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INOSITOL LIPID METABOLISM IN VASOPRESSIN STIMULATED HEPATOCYTES FROM RATS INFUSED WITH TUMOR NECROSIS FACTOR

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We studied the effect of i.v. infusion of human recombinant tumor necrosis factorα (rHuTNFα, Cetus, 15 μg/100 g bw over 3 h) on vasopressin (VP)-stimulated ^{32}P-inositol lipid turnover and the release of ^{3}H-inositol phosphates in isolated rat hepatocytes. The early VP-induced decrease (within 30 s) in ^{32}P-phosphatidylinositol 4-phosphate and ^{32}P-phosphatidylinositol 4,5-bisphosphate labeling was significantly reduced (-40%) and at the same time the uptake of ^{32}P into phosphatidic acid was 50% lower than in saline-infused (matched control) rats. Within 5 min of VP-stimulation, lower ^{32}P phosphatidylinositol (-40%) and higher ^{32}P-phosphatidic acid (+30%) labeling were observed in rHuTNFα-infused rats. Infusion of rHuTNFα also affected the VP-induced release of ^{3}H-inositol phosphates. The accumulation of ^{3}H-inositol-labeled water soluble products was decreased by 25% and 17% at 30 s and 10 min, respectively. These data show that rHuTNFα mimics early perturbations induced by Escherichia coli endotoxin infusion in VP-stimulated inositol lipid metabolism in rat hepatocytes.

Tumor necrosis factor (TNF) was first identified as a protein with tumor necrotizing activity present in the serum of mice sequentially injected with Bacillus Calmette Guerin (BCG) and bacterial endotoxin (1). TNF produced by macrophages in response to endotoxic stimuli is identical to cachectin and has pleiotropic activities on a wide variety of cells (2,3). TNF has been implicated as a pivotal mediator of the large array of metabolic and hemodynamic responses associated with endotoxemia and sepsis (4-6). In addition,passive immunization against TNF decreases the toxicity

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ABBREVIATIONS

ET, Escherichia coli endotoxin; Human recombinant tumor necrosis factor, rHuTNFα; I, inositol; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; IP_4, inositol tetrakisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; Poly-PI, polyphosphoinositides; PA, phosphatidic acid; VP, vasopressin.

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and mortality induced by ET (7,8). Recent evidence also suggests that TNF may have a physiologic role in mediating the acute metabolic homeostatic adaptations to tissue injury and sepsis, via modulation of endogenous hormone action (9). The mechanism(s) by which TNF exerts its widespread and diverse activities is incompletely defined.

Several lines of experimental evidence obtained in the last few years support the idea that a decreased ability of cells to respond to Ca^{2+} mobilizing stimuli is central to the metabolic dysfunctions observed during endotoxicosis and sepsis (10-13). In our laboratory we have extensively analysed the responsiveness of hepatocytes from chronically endotoxemic and septic rats to hormones such as epinephrine and VP. Perturbations at different steps of the informational cascade (14-17) contribute to a lower hormone-induced activation of glycogen phosphorylase in the liver (12) and may underlie the commonly observed glucose dyshomeostasis in the host under ET challenge (18-20). The purpose of the present study was to determine if infusion of a non-lethal dose of human recombinant TNFα (rHuTNFα) into rats could mimic the early inhibitory effect triggered by ET infusion on VP-stimulated metabolism of inositol lipids in rat hepatocytes (21). The data presented here suggest that TNFα could be the mediator directly and/or indirectly involved in the observed lower ability of VP to stimulate ^{32}P-PI turnover and release ^{3}H inositol phosphates previously observed in hepatocytes from rats infused with ET for 3 h (21).

MATERIAL AND METHODS

Sources of materials. [2-^{3}H] myo-inositol was from ARC Chemical Inc. (St. Louis, Missouri). [2-^{3}H] labeled standards of I (1) P, I (1,4) P_2 and I (1,4,5) P_3 were from Amersham International. Anion-exchange resin Dowex 1-X8 (formate form, 200-400 mesh) was from Bio Rad Lab (Richmond, CA). ^{32}P-as $H_3{}^{32}PO_4$, carrier free, was from ICN Biomedicals, (Irvine, CA). [Arg^{8}] vasopressin (Pitressin) was from Parke-Davis, Morris Plains, NJ). Purified human recombinant TNFα was provided as a gift by Cetus Corp., (Emeryville, CA).

