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ENDOTOXIN AND TNFα DIRECTLY STIMULATE NITRIC OXIDE FORMATION IN CULTURED RAT HEPATOCYTES FROM CHRONICALLY ENDOTOXEMIC RATS

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We examined the effects of endotoxin on nitric oxide formation in isolated rat hepatocytes in primary culture. Endotoxin was administered either in vivo, by continuous infusion for 30 or 3 h, or in vitro, on cultured cells. The spontaneous production of nitrites in hepatocytes from in vivo ET-infused rats was lower than equivalent saline controls in the absence of added stimuli. However in vitro addition of endotoxin in culture to hepatocytes from 30 h ET-infused rats greatly enhanced production relative to saline controls. This effect was mimicked by TNFα, and activators of protein kinase C (PMA and Ca^{2+} ionophore A23187). The effects of ET were blocked by NMMA, dexamethasone and protein synthesis inhibitors Actinomycin D and cycloheximide. No in vitro effect of ET was observed in the 3 h infusion model. The results show that chronic exposure to sub-lethal levels of ET primes liver parenchymal cells for the production of nitric oxide, when exposed in vitro to ET or TNFα.

Arginine dependent nitric oxide formation appears to be an important metabolic modulator and nitric oxide is synthesized by numerous cell types [1] including, endothelial cells [2], macrophages [3] and liver cells [4-8]. At least two types of nitric oxide synthase have been characterized. The first, termed constitutive, is Ca^{2+} calmodulin dependent and is present in the brain and vascular endothelium. The second, termed inducible, is Ca^{2+} independent and is induced by various cytokines and bacterial lipopolysaccharide and is present in many cells including, macrophages, endothelial cells and liver cells [1]. Administration of LPS to rats induces nitric oxide synthase in the liver. This has been reported to occur either only in the parenchymal cells [5-6] or only in the non-parenchymal cells [4,8] of the liver. The latter authors also reported that LPS-activated non-parenchymal cells stimulate nitric oxide production in parenchymal cells if co-cultured [7-8]. In contrast they also show that C. parvum stimulates nitric oxide synthase in parenchymal cells, but not in non-parenchymal cells, suggesting two distinct enzymes in these cells [4].

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The aim of this study was to determine the effects of endotoxin either in vivo by continuous infusion, or in vitro, on nitric oxide synthase in cultured parenchymal cells of the liver.

MATERIALS AND METHODS

For chronic in vivo administration of endotoxin, rats were continuously infused with sub-lethal levels of endotoxin using subcutaneously implanted osmotic mini-pumps [9]. The pumps were set to deliver 1.0 mg endotoxin/kg body wt/24 hrs, or saline in an equivalent volume. For acute in vivo experiments arterial and venous catheters were implanted one day before the experiments.
Endotoxin was then infused in a non-lethal dose of 0.5mg endotoxin/kg body wt over a period of 3 h at a rate of 1 ml/h [10].

Hepatocytes were prepared by collagenase digestion as described [11-12] and parenchymal cells were plated on 23 mm plastic 12 well tissue culture plates in a modified Liebowitz L15 medium containing 10% newborn calf serum at a density of 0.5 X 10^6 cell/well [13-14]. Hepatocytes were maintained for 20 h in primary culture. The presence of nitrites, a stable inactive end product of nitric oxide production, was determined in the medium after 20 h using the Griess reagent [15]. Protein content of the attached cells was determined by the method of Bradford [16]. Nitrite production is expressed as nmoles nitrite released/mg cell protein /20 h. Endotoxin and other agents described were added at the time of plating. None of the agents tested affected the plating efficiency or viability of the cells as judged by trypan blue uptake and retention of lactate dehydrogenase activity.

LPS (E. Coli 026:B6, prepared by the Boivin method) was from Difco, Michigan; human recombinant TNFα was a generous gift from Cetus Corporation, Emeryville California; Tissue cultue plates were from Costar; tissue culture reagents and other hormones and chemicals were from Sigma.

RESULTS AND DISCUSSION

Endotoxin was administered in these experiments in 3 ways. Firstly in vivo by 30 h of continuous infusion with a non-lethal dose of endotoxin via implanted mini-pump as described. Many of the metabolic and hemodynamic perturbations associated with Gram negative septicemia are reproduced with this model [9,11,17-18]. Secondly in vivo by 3 h of continuous infusion of endotoxin delivered by venous catheters. This model has been used to determine some of the early metabolic effects of endotoxin [10]. Thirdly in vitro where endotoxin is added to isolated parenchymal cells in primary culture from either of the two in vivo models. TNFα, PMA and other agents as indicated were also tested in vitro where one can test the direct effect of a single agent on one cell type. Nitrite accumulation in the medium after 20 h in culture was determined as an indicator of nitric oxide formation.

