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THE RENAL MEDULLARY THICK ASCENDING LIMB AS A MODEL FOR UNDERSTANDING LIPID MEDIATORS IN SEPSIS

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Cytokines are an essential component in those mechanisms that depress renal function in response to inflammatory diseases of the kidney. We have addressed the role of tumor necrosis factor (TNF) in mediating changes in renal function by isolating a nephron segment, the medullary thick ascending limb of Henle's loop (mTAL), and studying the effects of TNF on ion transport *via* changes in the activity of two epoxygenases, cyclooxygenase (COX) and cytochrome P450 (CYP450) monooxygenase. This nephron segment generates 20-hydroxyeicosatetraenoic acid (20-HETE) as a principal arachidonate metabolite. However, when challenged with lipopolysaccharide (LPS), the mTAL expresses a considerable capacity to metabolize arachidonic acid (AA) *via* a COX pathway to form PGE_2.

Key words: *20-HETE, angiotensin II, arachidonate metabolites, cytochrome P450, lipopolysaccharide,* PGE_2*, renal medullary thick ascending limb, tumor necrosis factor-α.*

INTRODUCTION

Cytokines produced by infiltrating macrophages and T cells as well as by renal vascular and tubular elements in inflammatory diseases of the kidney, depress GFR and renal blood flow and alter tubular function (1, 2). Sepsis and hemorrhagic shock are also associated with activation of cytokines that have adverse effects on the circulation ans renal function (3, 4). Tumor necrosis factor-α (TNF), first described as a product of activated macrophages, occupies a central role in inflammation as evidenced by its chemotactic properties, and its ability to promote prostaglandin formation, synthesis of pro-coagulant factors and expression of adhesion molecules (5, 6). The effects of TNF are normally restricted to the local environment of the cell of origin which locus has been greatly expended from macrophages (6) to include vascular smooth muscle (7), renal mesangial cells (2) and epithelial cells (5).

SHOCK, TNF, AND NO

In immune-mediated glomerulonephritis, Baud *et al.* (2) have focused on the mesangium as the source of factors, particularly TNF, that promote glomerular injury. They have challenged rat cultured mesangial cells with bacterial lipopolysaccharide (LPS) to induce release of TNF. A negative feedback mechanism mediated by PGE_2 that controlled release/formation of TNF was identified, confirming the earlier study of Kunkel *et al.* (8) that production of TNF by macrophages was regulated by PGE_2. Thus, LPS-stimulated TNF release from mesangial cells was enhanced several-fold by inhibition of cyclooxygenase (COX) with indomethacin at μM concentrations and reduced dose-dependently by nM concentrations of PGE_2 *via* cAMP generation (8). TNF is also produced by the proximal tubules (9) and the medullary thick ascending limb of Henle's loop (mTAL) (10) where it affects transport (11) ans very likely cell growth (12). These studies on production of TNF by tubular segments that TNF acts in local mechanisms, functioning in a physiological setting, its production and effects being "tightly regulated to avoid systemic toxic injury" (5). Septic and hemorrhagic shock result in dysregulation of local production of TNF (5) causing a cascade of events involving NO production and eicosanoid synthesis that greatly affect the circulation (3, 4). In acute hemorrhagic shock in rats, TNF serum levels were increased many-fold and death followed rapidly (4). Anti-TNF antibodies increased survival, demonstrating a critical role for TNF in the pathogenesis of shock. Reduced vascular activity to pressor agents, a hallmark of hemorrhagic and septic shock (13), was demonstrated in this study to be dependent on TNF production as anti-TNF antiserum, which improved the survival rate, restored the contractile responsiveness of the aorta to phenylephrine (4).

