Control and production of leukotriene B₄ in rat tumour and testicular Leydig cells

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As part of an investigation into the role of leukotrienes in steroidogenesis, the formation of leukotriene B₄ was investigated in purified Leydig cells from rat testes and from a tumour by using a sensitive radioimmunoassay. Detectable levels were found in both Leydig cell types (70 pg/10⁶ cells) and these remain unchanged during incubation for 60 min at 32°C. Addition of the Ca²⁺ ionophore A23187 increased LTB₄ production more than 6-fold within 10 min whereas steroidogenesis was not increased until after 20 min. In the presence of luteinizing hormone or luteinizing hormone releasing hormone agonist no increase in LTB₄ was detected in the testis Leydig cells whereas luteinizing hormone stimulated testosterone production from 3.2 ± 0.1 to 148.9 ± 7.5 ng/10⁶ cells during the same time period. Similar results were obtained with the tumour Leydig cells. The LTB₄ was found to be rapidly secreted by the cells in all experiments. The basal and A23187-stimulated levels were inhibited by nordihydroguaiaretic acid, a lipoxygenase inhibitor. It is concluded that LTB₄ is produced in Leydig cells and can be stimulated by high calcium levels, but that it is probably not required for the control of steroidogenesis.

Previous work from this laboratory has shown that inhibitors of the lipoxygenase, but not the cyclo-oxygenase, pathway of arachidonic acid metabolism cause a potent inhibition of LH- and dibutyl cyclic AMP-stimulated steroidogenesis in rat Leydig cells (Dix *et al.*, 1984a), at a point between cyclic AMP production and the metabolism of cholesterol to pregnenolone. Preliminary evidence indicated that these inhibitors also inhibited the formation of arachidonic acid metabolites that had similar chromatographic properties to those of reported lipoxygenase products of other systems. Using a sensitive radioimmunoassay the control and production of one of these products, LTB₄, has now been investigated in rat

tumour and testicular Leydig cells. In addition to LH the effects of an LHRH agonist and the calcium ionophore A23187 have been determined. LHRH agonists have been shown to stimulate steroidogenesis directly in Leydig cells [Hunter *et al.*, 1982; Sharpe & Cooper, 1982; Cooke & Sullivan, 1985 (review)] and this stimulation is also prevented by inhibitors of the lipoxygenases (Cooke & Sullivan, 1985). The calcium ionophore was previously shown to potentiate arachidonic acid metabolism in Leydig cells (Dix *et al.*, 1984a).

Materials and methods

Leydig cells and tumour Leydig cells were prepared and purified by Percoll density gradient centrifugation as previously described (Aldred & Cooke, 1982; Dix *et al.*, 1982), except that in the final experiment (Table 2) the tumour Leydig cells were subjected to a second Percoll gradient before culture. This was done to remove any contaminating cell types not removed by the first gradient (see Dix *et al.*, 1984b). The cells were incubated in Dulbecco's Modified Eagle's Medium at 32°C at a concentration of approx. 200 000 cells/0.5 ml and

Abbreviations used: LTB₄, (5S,12R)-dihydroxyeicosatetraenoic acid, leukotriene B₄; 12-HETE, 12-L-hydroxyeicosatetraenoic acid; 5-HETE, 5-L-hydroxyeicosatetraenoic acid; LH, luteinizing hormone, lutropin; LHRH, luteinizing hormone-releasing hormone, luteinizing hormone; NDGA, nordihydroguaiaretic acid [4,4'-(2,3-dimethylbutan-1,4-diyl)bis(1,2-benzenediol)].

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then centrifuged at 100g for 5 min. The cells and supernatant media were separated and kept frozen at -20°C until assay for LTB_4 (Salmon *et al.*, 1982) or testosterone (Verjans *et al.*, 1973). The antibody for the LTB_4 radioimmunoassay was a gift from Dr. J. Salmon (Wellcome Research Laboratories). The LTB_4 assay used (Salmon *et al.*, 1982) has been shown to detect amounts of LTB_4 in the range 12.5–400 pg/ml, and to have low cross-reactivity (3.3%–0.03%) to other lipoxygenase and cyclooxygenase products. Sample volumes of 100 μl were used for LTB_4 assay, except with rat Leydig cells stimulated with A23187, when 50 μl was used. LH (NIH S20; 1.9 i.u. of NIH-S1/mg) was a gift from the Endocrinology Study Section, National Institute of Health, Bethesda, MD, U.S.A. Ca^{2+} ionophore A23187 and NDGA were purchased from Sigma Chemical Co. [5,6,8,9,11,12,14,15(*n*)- ^3H] LTB_4 (210 Ci/mmol) was purchased from Amersham International. LHRH agonist (ICI 118630) was a gift from ICI p.l.c.