Parenchymal cells in culture spontaneously produce nitrites in the medium [19]. Saline-treated rats from the 30 h pump model or 3 h infusion model produced similar amounts of nitrites in the medium after 20 h (42.7±7.5 and 39.2±17.6 nmoles/mg protein respectively). These values are similar to that seen in naive rats [19]. To our surprise hepatocytes from ET treated rats produced less nitrite in the medium then their saline equivalents (12.7±2.3 and 24.5±5.8 nmoles/mg protein respectively), especially in the case of the 30 h pump model. This was not a reflection of cell viability as the retention of lactate dehydrogenase and trypan blue uptake were the same for all groups (results not shown). Billiar et al [4] did not see changes in parenchymal cell nitric oxide

Table 1. Effects of endotoxin, TNFα and other agents on nitric oxide formation in cultured hepatocytes from chronically or acutely endotoxemic rats

Additions	30 h pump			3 h infusion		
	Sal	ET	(n)	Sal	ET	(n)
	% of basal nitrite release					
Endotoxin 10 μg/well	122±9*	706±94+	(13)	124±9*	169±10*	(5)
TNF 500 U/well	151±11+	374±35+	(9)	175±36	131±13	(4)
PMA 1 μM	124±18	317±42+	(8)	144±28	108±10	(4)
A23187 μM	44±13+	309±44+	(6)	71±12	67±17	(4)
NMMA 30 μM	10±1+	29±10+	(5)	26±15*	20±6*	(3)
Dexamethasone 10 nM	7±1+	24±6+	(6)	n.m.	n.m.	
Glucagon 10 nM	45±14*	48±11*	(5)	n.m.	n.m.	
Insulin 10 nM	116±8	212±30*	(5)	n.m.	n.m.	

Hepatocytes were prepared from rats infused chronically (30 h) or acutely (3 h), with either saline or endotoxin and were cultured for 20 h in the presence of agents as indicated. Nitrite accumulation in the medium was measured as an indicator of nitric oxide formation. Results are expressed as % of basal nitrite levels which were 42.7±7.5 and 12.7±2.3 nmoles released/mg protein for saline and endotoxic rats respectively from the 30 h pump animals, and 39.2±17.6 and 24.5±5.8 from the 3 h infusion animals. The significance of difference between the groups was calculated using a paired t test where : * $p<0.05$; + $p<0.001$. n.m. not measured.

formation 6 or 18 h following endotoxin administration (4 mg/kg). However Knowles et al [5-6] did see an induction of nitric oxide formation (2 or 4 mg/kg). Our non-lethal dosage of endotoxin (1mg/kg/24 hrs or 0.5 mg/kg/h for 30 h pump and 3 h infusion models, respectively) was administered as a continuous infusion as opposed to a bolus injection so peak endotoxin levels were considerably lower and more closely reflect Gram negative septicaemia as seen in humans [9].

Endotoxin produced a slight (although significant) increase in nitrite formation when added in cultures of saline control rats from both models (Table 1), in agreement with Billiar et al [4]. If endotoxin was added to hepatocytes from the 30 h pump model then a large stimulation of nitrite formation in the medium was seen (Table 1). Significant effects were seen with as little as 100-300 ng/well (Fig. 1). Endotoxin did not have significant effect in the 3 h infusion model (Table 1). It appears that in the endotoxic 30 h pump model the parenchymal cells, whilst not spontaneously producing nitric oxide, are primed to produce nitric oxide when exposed to a secondary stimulus. This priming effect was not seen in the 3 h infusion model, which suggests that an induction process is involved.

TNF has been implicated as a principal mediator of a wide range of metabolic responses associated with endotoxemia [20-21]. TNFα was added to parenchymal cell cultures to determine if it could mimic the effects of endotoxin. As seen in Table 1 TNFα added to saline control cells slightly increased nitric oxide formation. However if added to cell from the endotoxic 30 h pump model then a much larger stimulation was seen, to a degree mimicking the effects of endotoxin (Table 1). However this effect requires at least 100 units/well to be seen (Fig. 1). No effect of