The cardinal features of endotoxin shock (sepsis) in patients (14) can be reproduced in animal models with administration of bacterial LPS (3). LPS stimulates formation of TNF (11) and interleukin-1 (IL-1) (15) which in turn cause expression of inducible nitric oxide synthase (iNOS) and COX-2, that contributes decisively *via* production of NO and prostaglandins to the hemodynamic abnormalities that characterize endotoxin shock; viz., a hyper-dynamic circulation with high cardiac output and low peripheral vascular resistance (3, 14). Less well-recognized is the negative effect of TNF on cytochrome P450 monooxygenase (CYP450) activity, resulting in diminished production of the vasoconstrictor eicosanoid, 20-hydroxyeicosatetraenoic acid (20-HETE), a principal determinant of renal vascular resistance (16).

Activation of iNOS by cytokines in endotoxin shock has occasioned therapeutic strategies based on inhibition of nitric oxide synthase (NOS) (3). Wright, Ress and Moncada have distinguished the effects of inhibiting constitutive (c)-NOS from those produced by inhibiting iNOS (3). A beneficial outcome resulted from inhibition of iNOS as opposed to the adverse effects of

cNOS inhibition. The glucocorticoid, dexamethasone, which inhibits iNOS while sparing cNOS, proved protective when given to rabbits challenged with LPS. In contrast, administration of N^G-monomethyl-L-arginine (L-NMMA), which inhibits both iNOS and cNOS, exacerbated the deleterious circulatory responses to LPS. To underscore the necessity of sparing cNOS while inhibiting iNOS in order to achieve a favorable outcome in endotoxin shock, the beneficial effects of dexamethasone could be reversed by co-administration of L-NMMA, supporting an essential role of cNOS in maintaining circulatory integrity (3).

THE mTAL, TNF AND EICOSANOID

The mTAL which has a key role in the regulation of extracellular fluid volume has been recognized to possess immunoregulatory activity that involves cell-specific expression of a glycoprotein, Tamm-Horsfall, coating the apical and basolateral cell surface (17). Tamm-Horsfall protein is identical to uromodulin, an 85-kDa immunosuppressive factor which binds IL-1 and TNF and translocates cytokines perhaps influencing their activity on renal tubules (17). IL-1 and TNF can affect epithelial cell function by modifying arachidonic acid (AA) metabolism *via* affecting the activity of two oxygenases, COX and CYP450, enhancing the former and suppressing the latter (11) (*Fig. 1*). Stimulation of TNF production by the mTAL results in changes in

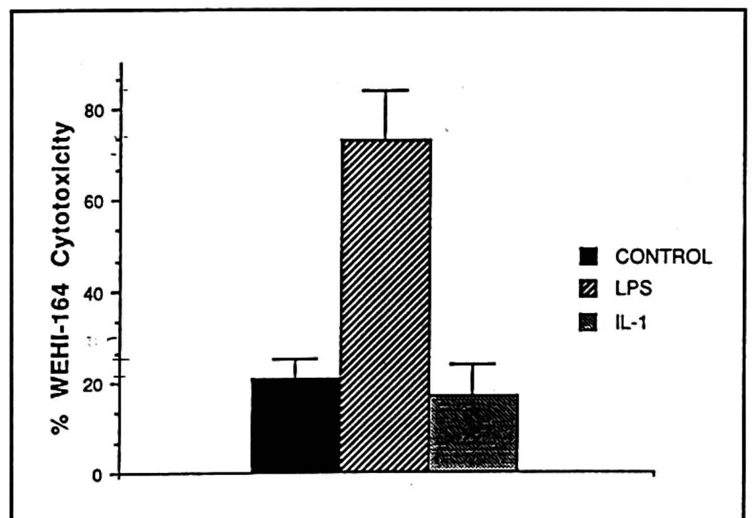

Fig. 1. Tumor necrosis factor-α (TNF) production by mTAL cells. Seven-day-old primary cultured mTAL cells were incubated in absence (*left bar*) or presence of lipopolysaccharide (LPS, 1 μg/ml; *middle bar*) or interleukin-1 (4×10^{-10} M; *right bar*) for 24 h at 37°C in 5% CO_2. Supernates were harvested, and TNF/leukotriene bioactivity was determined using WEHI-164 indicator cell line. Data are means ± SD from 3 separate experiments (Escalante *et al.*, Am J Physiol 266: C1568, 1994).

