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EFFECT OF TOXINS ON ARACHIDONIC ACID METABOLISM IN CULTURED RAT PULMONARY ALVEOLAR MACROPHAGES

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Summary: The action of a trichothecene (T-2), microcystin-LR and saxitoxin on arachidonic acid metabolism in cultured rat alveolar macrophages was studied. Pulmonary macrophages exposed to 1μM T-2 were stimulated to synthesize and increase in release thromboxane B2 (TxB_2) by 56% and 6-Keto$F_{1\alpha}$ (73%). Microcystin-LR induced a significant increase in release of prostaglandins $F_{2}\alpha$,(40%), PGE_2(75%) and TxB_2 (69%), as compared to controls. Saxitoxin increased TxB_2 release by 37%. Arachidonic acid release was stimulated by all three toxins tested. The release of arachidonic acid and its metabolites by alveolar macrophages exposed to T-2 toxin was partially blocked by fluocinolone (1 μM). These results suggest that macrophages synthesize and release inflammatory mediators in response to toxin exposure, and fluocinolone may protect against T-2 toxicosis.

Some natural toxins are potent and powerful inflammatory agents (1,2). For example, dermal administration of a T-2 toxin causes skin irritation (3), edema (4,5), and cellular damage (6). Intraruminal injection of *Microcystin aeruginosa* in sheep results in edema of the lung and neutrophil accumulation within the pulmonary arterial tree (7). In these studies the mechanism of irritant action was not determined nor were the inflammatory mediators

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identified, although it is possible that release of inflammatory mediators may be an early step in toxin-induced injury. In a recent study, dexamethasone increased the survival of rats exposed to a lethal dose of T-2 toxin (8). Similarily, hydrocortisone prevented the development of thrombocytopenia in mice treated with microcystin-LR (9). The authors speculated that glucocorticoids inhibit toxicosis by blocking the release of free arachidonic acid through inhibition of phospholipase A_2 activity, and consequently, block the synthesis and the release of both cyclooxygenase and lipooxygenase products (10).

Macrophages are known to release a wide array of inflammatory mediators, such as proteolytic enzymes, chemotactic proteins, and arachidonic acid metabolites (11-13). To study the possible role of macrophages in the inflammatory response to natural toxins, we examined the effect of T-2, microcystin-LR known inflammatory agents, and also included saxitoxin (not yet known to cause inflammatory reactions in any tissues) on arachidonic acid metabolism in rat alveolar macrophages. Alveolar macrophages were selected because they respond immediately to various physiological stimuli by synthesizing and releasing immunoregulatory substances including arachidonic acid cascade (14-15).

Materials and Methods

Macrophages

Lungs from 200-280 g Fisher 344 (Charles River, Wilmington, MA) rats were removed and macrophages were isolated by lavage and cultured in 35-mm culture plates (Falcon Labware, Division of Becton-Dickson Co., Oxford, CA) by the procedure described by Grundfest et al. (16). Non-adherent cells were removed by washing the cells twice with Hank's balanced salt solution (HBSS). The adherent cell population contained 95% alveolar macrophages as determined by morphology and biochemical criteria (16).

Macrophage Labeling and Stimulation

After overnight culture, the medium was removed and the cells washed twice with HBSS. [^{14}C]Arachidonic acid (^{14}C-AA, sp. act. 52.7 mCi/mmole, New England Nuclear, Boston, MA) in 0.1% lipid-free bovine serum albumin (BSA) in medium 199 was added to the cells. Cultures were then incubated for 60 min at 37°C under 5% CO_2 and 95% air. The cells were washed with HBSS, incubated for another hour with medium 199 containing 10% fetal calf serum (FCS), and then washed three times with HBSS to remove any unincorporated ^{14}C-AA. These ^{14}C-AA-prelabeled macrophages were used to study the release of arachidonic acid and its metabolites. Experiments with T-2 toxin, microcystin-LR and saxitoxin were done in 1 ml HBSS containing 0.1% BSA. The effect of fluocinolone on arachidonic acid metabolite release was determined by stimulating macrophages in the presence or absence of 1μM fluocinolone for 16 hr in culture medium. Cultures were incubated for 2 hr, with final concentrations of toxins as indicated in the Tables. N-ethylmaleimide (NEM, Sigma Chemical Co., St. Louis, Mo), and calcium ionophore A23187 (Calbiochem, Los Angeles, CA) were used as positive controls (16). All experiments were done at least 3 times in duplicate. Cell viability was monitored by trypan blue exclusion and plates were checked by phase contrast microscopy for floating and dead cells.

