Inhibition of steroid production in Leydig cells by non-steroidal anti-inflammatory and related compounds: evidence for the involvement of lipoxygenase products in steroidogenesis

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The effect of inhibitors of the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism on steroidogenesis in rat testis Leydig cells and rat tumour Leydig cells has been investigated. In the presence of nordihydroguaiaretic acid {NDGA; 4,4'-(2,3-dimethylbutan-1,4-diyl)bis[1,2-benzenediol]}, 5,8,11,14-eicosatetraenoic acid (ETYA), BW755C {3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline hydrochloride} and benoxaprofen [Opren; 2-(2-*p*-chlorophenylbenzoxazol-5-yl)propionic acid] (which inhibit lipoxygenase activity), but not indomethacin and aspirin (which inhibit cyclo-oxygenase activity), a dose-related inhibition of lutropin (LH)-stimulated testosterone and pregnenolone production was obtained (ID_{50} values of 2.5, 30, 25 and 30 μ M for NDGA, ETYA, BW755C and benoxaprofen were obtained, respectively). BW755C and benoxaprofen had no significant effect on LH-stimulated cyclic AMP production except at the highest concentrations examined (330 and 380 μ M, respectively), whereas NDGA and ETYA inhibited LH-stimulated cyclic AMP production in a dose-dependent manner (ID_{50} 7.0 and 22 μ M respectively). However, NDGA and ETYA also caused a dose-dependent inhibition of dibutyryl cyclic AMP-stimulated testosterone and pregnenolone production. The metabolism of exogenous (22*R*)-hydroxycholesterol or pregnenolone to testosterone by Leydig cells was not inhibited by either NDGA, ETYA or indomethacin. At low concentrations of NDGA and ETYA a significant increase in the conversion of both pregnenolone and (22*R*)-hydroxycholesterol to testosterone was obtained. Studies in which the metabolism of [14 C]arachidonic acid by purified rat tumour Leydig cells was investigated indicate that products are formed by tumour Leydig cells that have similar mobilities in a thin layer chromatography system to 5-L-hydroxy-6,8,11,14-eicosatetraenoic acid, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid and leukotriene B_4 . The formation of these products was inhibited to varying degrees by NDGA, BW755C and benoxaprofen but not by aspirin and indomethacin. These studies demonstrate for the first time that inhibition of lipoxygenase activity but not cyclo-oxygenase activity causes an inhibition of LH- and dibutyryl cyclic AMP-stimulated steroid production and suggest a stimulatory role for products of the lipoxygenase pathway of arachidonic acid metabolism in steroidogenesis. The site of this stimulation is apparently distal to the production of cyclic AMP and before the side chain cleavage of cholesterol.

Abbreviations used: LH, lutropin; NDGA, 4,4'-(2,3-dimethylbutan-1,4-diyl)bis[1,2-benzenediol]; ETYA, 5,8,11,14-eicosatetraenoic acid; BW755C, 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline hydrochloride;

benoxaprofen, 2-(2-*p*-chlorophenylbenzoxazol-5-yl)propionic acid; 5-HETE, 5-L-hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid.

It is now over a decade since cyclic AMP and cyclic AMP-dependent protein kinases were shown to be involved in hormone-stimulated steroidogenesis (Catt & Dufau, 1973; Rommerts *et al.*, 1974). Yet, to date the exact site(s) at which steroidogenesis is controlled and the mechanisms involved are still uncertain. Most of the data obtained suggest three possible sites: (i) the conversion of cholesterol esters to cholesterol in the lipid droplets, (ii) the transport of cholesterol from the lipid droplet to the inner mitochondrial membrane and (iii) the conversion of cholesterol to pregnenolone by the cholesterol side chain cleavage enzyme (see review by Simpson, 1979). Recent studies have suggested that a calcium/calmodulin system may control the transport of cholesterol from lipid droplets to the mitochondria (Hall *et al.*, 1981) and the involvement of phosphatidylinositol metabolism/turnover in steroidogenesis has also been implicated (Farese *et al.*, 1980). In this report we present evidence which strongly suggests that products of the recently discovered lipoxygenase pathway of arachidonic acid metabolism (Borgeat & Samuelsson, 1979) may play an essential part in trophic hormone-stimulated steroidogenesis.