Results

The time course over 1 h of LTB_4 production in the testicular Leydig cells in the presence of LH, A23187 or medium alone was determined and compared with testosterone production (Fig. 1). It was found that the levels of LTB_4 produced by control or LH-stimulated cells were unchanged during a 1 h incubation whereas LH increased testosterone production 19-fold above basal levels during this time period. In contrast A23187 stimulated LTB_4 production more than 6-fold in 10 min but did not stimulate testosterone production until after 20 min of incubation. LTB_4 was found to be rapidly secreted; only approx. 14% was intracellular at all the time points studied even

when LTB_4 production was increased by A23187 (Table 1).

Similar studies were carried out with the tumour Leydig cells. The total LTB_4 levels were found to be similar to those obtained in the testicular Leydig cells (70 pmol/ 10^6 cells), although intracellular levels were higher during the first 30 min (Table 1). Again LH did not increase LTB_4 levels and in fact decreased extracellular levels ($P < 0.05$) without affecting intracellular levels. The ionophore A23187 stimulated LTB_4 production by approx. 3-fold at the three time points studied. The LHRH agonist had no detectable effect on LTB_4 levels in the testicular or tumour Leydig cells (Table 1).

The effect of the lipoxygenase inhibitor NDGA on LTB_4 production in the presence of LH and A23187 was determined (Table 2). A concentration of 5 μM -NDGA was chosen; this is twice the amount required to give 50% inhibition of LH-stimulated testosterone production (Dix *et al.*, 1984a). The basal levels of LTB_4 remained constant during 60 min incubation, and NDGA decreased these levels by approx. 30%. The LTB_4 levels were also decreased in the presence of LH and LH + NDGA, although to a lesser extent in the LH-stimulated cells than in Table 1. The reason for this is unknown. The stimulated production caused by A23187 was decreased by approx. 60% in the presence of NDGA. These results are consistent with a potent effect of A23187 on LTB_4 production and inhibition of the lipoxygenase pathway involved in its production by NDGA.

Discussion

In this study we have shown for the first time that steroidogenic cells have the capacity to produce a leukotriene, LTB_4 , and that the produc-

Fig. 1. Effects of media, LH (100 ng/ml) and ionophore A23187 (1 μM) on LTB_4 (●) and testosterone (○) production in rat Leydig cells

All data are means $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ versus media (control).

Table 1. *Effect of various stimulants on LTB₄ production by rat and tumour Leydig cells*
 Rat Leydig cell data are means $\pm$ range ($n = 2$). Tumour Leydig cell data are means $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ versus control.

Incubation conditions	Incubation time (min)	LTB ₄ (pg/10 ⁶ cells)	
		In medium	Cellular
Rat Leydig cells			
Control	30	69.5 ± 13.9	12.5 ± 0.7
+LH		61.5 ± 1.4	10.4 ± 3.1
+A23187		807.0 ± 57.1	89.2 ± 3.0
+LHRH agonist		83.0 ± 10.7	7.3 ± 2.9
Control	60	85.5 ± 1.7	13.0 ± 6.1
+LH		70.0 ± 1.7	17.2 ± 2.4
+A23187		687.0 ± 53.9	51.7 ± 6.1
+LHRH agonist		73.5 ± 0.3	20.5 ± 6.7
Tumour Leydig cells			
Control	10	51.5 ± 9.5	36.0 ± 4.0
+LH		1.6 ± 1.6*	31.1 ± 2.9
+A23187		134.2 ± 13.4*	66.8 ± 8.2*
+LHRH agonist		47.8 ± 2.0	28.6 ± 8.9
Control	30	32.8 ± 5.7	35.3 ± 4.2
+LH		0 ± 0*	46.2 ± 3.6
+A23187		140.9 ± 4.3*	56.3 ± 3.7*
+LHRH agonist		41.1 ± 6.3	34.0 ± 4.4
Control	60	48.4 ± 4.6	19.3 ± 0.5
+LH		12.0 ± 8.7*	16.8 ± 1.0
+A23187		147.2 ± 11.9*	57.5 ± 6.7*
+LHRH agonist		40.0 ± 2.1	19.4 ± 2.5

Table 2. *Effect of NDGA on LTB₄ production in tumour Leydig cells*
 All data are means $\pm$ S.E.M. of total LTB₄ levels ($n = 3$). * $P < 0.05$ versus control.