Animals: Male Sprague-Dawley rats (Charles River Breeding Lab., Wilmington, MA) weighing 330-360g were used in this study. Animals were anesthetized with ether and arterial and venous catheters were implanted using aseptic surgical techniques. They were kept in individual cages with free access to water until the next morning when the experiment was performed. The experimental group was infused with rHuTNFα 15μg/100g b.w. corresponding to 6 x 10^{5} U diluted in 4 ml of sterile saline (0.9% NaCl). The specific activity of TNF was 2.2 x 10^{7} units/mg of protein and the preparation contained less than 0.1 ng of endotoxin/mg of protein. A priming dose of 1 ml was injected rapidly, and the rest infused over 3 h at a rate 1 ml/h. The control group was infused with the same volume of saline. The heart rate and mean arterial blood pressure were unchanged during the three hour infusion and did not differ from the values observed in saline-infused controls.

Methods: The procedure for hepatocyte isolation and incubation was previously described (14). The cell suspension (100 mg ww/ml) was prepared

in Krebs Ringer Bicarbonate HEPES buffer pH 7.4, containing 1.3mM $CaCl_2$, 10mM glucose and 2% (w/v) dialyzed bovine serum albumin and continuously gassed with oxygen. Hepatocytes were incubated in bulk at 37° for 90 min in the presence of [2-^{3}H] myo-inositol (40 µCi/ml). The cells were then washed twice with buffer containing 5mM inositol, resuspended at a concentration of 5mg dry weight/ml in Krebs-Ringer Bicarbonate HEPES buffer containing 10mM LiCl and incubated for a further 20 min. Aliquots in triplicates (0.5ml) were pipetted into plastic tubes and the incubation continuted for 30s and 600s in the presence and absence of VP (10^{-7}M). The reaction was stopped by addition of 250µl of cold perchloric acid 12% (w/v) containing 3mM EDTA. Tubes were kept on ice for 15 min, then centrifuged at 2,000g for 10 min and the supernatant neutralized with 1.8M KOH in 60mM HEPES. Phytic acid hydrolysate (25µg P per sample) was added to improve the recovery of inositol phosphates (22). The neutralized extracts were applied to a Dowex 1-X8 (formate form) columns and water soluble products eluted with a gradient of ammonium formate following the procedure of Berridge et al (23) as previously described (21). The recovery of exogenous added [^{3}H]-inositol phosphates to non-labeled samples exceeded 90%. For the analysis of [^{3}H] inositol lipid labeling, parallel incubations were prepared and lipid extracted with an acidic solvent system (14). When ^{32}P was used as the labeled precursor, labeling of the cells, lipid extractions and TLC isolation were performed as described elsewhere (14). Polyphosphoinositides (Poly-PI) were isolated by a monodimensional TLC on K-oxalate precoated high performance plates (Silica Gel 60, Merck) using chloroform/methanol/4M ammonium hydroxide (9:7:2, by vol) as a solvent system (14). Poly-PI standards were cochromatographed with each sample. Plates were developed in iodine, the spots scraped into vials that contained 0.5ml of water and the radioactivity determined using 10ml of Ready Value cocktail (Beckman Instruments, Fullerton, CA.) mixed with Triton X100 (20%).

RESULTS AND DISCUSSION

Circulating serum TNF levels were determined in the laboratory of Dr. G.J. Bagby of the Department of Physiology, LSU Medical Center by the L929 cytotoxicity assay. Serum TNF levels were detectable by 30 min of infusion and reached peak values between 60 and 90 min of continuous infusion. There was no detectable TNF in the serum of saline-infused control rats.

Figure 1 shows the changes induced by VP (10^{-7}M) in the ^{32}P-labeling of PA, PI and poly-PI as a function of time in hepatocytes from saline-(matched control) and rHuTNFα-infused rats. The data indicate that as a consequence of rHuTNFα infusion the earliest (within 30 s of VP stimulation) decrease in poly-PI labeling was significantly lower (-40%). This was followed by a sustained decrease in [^{32}P] PIP_2 labeling up to 60s at the same time when cells from saline-infused rats have already reached a steady state level at poly-PI labeling. At a later time (5 min) the turnover of ^{32}P-PI was 40% lower than in saline-infused rats concomitantly with a higher accumulation of ^{32}P labeling into PA. A similar perturbation in the PA-PI pathway was also noted in one experiment when 10 µg of TNF, corresponding to 2.2×10^5 U was infused over a 30 min period. In this case also, PI turnover was decreased 40%, concomitantly with a proportionately higher labeling of PA, while the total VP-stimulated