It is established that non-steroidal anti-inflammatory drugs block the formation of prostaglandins by inhibiting the cyclo-oxygenase pathway of arachidonic acid metabolism (Ferreira & Vane, 1979). Recently it has been shown that some of these non-steroidal anti-inflammatory compounds also inhibit the lipoxygenase pathway of arachidonic acid metabolism (Higgs *et al.*, 1979; Walker & Dawson, 1980). We have investigated the effects of two of these compounds, BW 755C and benoxaprofen (Opren) on steroidogenesis. In addition, NDGA and ETYA, which are potent inhibitors of lipoxygenase activity, and two inhibitors of cyclo-oxygenase activity, indomethacin and aspirin (Ferreira *et al.*, 1971) have been studied. The effect of inhibition of arachidonic acid metabolism by these compounds has been investigated by using Leydig cells purified from rat testes and from a Leydig cell tumour.

Materials and methods

Sheep LH (NIH-S20, 1.9 i.u. NIH-SI/mg) was a gift from the Endocrinology Study Section, National Institute of Health, Bethesda, MD, U.S.A. Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], bovine serum albumin (fraction V), aspirin (2-acetoxybenzoic acid), indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-ylacetic acid] the calcium ionophore A23187 and NDGA were purchased from Sigma Chemical Co. BW 755C was a gift from Wellcome

Research Laboratories (Beckenham, Kent, U.K.), ETYA (Ro 3-1428) was a gift from Hoffmann-La Roche Inc. (Nutley, NJ, U.S.A.) and benoxaprofen was a gift from Lilly Research Centre (Windlesham, Surrey, U.K.). Percoll was purchased from Pharmacia Fine Chemicals and 3-isobutyl-1-methylxanthine was purchased from Aldrich Chemical Co. Dulbecco's modified minimum essential media either with or without NaHCO_3 were purchased from Gibco Biocult (Paisley, Scotland, U.K.). $[1-^{14}\text{C}]$ Arachidonic acid (58.4 mCi/mmol) and $[5,6,8,9,11,12,14,15(n)-^3\text{H}]$ -leukotriene B_4 (210 Ci/mmol) were purchased from Amersham International. 12-L- $[5,6,8,9,11,12,14,15-^3\text{H}(n)]$ Hydroxy-5,8,10,14-eicosatetraenoic acid (48 Ci/mmol) and 5-L- $[5,6,8,9,11,12,14,15-^3\text{H}(n)]$ Hydroxy-6,8,11,14-eicosatetraenoic acid (80 Ci/mmol) were purchased from New England Nuclear. All radiochemicals were stored under N_2 at -21°C and used without further purification.

Cell isolation and purification

Leydig cells from rat testes were isolated and purified on 0–90% Percoll gradients as previously described (Hunter *et al.*, 1982; Aldred & Cooke, 1982). Leydig tumour cells used for this study have been characterized previously (Cooke *et al.*, 1979). The Leydig tumours were grown in Fischer rats and the cells isolated and purified using 0–90% Percoll gradients as previously described (Dix *et al.*, 1982). Cells with an average density of 1.070 g/ml were used in all studies.

Cell incubations

Purified rat testis or tumour Leydig cells were suspended in Dulbecco's minimum essential media containing 0.1% bovine serum albumin. When cyclic AMP was to be determined the media also contained the phosphodiesterase inhibitor, isobutylmethylxanthine (0.5 mM). The cells (100 000/well) were preincubated for 2 h at 32°C in an atmosphere of air/ CO_2 (9:1) and then for a further 15 min with inhibitors of arachidonic acid metabolism followed by a 1 or 2 h incubation in the absence or presence of LH (100 ng/ml for testis Leydig cells and 1.0 $\mu\text{g}/\text{ml}$ for tumour Leydig cells). Incubations were stopped by the addition of HClO_4 (final concentration 0.5 M) and neutralized with K_3PO_4 (final concentration 0.23 M). Testosterone, pregnenolone and cyclic AMP were determined by specific radioimmunoassay as previously described (Verjans *et al.*, 1973; van der Vusse *et al.*, 1975; Steiner *et al.*, 1972; Harper & Brooker, 1975). In experiments where pregnenolone production was determined cyanoketone (5×10^{-9} M) and SU 10603 (2×10^{-8} M) were also added to inhibit the metabolism of pregnenolone to testosterone.

