoxin challenge (20 mg/kg, iv). Survival was recorded at 1, 2, 4, 6, 12, 18, 24, and 48 hr after LPS injection.

The data from *in vitro* experiments were subjected to statistics by the Student *t* test for unpaired data. The χ^2 test was used for statistical analysis of survival results.

Results. Rat peritoneal macrophages tested with 1 m*M* of picotamide in the culture medium underwent a significant ($P < 0.05$) reduction of $iTxB_2$ and an increase ($P < 0.001$) of i6-keto-$PGF_{1\alpha}$ release (from 7465 ± 2568 to 1622 ± 158 pg/ml and from 8600 ± 1267 to 18303 ± 610 pg/ml, respectively), compared to cells incubated with medium containing vehicle alone (Fig. 1). Macrophage cultures with LPS plus picotamide vehicle showed a significant increase of $iTxB_2$ concentration ($P < 0.05$ vs basal values). $iTxB_2$ concentration was significantly reduced ($P < 0.01$) in LPS-stimulated picotamide-treated (both of the doses) culture medium in comparison to the amount found in the media containing LPS plus picotamide vehicle (Fig. 1).

In LPS-stimulated cell cultures i6-keto-$PGF_{1\alpha}$ concentrations were 10460 ± 3715 in the vehicle-treated group, and 26651 ± 3093 and 16872 ± 3481 pg/ml, respectively, in the 0.5 and 1 m*M* picotamide-treated groups. Thus 0.5 m*M*, but not 1 m*M*, of the drug significantly ($P < 0.05$ vs LPS plus vehicle group) enhanced 6-keto-$PGF_{1\alpha}$ in the cell culture media containing LPS.

At 48 hr following LPS (20 mg/kg, iv) administration in picotamide vehicle-pretreated rats, a progressive decrease of survival rate was observed, resulting in a 10.5% survival at 48 hr after LPS (Table I). However, LPS-injected animals pretreated with

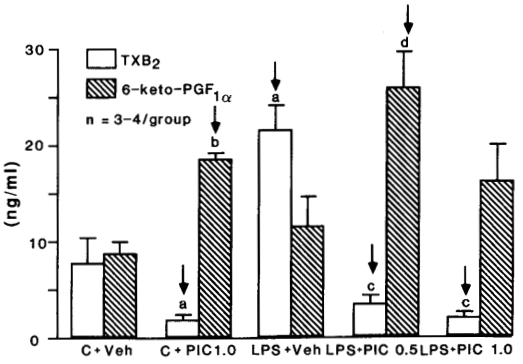

FIG. 1. Macrophage *in vitro* synthesis of $iTxB_2$ and i6-keto-PGF_{1a} in control (C) and *S. enteritidis* endotoxin (LPS, 50 μg/ml) stimulated cells, treated either with picotamide vehicle (Veh) or with picotamide 0.5 m*M* (PIC 0.5) and 1.0 m*M* (PIC 1.0). Data are expressed as means ± S.E. (a) $P < 0.05$ vs C + Veh; (b) $P < 0.01$ vs LPS + Veh; (c) $P < 0.001$ vs C + Veh; (d) $P < 0.05$ vs LPS + Veh.

TABLE I. SURVIVAL AT SEVERAL TIMES AFTER ENDOTOXIN ADMINISTRATION[a]

Group	Time (hr): 0	% Survival 1	2	4	6	12	18	24	48
Controls (N = 19)	100	100	94.7	73.6	52.6	26.3	21.0	13.7	10.5
PIC (mg/kg)									
75 (N = 9)	100	100	100	88.8	77.7[b]	33.3	22.2	11.1	0
150 (N = 10)	100	100	100	100	80.0	50.0	30.0	30.0[b]	30.0[b]
300 (N = 12)	100	100	100	100	75.0	58.0	41.0	41.0[c]	41.0[c]
600 (N = 15)	100	100	100	93.3	60.0	40.0	20.0	13.3	6.6

[a] Rats pretreated with picotamide (PIC; 75, 150, 300, 600 mg/kg), or its vehicle (Controls), were orally administered 2 hr before *S. enteritidis* endotoxin (20 mg/kg, iv) injection.
[b] $P < 0.05$ vs Controls.
[c] $P < 0.01$ vs Controls.

150 or 300 mg/kg of picotamide exhibited significant ($P < 0.05$ and $P < 0.01$, respectively) protection against LPS-induced lethality at 48 hr. The lowest dose (75 mg/kg) demonstrated some beneficial effects, only within 12 hr from LPS injection. Pretreatment with 600 mg/kg of picotamide did not significantly improve the survival rate in our experiments (Table I).

Discussion. This study demonstrates that picotamide is a highly selective thromboxane synthetase inhibitor *in vitro*. Both basal $iTxB_2$ synthesis and endotoxin-stimulated synthesis of $iTxB_2$ by peritoneal macrophage were significantly reduced at the two concentrations of picotamide tested (0.5 and 1 mM). Indeed, $iTxB_2$ in the 1 mM picotamide-treated macrophages was reduced to 7.2% compared to that seen in control cells stimulated with endotoxin. On the other hand, both basal and endotoxin-stimulated 6-keto-$PGF_{1\alpha}$ levels were significantly enhanced in picotamide-treated cells. The latter data demonstrate both selectivity of picotamide on inhibition of thromboxane synthetase and cellular shunting of endoperoxide metabolites through prostacyclin synthetase. This shunting phenomenon, following thromboxane inhibition in macrophages, concurs with previous *in vitro* studies with dazoxiben (10) and pyridine derivatives (12).

Our observations also demonstrate that pretreatment of rats *in vivo* with picotamide 2 hr prior to induction of endotoxic shock significantly improved both survival time and overall mortality. These observations are thus consistent with previous studies demonstrating improved survival in rats with thromboxane synthetase inhibitors such as imidazole (9), 7-IHA (9), dazoxiben (10, 11), pyridine derivatives (12), and OKY-1581 (13). Therefore, these results, with a number of structurally dissimilar thromboxane synthetase inhibitors, would appear to further corroborate a pathogenic role of TxA_2 in endotoxic shock in the rat. It is possible that preservation of PGI_2 or actual endoperoxide shunting to PGI_2 in the presence of thromboxane synthetase inhibition may be beneficial. However, a previous study has shown that the stable PGI_2 analog, iloprost, is not protective in rats during endotoxic shock (21). The effect of picotamide on LPS-induced lethality was also dose dependent, with 150 and 300 mg/kg providing optimal protection determined by 48-hr survival. The highest dose of the compound was less effective, suggesting the possibility that other pharmacologic effects may be manifested at these doses. Similar dose-dependent effects of aspirin (20) and ibuprofen (23) have been reported on LPS-induced pathophysiologic sequelae and lethality in rats.

In conclusion, picotamide is a selective thromboxane synthetase inhibitor as demonstrated by its selective inhibition of macrophage $iTxB_2$ production. The observations that this compound also improved endotoxin lethality in rats, similar to that seen with structurally dissimilar thromboxane synthetase inhibitors, further supports the notion that this pharmacologic agent is an efficacious inhibitor. These findings thus suggest picotamide may be a useful drug in

disease states characterized by increased TxB_2 synthesis.

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