Extraction of Prostaglandins

After incubation with toxins or other agents, the cultures were placed on ice. The culture medium (HBSS with 0.1% BSA) was removed, the plates were washed with 1 ml HBSS media and washes were pooled and were extracted with chloroform:methanol (1:2 v/v) containing 0.005% butylated-hydroxy toluene (BHT, Sigma Chem. Co., St. Louis, MO) for prostaglandin measurement. In order to partition the extract, additional chloroform and water were added. The final volume of solvents was chloroform:methanol:water (2:2:1.8 v/v). The aqueous phase was washed with 2 ml of chloroform. The chloroform phases were pooled, their total lipid extract evaporated to dryness under nitrogen, and they were stored under nitrogen at -20°C.

Determination of Prostaglandins

Precoated Silica Gel-60 thin layer chromatographic (TLC) plates (E. Merck, American Scientific Products, Columbia, MD) were washed with ethanol and dried overnight at room temperature. The plates were activated at 100°C for 60 min before use. Lipid residue from media was dissolved in 0.1 ml chloroform:methanol (2:1) and quantitatively applied to plates. TLC plates were developed in ethylacetate and formic acid (80:1 v/v) to separate arachidonic acid and its metabolites. Arachidonic acid metabolites produced by alveolar macrophages were identified by comparing the Rf values to known standards (Sigma Chem. Co., St. Louis, MO). The standards and their corresponding Rf values were 6 Keto $F_{1\alpha}$, 0.24; $PGF_{2}2\alpha$, 0.36; TxB_2, 0.57; PGE_2 0.63; and arachidonic acid, 0.95. Recovery of these products was always >80% as determined by co-migration of radiolabeled prostaglandin standards. Protein was measured by the Lowry method (17).

Scintillation Spectrometry

Thin-layer chromatograms were exposed to iodine vapor, and lipid spots were identified by their co-migration with their respective standards.

Resolved and identified compounds were scraped, extracted with ethanol for 30 min, and counted for radioactivity in a Beckman scintillation counter. The counting efficiency for ^{14}C was always >95%.

T-2 toxin [3α-hydroxy-4β, 15-diacetoxy-8α-(3-methylbutyryloxy) 12, 13 - epoxy trichothe-9-ene) was purchased from Romer Laboratory, St. Louis, MO. Microcystin-LR and saxitoxin were obtained from Dr. Wayne Carmichael, Wright State University, Dayton, OH, and Dr. Samuel Page, Food and Drug Administration, Washington, D.C., respectively.

Statistical Analysis. Statistical significance was evaluated by student "t" test. Relationship between different toxins was estimated by using SAS procedures of one-way analysis of variance with Tukey's method for comparison of means.

Results

Treatment of alveolar macrophages in culture with T-2, microcystin-LR or saxitoxin for 2 hr resulted in toxin induced release of arachidonic acid metabolites with some toxin specificity (Fig. 1). T-2 toxin treatment of macrophages resulted in significant stimulation of 6-Keto $F_1\alpha$ production when compared to control, microcystin-LR or saxitoxin exposure ($p < 0.05$, one-way analysis of variance). Similar doses of saxitoxin and microcystin-LR showed no effect as compared to control. Microcystin-LR induced a significant release of $PGF_2\alpha$ and PGE_2, but neither T-2 nor saxitoxin affected the release of these compounds. Thromboxane (TxB_2) and arachidonic acid were released by all the toxins, though the magnitude of effect by saxitoxin was less than that of T-2 or microcystin-LR (Fig. 1). The calcium ionophore A23187, stimulated the release of all arachidonic acid metabolites except $PGF_2\alpha$, while NEM failed to induce the release of $PGF_2\alpha$ and 6-Keto $F_1\alpha$, (Table 1).

Pretreatment of macrophages with fluocinolone, a potent fluorinated glucocorticoid, completely abolished the stimulatory effect of T-2 on TxB_2, and partially reduced the release of 6-Keto $F_1\alpha$ and free arachidonic acid (Table 2). Other metabolites were not significantly affected by steroid pretreatment in T-2 exposed cultures (data not shown).

Discussion

Recent studies with inhaled T-2 toxin demonstrate that several trichothecene toxins can reduce the phagocytic activity of pulmonary macrophages (18). Reduction in phagocytic activity could result in suppression of antibody synthesis (19) and pulmonary immune function (18) which suggest an important pathophysiologic role of toxins. We compared the effect of T-2-induced release of cyclooxygenase products with that of saxitoxin and microcystin-LR, as well as with known irritants such as calcium ionophore A23187 and sulfhydryl NEM. Stimulation of T-2, induced release of arachidonic acid metabolites in alveolar macrophages was comparable to microcystin-LR but varies in specificity (Fig. 1). Saxitoxin was least effective in inducing the release of arachidonic acid metabolites, probably due to non-inflammatory nature of the toxin. We observed a large increase in release of TxB_2 and 6-Keto $F_{1\alpha}$ and moderate stimulation of PGE_2 by T-2 toxin in alveolar macrophages. Shohami et al. (2, 8) reported a similar results of arachidonic acid metabolism by T-2 toxin in regions of the brain.