[¹⁴C]Arachidonic acid metabolism

[¹⁴C]Arachidonic acid metabolism was studied in purified tumour Leydig cells. Cells (10×10^6) were incubated in minimum essential medium (0.5 ml) in the presence of various inhibitors and [¹⁴C]arachidonic acid (1000000 d.p.m.) for 30 min at 32°C. At the end of the incubation period the samples were transferred to an iced water bath and the products extracted by the addition of acidified acetone [1 ml of 1% (v/v) 5M-HCl in acetone at -21°C]. This mixture was shaken for 1 min then chloroform (2 ml at -21°C) was added and the mixture shaken vigorously for 1 min and then centrifuged (800g for 10 min at 4°C). The bottom phase (chloroform) of this two-phase extraction was then saved and the chloroform evaporated to dryness under N₂ at 50°C. The extracted products were then redissolved in chloroform/methanol (1:1, v/v; 50–100 µl) and spotted onto a t.l.c. plate and developed in a diethyl ether/hexane/acetic acid (60:40:1 by vol.) solvent system at 4°C. Once the solvent front had reached the top of the plate it was removed from the solvents and allowed to dry and then redeveloped in the same solvent system. This double running procedure gave an increased resolution. When standards were also run, these were spotted directly on the plates and developed alongside the Leydig cell products. The t.l.c. plate was then autoradiographed with Kodak NS-2T film with an exposure time of 7–14 days at -21°C. The film was developed with Kodak DX80 developer and fixed in a solution of sodium metabisulphite (20 g/l), NH₄Cl (50 g/l) and sodium thiosulphite (200 g/l). After autoradiography each lane of the t.l.c. plate was cut into 0.5 cm sections and each section was dissolved in scintillation fluid (Scintran, Cocktail T, BDH Chemicals) and counted in a Packard Tricarb (3255) liquid-scintillation spectrometer.

Statistical analysis

Statistical analysis was by Student's *t*-test.

Results

As can be seen in Fig. 1(a), LH-stimulated testosterone production is inhibited in testis Leydig cells in a dose-related manner by NDGA, ETYA, BW 755C and benoxaprofen with ID₅₀ values of 2.5, 30, 25 and 30 µM, respectively. However, indomethacin at all concentrations tested (10–200 µM) had no inhibitory effect on LH-stimulated testosterone production. Indomethacin also had no significant effect on LH-stimulated cyclic AMP production (Fig. 1b) and BW 755C and benoxaprofen only inhibited at the highest concentrations tested (330 and 380 µM, respectively; Fig. 1b). NDGA and ETYA inhibited LH-stimulated

cyclic AMP production over the same concentration range that inhibited testosterone production. However, if steroidogenesis was stimulated with dibutyryl cyclic AMP, NDGA and ETYA still inhibited testosterone production with similar potency to that seen with LH-stimulated steroidogenesis (Fig. 2a and Table 1).