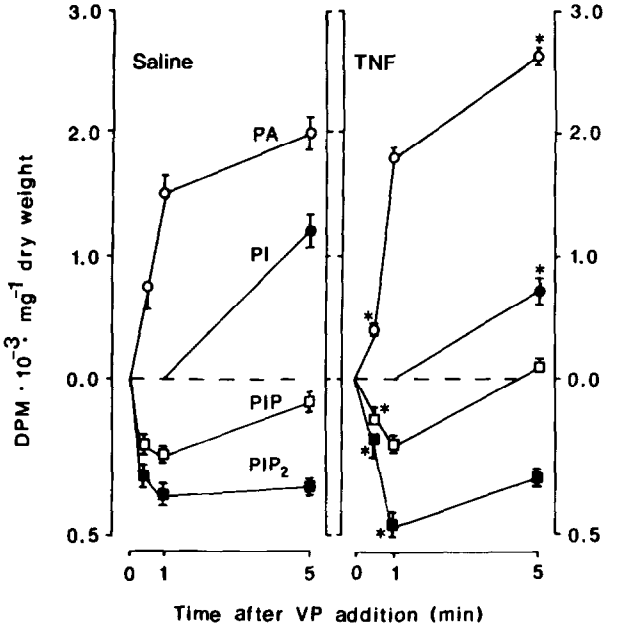

Figure 1
Time course of vasopressin-stimulated changes in ^{32}P-labeled PA and inositol lipids in hepatocytes from saline and rHuTNFα-infused rats. Changes in total ^{32}P-labeling of lipids were calculated as the difference between samples incubated for the same period of time in the presence and absence of vasopressin (10^{-7}M). Values are mean ± SD for triplicate determinations in one of three similar experiments. The scale of the axis below 0.0 was amplified by 2 in order to show more clearly the changes in poly-PI labeling. Asterisks denote statistically significant differences from saline values. ($p<0.05$)

increase in ^{32}P uptake was similar (1,150 vs 1,300 dpm/mg dry wt) in hepatocytes of saline- and TNF-infused rats. Thus PI turnover in rat hepatocytes may be a sensitive, early target for ET action resulting in altered metabolic function.

Considering that the changes in ^{32}P-inositol lipid labeling during the time course of agonist stimulation reflect the net balance between degradation and resynthesis (24-25), the rHuTNFα-induced changes could result from perturbations in one or both metabolic pathways. The lower uptake of ^{32}P into PA 30 s after the addition of VP (Fig 1) is likely to be a more sensitive index of the early receptor-activated hydrolysis of poly-PI. At this time no changes were observed either in ^{32}P-PI labeling (Fig 1) or in ^{3}HI(1)-P levels (21-26). An impaired phosphodiesteratic cleavage of poly PI will lead to 1) a lower release of diacylglycerol and subsequent lower accumulation of its phosphoylated product ^{32}P PA (Fig 1) and 2) a lower release of ^{3}H-IP$_3$ and other related water soluble products, as shown in Table 2. Cells prelabeled with ^{3}H inositol and stimulated with VP in the presence of 10 mM LiCl for 30 s showed a 25% lower accumulation

of ^{3}H-IP_2, ^{3}H-IP_3 and ^{3}H-IP_4 in rHuTNFα than in saline-infused rats (Table 1). On the other hand, the sustained decrease in ^{32}P-PIP_2 labeling up to 60 s (Fig 1) could be the consequence of an impairment in the replenishment of the hormone-sensitive ^{32}P-PIP_2 pool during sustained hormonal stimulation, thus contributing to the modulation of the duration and intensity of the intracellular hormonal signal.

A lower accumulation of ^{3}H-inositol phosphate was also observed 10 min after VP stimulation (Table 2). The $^{3}HI(1)$-P fraction eluted from the Dowex 1 X8 column contains, in addition to $^{3}HI(1)$-P, other monophosphate isomers not resolved by this procedure. This contributes to the overestimation of the amount of $^{3}HI(1)$-P released from the phosphodiesteratic cleavage of PI. Nevertheless, the results suggest that the observed lower ^{32}P PI turnover in rHuTNFα-infused rats could be the result of a) a lower phospholipase C-mediated hydrolysis of PI and b) an impairment in PI resynthesis reflected in a higher accumulation of ^{32}P-PA. Similar results were previously obtained with rats infused for 3 h with a non-lethal dose of ET (21). Moreover, both ET and rHuTNFα infusion affected basal PI metabolism to a similar extent. PI labeling was significantly lower both when ^{32}P (saline: 1200 ± 50 and rHuTNFα: 940 ± 40 dpm/mg.dw) or ^{3}H inositol (legend to Table 2) were used as precursors with no significant changes in poly-PI labeling. Using a similar experimental protocol, Meszaros et. al (27) and Bagby et. al (6) have shown that infusion of rHuTNFα in rats could mimic changes induced by ET in glucose homeostasis. The key role of TNF as an endogenous mediator of the early metabolic responses of normal cells to sepsis and endotoxemia has been