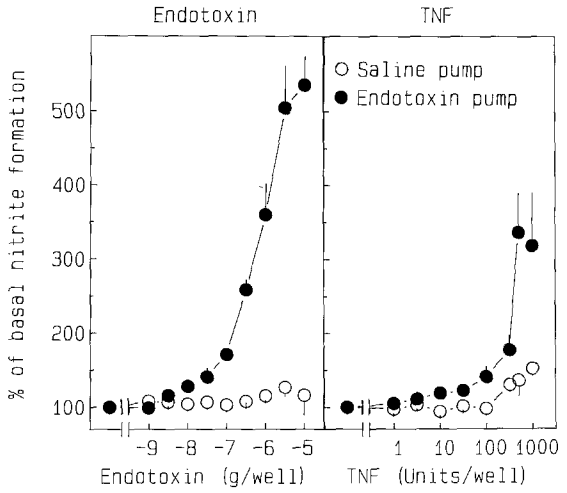

Figure 1. Effects of endotoxin and TNF on nitric oxide formation in cultured hepatocytes from chronic endotoxemic rats. Hepatocytes were prepared from chronically infused for 30 h with either saline or endotoxin. Hepatocytes were incubated in the presence of endotoxin or TNF as indicated and nitrite accumulation in the medium was determined after 20 h. Results are expressed as % if basal nitrite formation and are means±SE from 3 independent experiments.

TNFα was seen on the 3 h infusion model. This suggests that TNFα may be a direct mediator of the metabolic changes seen with chronic endotoxin administration.

A similar effect to endotoxin and TNFα however was also seen with addition of PMA, an activitor of protein kinase C (Table 1). However because of the 20 h length of incubation it is not known whether activation of protein kinase C or the effects of down regulation of protein kinase C is responsible for the enhancement of nitrite formation in the primed hepatocytes of the 30 h pump infused rats. Protein kinase C is markedly down regulated following PMA administration within as little as 6 h [22]. The constitutive form of nitric oxide synthase is Ca^{2+} calmodulin dependent, whilst the inducible form is Ca^{2+} independent [1,6]. The addition of calcium ionophore A23187 at low doses, inhibited nitrite formation in hepatocytes of 30 h saline pump-infused rats. If the constitutive enzyme was present then an increase in nitrite formation would be expected. However in ET primed 30 pump hepatocytes, A23187 enhanced nitrite formation to the same extent as was seen with PMA or TNFα (Table 1). Whether this is due to the influx of Ca^{2+} into the cells or activation of protein kinase C remains to be determined.

As expected [1,5,24-25], dexamethasone and NMMA, a synthetic analogue of arginine and competitive inhibitor of nitric oxide synthase, inhibited the spontaneous release of nitrite in both saline and endotoxic 30 h pump rats (Table 2). Indeed, NMMA, dexamethasone and the protein synthesis inhibitors cycloheximide and Actinomycin D were able to inhibit both spontaneous and endotoxin induced nitrite formation in the endotoxic 30 h pump hepatocytes. Similar effects of the protein synthesis inhibitors has been reported in aortic rings [23]. Whilst the effects of NMMA demonstrate that nitric oxide synthase is responsible for enhanced nitrite formation, the effects of the protein synthesis inhibitors suggests that the stimulation of nitrite formation is due to the

Table 2. Inhibition of the effects of endotoxin by NMMA, dexamethasone and protein synthesis inhibitors

Additions	Basal	+ET 10μg/well
	% of basal	
None	100	712 ± 195
NMMA 30 μM	43 ± 20	39 ± 1
Dexamethasone 10 nM	30 ± 12	11 ± 5
Cycloheximide 5μg/well	25 ± 16	3 ± 1
Actinomycin D 1μg/well	23 ± 13	2 ± 1

Hepatocytes were prepared from rats chronically infused for 30 h with endotoxin as described in the Methods. The cells were cultured for 20 h in the presence or absence of endotoxin and in the presence of the other agents as indicated. Results are expressed as the % change of nitrite accumulation compared to cultures containing no additions, which were 11.4±4.1 nmoles released/mg protein and are means±ranges from 2 independent experiments.

induction of nitric oxide synthase, a mechanism that has been suggested for glucocorticoids [1]. It is interesting to note that glucagon had similar effects to those seen with dexamethasone and suppressed spontaneous nitrite formation, whilst insulin actually enhanced nitrite formation in the endotoxic 30 h pump hepatocytes (Table 1).

In summary the results show that in the chronically endotoxemic rat parenchymal cells are primed for the production of nitric oxide. However this is not expressed without the addition of a secondary stimulus. This priming effect was not seen with the 3 h infusion model. The results also show that TNFα, like endotoxin, can directly stimulate nitric oxide formation in the primed hepatocyte without the requirement of a secondary cytokine. Since TNFα is produced by Kupffer cells of the liver in response to endotoxin one can speculate that in vivo parenchymal cells do indeed produce nitric oxide in response to endotoxin. The mechanism of action of endotoxin or TNF may be through protein kinase C since production of nitric oxide is also affected by agents that activate protein kinase C (PMA and A23187). The results also show that nitric oxide synthase in the liver may well be regulated in part by the systemic hormones, glucocorticoids, glucagon and insulin.

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