The inhibition of T-2 toxin-induced arachidonic acid release in fluocinolone-treated macrophages is consistent with the prevailing hypotheses concerning the mechanism by which glucocorticoids inhibit prostaglandin synthesis, contributing to their in-vivo, anti-inflammatory effect (19, 20). Pretreatment of macrophages with steriod significantly augmented the T-2 induced release of TxB_2 and partially inhibited the release of 6-Keto F1α and free arachidonic acid from cellular membrane. This suggests the possibility that fluocinolone may modify arachidonic acid metabolism beyond phospholipase activation by T-2 toxin, and probably also regulate cyclooxygenase synthetic and/or degradative enzymes (22) which nas yet to be determined. At any rate,

the data presernted here are compatible with other reports (23) that the effects of steriods are not limited to induction of phospholipase A_2 inhibitory protein.

Dunham et al. (24) reported that leukotriene B_4 (LTB_4) and LTD_4 influence the biosynthesis and release of TxB_2. Earlier studies indicate that sulfhydryl reagents also stimulate the release of TxB_2 and LTB_4, but fail to influence PGE_2 and LTC_4 synthesis by inhibiting endoperoxide PGE_2-isomerase and leukotriene C_4 synthetase, preventing the conversion of arachidonic acid to PGE_2 and LTC_4, promoting the formation of TxB_2 in alveolar macrophages (16, 25). In this study, another sulfhydryl reagent, NEM, stimulated the release of TxB_2 by 92%. The TxB_2 production by macrophages may be mediated via a sulfhydryl reaction, thus favoring the synthesis of TxB_2, which is probably controlled by LTB_4 production (26). These results suggest that toxins and sulfhydryl agonists influenced alveolar macrophages in similar fashion to release certain inflammatory materials.

The data from this study together with data from Shahami *et al.*, (8) and Adams *et al.*, (9) suggest that a possible pathogenesis of toxicity of T-2 and microcystin-LR may involve arachidonic acid metabolism.

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Fig. 1. Cultured alveolar macrophages were labeled with ^{14}C-arachidonic acid for 60 min, washed cells were then exposed to 1.0 μM T-2 toxin, saxitoxin, or microcystin-LR for 2 hr. The media were collected, extracted, and subjected to TLC for prostaglandin separation and measurement. Data represent mean ± SEM of 3 separate cultures in duplicate.

* P-value significant at least <0.05 from control by student "t" test.

Values estimated by one-way analysis of variance test indicate T-2 treatment significantly different at <0.05 level in 6-Keto F1α production from microcystin-LR and saxitoxin, and arachidonic acid release from saxitoxin. Microcystin-LR treatment stimulated the release of PGE_2 as compared to T-2 and saxitoxin ($p < 0.05$).

Table 1
Effect of NEM and A23187 Ionophore on Prostaglandin Release
by Alveolar Macrophages in Culture

DPM/Plate

Treatment	6-Keto F1α	$PGF_{2\alpha}$	PGE_2	TxB_2	AA
Control	309 ± 72	449 ± 49	311 ± 29	448 ± 29	2,654 ± 379
NEM (5 μM)	529 ± 93 (NS)	476 ± 32 (NS)	490 ± 23 (< 0.05)	860 ± 82 (< 0.005)	5,158 ± [illegible] (<0.05)
A23187 (10 μM)	872 ± 42 (<0.005)	531 ± 51 (NS)	614 ± 68 (< 0.01)	820 ± 75 (< 0.005)	5,029 ± 427 (< 0.01)