TABLE 1

Accumulation of [^{3}H]inositol phosphates in hepatocytes from saline- and hr TNFα-infused rats after 30 sec of vasopressin stimulation

IPs Fraction	Saline	(%)	rHuTNFα	(%)
IP_2	6,730 ± 370	(84.7 ± 4.7)	5,060 ± 80^{c}	(62.6 ± 1.2^{c})
IP_3	1,310 ± 240	(16.5 ± 3.0)	915 ± 50^{a}	(11.3 ± 2.6^{a})
IP_4	189 ± 24	2.4 ± 0.2)	114 ± 10^{b}	(1.4 ± 0.1^{c})
Total	8,345 ± 600	(105.0 ± 7.8)	6,070 ± 104^{c}	(75.1 ± 1.3^{c})

Values are dpm/mg dry weight ± S.D. from triplicates and represent the VP-induced increment in radioactivity in inositol polyphosphate fractions above basal values (i.e. in the absence of VP 10^{-7}M). The basal labeling of inositol phosphates and poly-PI (PIP and PIP_2) for Saline and rHuTNF samples were (in dpm/mg of dry weight): 405 ± 92 and 973 ± 31 (IP_2); 282 ± 14 and 325 ± 30 (IP_3); 141 ± 5 and 110 ± 4 (IP_4); 4,795 ± 378 and 4,795 ± 313 (PIP); 3,154 ± 158 and 3,295 ± 399 (PIP_2). (%), percent of radioactivity recovered in the inositol phosphate fractions from total basal labeling of poly-PI. Similar results were obtained in two additional experiments. Statistically significant difference compared to the Saline value: a, $p< 0.05$; b, $p< 0.01$; c, $p< 0.005$.

TABLE 2

Influence of in vivo rHuTNFα infusion in the accumulation of [^{3}H]inositol phosphates after 10 min of vasopressin stimulation

IPs Fraction	Saline	(%)	rHuTNFα	(%)
IP	57,480 ± 3,160	(10.04 ± 0.56)	41,995 ± 640[b]	(9.09 ± 0.14[a])
IP_2	61,555 ± 100	(10.76 ± 0.01)	39,215 ± 1,410[c]	(8.49 ± 0.31[c])
IP_3	7,450 ± 45	(1.30 ± 0.01)	3,940 ± 180[c]	(0.85 ± 0.04[c])
IP_4	346 ± 150	(0.06 ± 0.02)	175 ± 44	(0.04 ± 0.01)
Total	126,720 ± 3,450	(22.20 ± 0.60)	85,330 ± 1,720[c]	(18.47 ± 0.37[c])

Values are dpm/mg dry weight ± S.D. from triplicates. (%), percent of radioactivity recovered in the inositol phosphate fractions from total basal labeling of inositol phospholipids (PI plus PIP plus PIP_2). The basal labeling of total inositol phospholipids was (in dpm/mg dry weight): 572,250 ± 24,940 (Sal) and 462,050 ± 22,680 (TNF). Statistically significant difference compared to the Saline Value: a, $p < 0.05$; b, $p < 0.005$; c, $p < 0.001$.

supported by several studies (3-5, 28,29). Moreover, receptors for TNF are present in hepatocyte membranes (30) and the liver has been reported to be one of the major tissue targets of TNF (31).

In summary, our present results show that infusion of rHuTNFα can mimic the previously observed perturbations induced by ET-infusion in hepatocyte inositol lipid metabolism both under basal conditons and after VP stimulation. This suggests that TNFα may be a direct and/or indirect mediator triggering some of the early metabolic alterations induced by ET in the liver. Furthermore, recent experimental evidence demonstrates a permissive effect of TNF on glucagon action in isolated rat hepatocytes (9). Our data indicating the modulating influence of TNF in the same cell type, but involving the action of another hormone namely VP, support the concept of an interrelationship between the immune and endocrine systems as a means of altered metabolic regulation in response to an endotoxin challenge.

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