In order to investigate further the site of action of these inhibitors, experiments were performed in which the conversion of pregnenolone to testosterone by the Leydig cells was inhibited with SU 10603 (an inhibitor of 17β-hydroxylase, EC 1.14.1.7) and cyanoketone (an inhibitor of 3β-hydroxysteroid dehydrogenase, EC 1.1.1.51) and the dibutyryl cyclic AMP-stimulated production of pregnenolone was measured. Again NDGA (Fig. 2b) produced a dose-related inhibition of pregnenolone production with a similar ID₅₀ as seen for testosterone production (2–3 µM). BW 755C (75 µM) and benoxaprofen (70 µM) also inhibited pregnenolone production to 27 ± 4.7 and $30 \pm 4.7\%$, respectively of the LH-stimulated pregnenolone production (190 ± 24 ng/10⁶ cells per 2 h). When pregnenolone was added to the Leydig cells its conversion to testosterone was not inhibited by NDGA using concentrations up to 10 µM; with 1.25 µM-NDGA a significant increase ($P < 0.001$) in the conversion of pregnenolone to testosterone was found (Table 2). Indomethacin again had no significant effect on this conversion at concentrations up to 100 µM. When (22R)-hydroxycholesterol was used instead of pregnenolone the results were essentially the same (Table 2), again with the only significant effect being an increased conversion of (22R)-hydroxycholesterol to testosterone with 1.25 µM-NDGA ($P < 0.05$). The increased conversion of pregnenolone to testosterone was seen in a more detailed study with several concentrations of both NDGA and ETYA (Table 3). This increase was still evident when indomethacin (50 µM) was present along with either NDGA or ETYA (results not shown). In order to study the possible production of lipoxygenase products by Leydig cells, the Leydig tumour was used since a large number of pure tumour cells can be obtained, which is essential for the detection of these metabolites of arachidonic acid. Tables 4 and 5 show the results of the effects of aspirin, indomethacin, BW 755C, benoxaprofen and NDGA on pregnenolone production in the purified rat tumour Leydig cells. These results are essentially the same as those for the rat testes Leydig cells. Both aspirin and indomethacin at concentrations up to 80 µM had no significant effect on LH-stimulated pregnenolone production (Table 4), whereas both BW 755C and benoxaprofen produced a dose-dependent inhibition (Table 4) over the same range of concentrations as that seen for the rat testes

Fig. 1. Effect of inhibitors of arachidonic acid metabolism on LH-stimulated testosterone and cyclic AMP production in rat Leydig cells

Leydig cells were preincubated for 2 h in minimum essential medium + 0.1% bovine serum albumin and isobutylmethylxanthine (0.5 mM) and then incubated in the presence of various concentrations of (▲) indomethacin, (○) benoxaprofen, (●) BW 755C, (□) NDGA and (■) ETYA for 15 min followed by a 2 h incubation in the presence of LH (100 ng/ml) at 32°C in an atmosphere of air/CO₂ (9:1). Incubations were stopped and (a) testosterone and (b) cyclic AMP were determined as described in the Materials and methods section. Values represent percentages $\pm$ S.D. of duplicate determinations carried out on triplicate samples. In a typical experiment LH increased testosterone production from 11.6 ± 1.3 to 103 ± 2.9 ng/10⁶ cells per 2 h and cyclic AMP production from 13.7 ± 2.3 to 225.1 ± 8.4 pmol/10⁶ cells per 2 h (means $\pm$ S.D., $n = 3$).

Leydig cells. NDGA again inhibited LH-stimulated cyclic AMP production in the tumour Leydig cell to the same extent as in the testis Leydig cell (results not shown), but still inhibited dibutyryl

cyclic-AMP-stimulated pregnenolone production with the same order of potency as seen for the rat testis Leydig cell (Table 5).

When tumour Leydig cells were incubated with

Fig. 2. Effect of NDGA on dibutyl cyclic AMP-stimulated rat Leydig cell testosterone and pregnenolone production. Rat Leydig cells were preincubated in minimum essential medium + 0.1% bovine serum albumin for 2 h and then incubated for 15 min with various concentrations of NDGA followed by 2 h incubation in the presence of dibutyl cyclic AMP (2 mM) at 32°C under an atmosphere of air/CO₂ (9:1) and (a) testosterone and (b) pregnenolone production was determined as described in the Materials and methods section. When pregnenolone production was measured SU-10603 (2 × 10⁻⁸ M) and cyanoketone (5 × 10⁻⁹ M) were also present in the incubation media. Values represent means ± s.d. of duplicate determinations on triplicate samples.