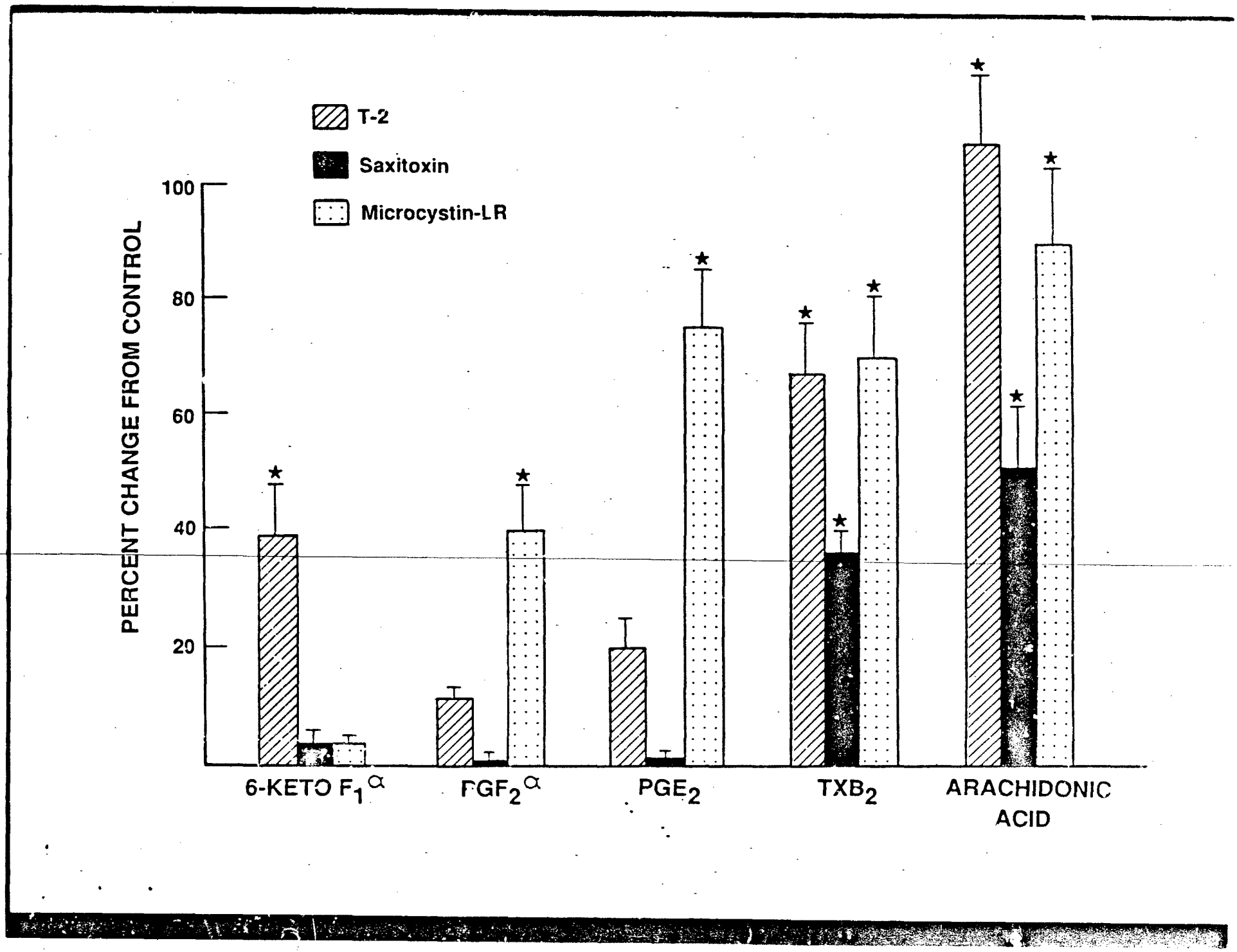
Alveolar macrophages were incubated with NEM or A23187 for 60 min in 1 ml HBSS. Media were collected, extracted and analyzed by TLC. Results represent mean ± SEM of 3 separate experiments in duplicate.

Table 2
Effect of T-2 Toxin and Fluocinolone on Arachidonic Acid and its Metabolite Release in Alvealar Macrophages

Treatment		DPM/mg Protein 6-Keto $F_{1\alpha}$	TxB_2	AA
Control	(4)	437 ± 48	544 ± 69	2,879 ± 432
0.1 µM T-2	(3)	554 ± 64 (NS)	768 ± 82 (<0.05)	6,590 ± 978 (<0.01)
1.0 µM T-2	(3)	683 ± 51 (<0.025)	942 ± 61 (<0.01)	6,874 ± 1,124 (<0.01)
1.0 µM T-2 + 1.0µM Fluocinolone	(3)	523 ± 44 (NS)	580 ± 61* (NS)	4,592 ± 457 (<0.01)

Macrophages were incubated as described in "Materials and Methods." 1.0µM fluocinolone itself had no effect on release of arachidonic acid or its metabolites. Results represents mean ± SEM of number of experiments in duplicate indicated in the table.

*P-Value significantly different (<0.05) from 1.0 µM T-2 toxin-treated cultures.


T-2
Saxitoxin
Microcystin-LR
PERCENT CHANGE FROM CONTROL
100
80
60
40
20
6-KETO F_1^{α}
FGF_2^{α}
PGE_2
TXB_2
ARACHIDONIC ACID