ion transport as reflected in ^{86}Rb uptake, an enticipated effect, as TNF, like IL-1, has been reported to modulate ion fluxes in a variety of cells: lymphocytes (18), skeletal muscle (19) and collecting ducts (20). There is abundant evidence that the diuretic and natriuretic effects of cytokines are dependent on production of eicosanoids. For example, the diuretic response to IL-1 was shown to be dependent on production of prostaglandins as it was abrogated by inhibition of COX (15). In keeping with these findings in the whole kidney, at

the local level a prostaglandin related mechanism (stimulated by IL-1) has been identified in the rat papillary collecting duct that promotes salt and water excretion by inhibiting Na^{+}–K^{+}–ATPase (21).

Our recent studies on the rat mTAL have illuminated the interactions of TNF with COX and CYP450, initiated by challenging mTAL cells with LPS, sufgesting a reciprocal relationship between COX and CYP450-dependent AA metabolism initiated by TNF and, presumably, mediated by mTAL production of NO after activation of iNOS by the cytokine (11, 22). This mechanism is responsible for expression of COX-2 and suppression of CYP450 activities, resulting in increased production of PGE_2 and suppression od 20-HETE by the mTAL. Extrapolation of these findings to the systemic circulation uncovers a key component, 20-HETE, in the pathogenesis of endotoxin shock. This eicosanoid is essential to the regulation of the renal circulation and, presumably, other regional vasculatures. 20-HETE is responsible for the myogenic arterial response to increased transmural pressure, the Baylis Phenomenon (23). 20-HETE is also the mediator of renal autoregulation (24).

The studies which identified the interplay of eicosanoids and cytokines in the mTAL began with the recognition of the immunomodulatory function of the mTAL (17). A key finding was the ability of the mTAL to synthesize TNF as well as to bind TNF to the Tamm-Horsfall glycoprotein that coats the mTAL (10). In view of the demonstrated ability of LPS and IL-1 to stimulate production of TNF in mesangial cells (2) and proximal tubules (9), respectively, these stimuli were tested on rat primary cultured mTAL cells. LPS, but not IL-1, stimulated TNF production several-fold by mTAL cells during a 24 h period of incubation associated with enhanced synthesis of PGE_2 (11) (*Fig. 1*). TNF production by mTAL cells was associated with diminished ion transport as indicated by measuring ^{86}Rb uptake, as index of changes in potassium transport in these cells. Initially, the effects of exogenous TNF on mTAL transport were determined. An important temporal relationship between TNF and functional effects was established; viz., incubation of TNF for 1 or 4 hours did not affect ^{86} Rb uptake, , whereas on 24-hour incubation, TNF (10^{-9} M) greatly reduced ^{86}Rb uptake (11). Moreover, the inhibitory action of TNF on transport was prevented by inhibiting COX with indomethacin but was not blocked by inhibiting CYP450 with SKF-525 A (*Fig. 2*). It should be noted that under basal conditions, CYP450-AA metabolites predominate in the mTAL (25), although there is evidence that COX-2 is present in the mTAL, being scattered amongst less than 20% of mTAL cells (26). The time lag of greater than 4 hours in the mTAL response to TNF and the ability of indomethacin to prevent the action of the cytokine are in keeping with the induction of COX-2 expression and production of PGE_2 initiated by the cytokine through induction of NOS and production of NO. Either NO or a product such as peroxynitrite can stimulate synthesis of

prostaglandins (27). Stokes had reported, in 1979, thah PGE_2 inihibited ion transport by the rabbit mTAL (28). A recent study has identified the Na^+–K^+–2 Cl^- cotransporter as the target of PGE_2 in the mTAL (29).