Incubation conditions	Time (min)	LTB ₄ (pg/10 ⁶ cells)			
		10	20	30	60
Control		60.5 $\pm$ 6.9	62.0 $\pm$ 2.0	66.4 $\pm$ 2.4	65.7 $\pm$ 4.8
+NDGA		53.4 $\pm$ 3.5	47.8 $\pm$ 0.9*	45.8 $\pm$ 4.6*	45.6 $\pm$ 2.1*
+LH		52.8 $\pm$ 2.8	48.7 $\pm$ 2.0*	54.9 $\pm$ 2.6*	55.5 $\pm$ 0.8
+LH + NDGA		55.7 $\pm$ 2.0	56.4 $\pm$ 2.0	48.7 $\pm$ 2.6*	48.3 $\pm$ 2.1*
+A23187		292.0 $\pm$ 24.5*	377.7 $\pm$ 16.6*	363.6 $\pm$ 37.9*	283.7 $\pm$ 36.6*
+A23187 + NDGA		132.7 $\pm$ 7.5*	116.1 $\pm$ 5.2*	120.2 $\pm$ 2.1*	129.0 $\pm$ 11.9*

tion of this leukotriene can be stimulated by the calcium ionophore A23187. Endocrine function in the pancreas (Metz *et al.*, 1982, 1983; Falck *et al.*, 1983), the hypothalamus (Capdevila *et al.*, 1983) and the pituitary (Naor & Catt, 1981; Snyder *et al.*, 1983) also appears to depend on lipoxygenase activity but only in the latter tissue has a product (12-HETE) previously been identified (Pilote *et al.*, 1982).

The pathway to LTB₄ via the 5-lipoxygenase and 12-lipoxygenase enzymes has been shown to be calcium-dependent (Borgeat & Samuelsson, 1979; Parker & Aykent, 1982; Rubin *et al.*, 1982); the results in the present study are therefore consistent with an ionophore-mediated calcium flux which

stimulates lipoxygenase activity in Leydig cells. In order to overcome the problem of effects of the ionophore on cell types other than Leydig cells the latter were purified by Percoll density gradient centrifugation (Aldred & Cooke, 1982). Results obtained by Dix *et al.* (1984b) showed that a crude tumour cell preparation responded to isoprenaline and prostaglandin E₁ by producing cyclic AMP. A single purification on a Percoll gradient removed these responses, so therefore the single, and in one experiment double, gradient purification employed in the present study ensured that only tumour Leydig cells were present. In addition it was shown that macrophages, which do metabolize arachidonic acid, are absent from this purified

tumour Leydig cell preparation (M. Niemi, C. J. Dix & B. A. Cooke, unpublished work).

LH and the LHRH agonist were found to be have no detectable stimulating effect on LTB_4 production; in fact LH decreased basal levels of this leukotriene in the tumour cells. The latter indicates that arachidonic acid may be metabolized to different lipoxygenase products in the presence of LH; the decrease in LTB_4 production is presumably due to diversion of arachidonic acid to another pathway of metabolism. Both LH- and LHRH agonist-stimulated steroidogenesis are however dependent on calcium (see the review by Cooke & Sullivan, 1985) so presumably the differences between the calcium ionophore and these hormones on LTB_4 production reflect their differential effects on calcium levels. The ionophore will cause large increases in calcium levels and extensive arachidonic acid metabolism to LTB_4 , whereas in the presence of lower levels of calcium arachidonic acid may be metabolized mainly to products earlier in the pathway, e.g. 5- and 12-HETE. Preliminary evidence for the formation of these products was previously obtained (Dix *et al.*, 1984a).

LHRH agonist and Ca^{2+} ionophore A23187 have similar effects on testosterone production (Sullivan & Cooke, 1984), but there are a number of differences between these two compounds on LH-stimulated testosterone and cyclic AMP production (Sullivan & Cooke, 1983), indicating different mechanisms of action. One of these differences appears to be at the level of arachidonic acid metabolism, in that ionophore A23187 is a potent stimulation of LTB_4 production, whereas LHRH agonist is not.

The results of the present study indicate that LTB_4 is not required for the stimulation of steroidogenesis. This is clearly seen in the different time courses of testosterone and LTB_4 production when the Leydig cells were stimulated by the calcium ionophore and LH.

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