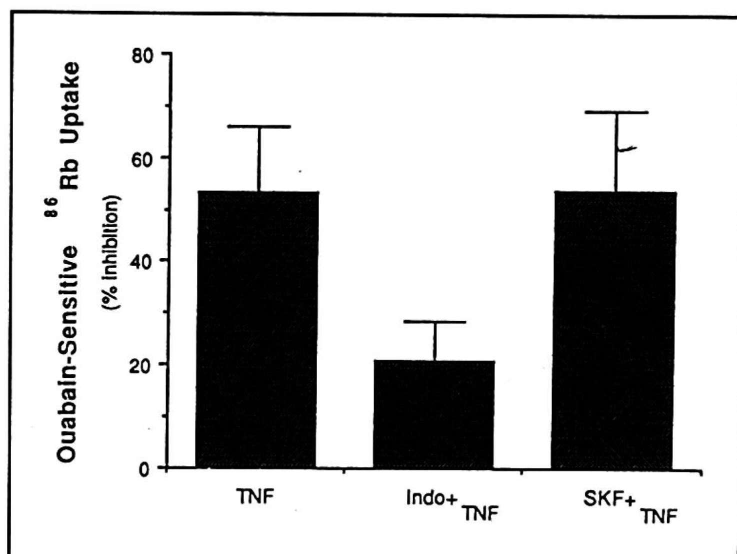

Fig. 2. Effect of TNF on ^{86}Rb uptake by mTAL cells. Cells were pretreated for 24 h with TNF alone (10^{-9} M), indomethacin (indo, 10^{-6} M) and TNF (10^{-9} M), or SKF-525 A (SKF, 5×10^{-5} M) and TNF (10^{-9} M). Experiments were run in pairs, with 1 group of cells incubated with vehicle and the other with combination indicated above. ^{86}Rb uptake was measured. Each bar represents mean ±SE of 5 different cultures (Escalante *et al.*, Am J Physiol 266: C1568, 1994).

IL-1, despite its inability to stimulate TNF production by the mTAL, inhibited ^{86}Rb uptake by this tubular segment and, like the response of the mTAL to exogenous TNF, this effect of IL-1 required prolonged contact of the cytokine with mTAL cells and was antagonized by COX inhibition (11). We concluded that both IL-1 and TNF inhibit ion transport in the mTAL segment by stimulating prostaglandin synthesis consequent to induction of expression of the COX-2 gene. We hypothesize, without direct evidence as yet, that an iNOA component links TNF to production of PGE_2.

As the study cited above was based on added (exogenous) TNF, we determined whether endogenous TNF, that produced by the mTAL, could modify ^{86}Rb uptake, using LPS to stimulate TNF production (11). LPS effected a significant decrease in ^{86}Rb uptake that was blocked by a polyclonal antibody against TNF (*Fig. 3*). The latter also blocked the inhibitory effects

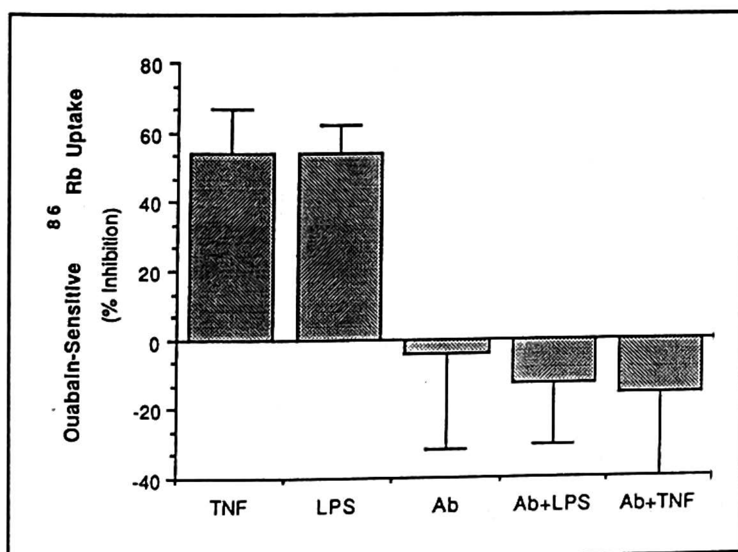

Fig. 3. Effects of anti-TNF antibody on LPS-induced ^{86}Rb uptake inhibition in mTAL cells. Cells were incubated with TNF (10^{-9} M), LPS (10 µg/ml), antibody (Ab), a combination of antibody plus TNF (10^{-9} M, ab+TNF), or a combination of antibody plus LPS (10 µg/ml, Ab+LPS) for 24 h. Experiments were run in pairs, with 1 group incubated with vehicle alone and the other with combinations mentioned above. ^{86}Rb uptake was measured. Each bar represents mean ± SE of 6 different cultures (Escalante *et al.*, Am J Physiol 266: C1568, 1994).

of exogenous TNF on ^{86}Rb uptake by mTAL cells, strongly suggesting that LPS-stimulated-TNF production by the mTAL affected transport with this tubular segment and indicating an autocrine function for TNF in the mTAL.

A neglected facet of these studies, that impacts directly on the pathophysiology of endotoxin shock, is the suppression of CYP450-AA metabolism by TNF, particularly, as the principal product of this pathway in the kidney, as noted above, is 20-HETE, a potent constrictor of the rat renal vasculature (30). We suggest that in any comprehensive construct of endotoxin shock as it relates to the important and critical role of lipid mediators, the inclusion of diminished production of CYP450-dependent AA metabolites is mandated. The key element in the oxygenase response initiated by a cytokine surge in endotoxemia is, likely, increased NO production, resulting primarily from stimulating iNOS expression by the cytokine (31). NO promotes COX-2 activity while stifling CYP450 activity. Indeed, the mTAL has been demonstrated to express NOS (iNOS) to a high degree — one of the highest of renal structures — when challenged with LPS (32). Many of the major components of endotoxin shock can be demonstrated on exposure of the isolated mTAL cells to LPS; namely, TNF, iNOS and lipid mediators. The mTAL, then, can serve as a microcosm for the principal components set in motion by either hemorrhagic or endotoxin shock. It has the advantage of facilitating the analysis of the contributions of single components — TNF, NO, prostaglandins and 20-HETE — to the homeostatic disturbances of endotoxin shock.

TNF MODULATES ANG II

We have also demonstrated that angiotensin II (ANG II) simulates production of TNF associated with accumulation of TNF mRNA and inhibition of ^{86}Rb uptake when the peptide was incubated for several hours with freshly isolated mTAL tubules (33). These findings suggest that TNF production might also be increased in experimental models of hypertension in which ANG II levels are elevated. A model of severe, rapidly developing angiotensin-dependent hypertension was used to test this hypothesis, based on infusion of ANG II for 10 days (34). On day 10, mTAL tubules were isolated from ANG II-infused and sham-operated rats, and incubated for 3 hr, without addition of exogenous ANG II. Supernates were harvested, and the concentrations of TNF and PGE_2 determinated. TNF and PGE_2 production by mTAL tubules was significantly greater in those tubules isolated from ANG II-treated rats compared to sham-infused normotensive control rats. This *in vivo* demonstration, that administration of ANG II for 10 days increased both TNF and PGE_2 production by isolated mTAL tubules, is in accord with the *in*

vitro findings that exposure of the mTAL to ANG II for several hours increased TNF and PGE_2 production (33).

On day 10, rats were anesthetized and blood pressure was measured. In rats made hypertensive by infusion with ANG II, mean arterial pressure (MAP), which was 150 ± 3 mmHg, was significantly elevated compared to the MAP of 90 ± 5 mmHg in sham-operated normotensive rats. Administration of anti-TNF antisera to ANG II-infused hypertensive rats increased blood pressure from 150 ± 3 mmHg within 90 min (34). As anti-TNF antisera increased MAP in rats made hypertensive by chronic ANG II infusions, we suggested that TNF participates in a counter-regulatory mechanism that opposes the pressor effects of ANG II. Further, this mechanism, when unopposed, as in endotoxin and hemorrhagic shock, can account, in large part, for the circulatory and renal abnormalities that characterize septic shock.

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