Table 1. Effect of ETYA on dibutyl cyclic AMP-stimulated testosterone production by purified rat Leydig cells

Rat Leydig cells were preincubated in minimum essential medium + 0.1% bovine serum albumin for 2 h and then for 15 min with various concentrations of ETYA followed by a 2 h incubation in the presence of dibutyl cyclic AMP [2 mM at 32°C under an atmosphere of air/CO₂ (9:1)]. Testosterone was determined by specific radioimmunoassay as described in the Materials and methods section. Values represent means ± s.d. of duplicate determinations on triplicate samples. Basal testosterone production was 12.9 ± 0.58 ng/10⁶ cells per 2 h (means ± s.d., n = 3). Results from various ETYA concentrations were compared with control values by Student's *t*-test: **P* < 0.001.

[ETYA] (μM)	Testosterone production (ng/10 ⁶ cells per 2h)
0	249.3 ± 13.6
6.25	204.8 ± 3.2*
12.5	184.5 ± 5.4*
25.0	146.6 ± 1.6*
50.0	38.9 ± 5.4*
100.0	9.5 ± 0.5*
200.0	8.5 ± 0.1*

[¹⁴C]arachidonic acid and the extracts subjected to t.l.c. followed by autoradiography, a number of products were evident. Fig. 3 shows a typical autoradiograph of these products and indicates the mobilities of arachidonic acid, leukotriene B₄, 5-HETE and 12-HETE. Compared with the control product pattern seen in lane 1, LH had variable and inconsistent effects on the products in fractions 2,3 and 4 (Fig. 3, lane 2); however, in all experiments there was an increased conversion of arachidonic acid to products found in fractions 1 and 6 (results not shown). In contrast the calcium ionophore, A23187, increased the metabolism of arachidonic acid to products found in fractions 2, 3 and 4 (Fig. 3, lane 3); these fractions contain products which have similar mobilities to leukotriene B₄, 5-HETE and 12-HETE respectively. The results seen for the calcium ionophore are typical of those seen in several experiments. In contrast, although indomethacin caused an increase in the formation of products in fractions 2, 3 and 4, this effect was inconsistent and never reached levels of significance (Table 6). NDGA

Table 2. *Effect of NDGA and indomethacin on the formation of testosterone from exogenous (22R)-hydroxycholesterol and pregnenolone by purified rat Leydig cells*

Rat Leydig cells were incubated in minimum essential medium + 0.1% bovine serum albumin in the presence of (22R)-hydroxycholesterol (10 μ M) or pregnenolone (500 ng/ml) and various concentrations of NDGA or indomethacin for 2 h at 32°C under an atmosphere of air/CO₂ (9:1). Testosterone was measured by radioimmunoassay in duplicate on triplicate samples. (22R)-Hydroxycholesterol (10 μ M) and pregnenolone (500 ng/ml) were undetectable in the testosterone radioimmunoassay. Values represent means $\pm$ S.D.. Results for each inhibitor were compared with the relevant control by Student's *t*-test: **P* < 0.05, ***P* < 0.001, all other values *P* > 0.05.

Inhibitor	Concentration (μ M)	Testosterone production (ng/10 ⁶ cells per 2h)	
		+ (22R)-Hydroxycholesterol	+ Pregnenolone
None	—	601.3 $\pm$ 28.9	505.8 $\pm$ 35.2
NDGA	1.25	689.3 $\pm$ 38.4*	659.4 $\pm$ 23.5**
	10.0	613.4 $\pm$ 21.9	582.4 $\pm$ 15.6
Indomethacin	12.5	621.1 $\pm$ 23.4	525.6 $\pm$ 29.7
	100.0	636.3 $\pm$ 19.8	620.0 $\pm$ 70.9

Table 3. *Effect of NDGA and ETYA on the formation of testosterone from exogenous pregnenolone by purified rat Leydig cells*

Rat Leydig cells were incubated in minimum essential medium + 0.1% bovine serum albumin in the presence of pregnenolone (500 ng/ml) and various concentrations of ETYA and NDGA for 1 h at 32°C under an atmosphere of air/CO₂ (9:1). Testosterone was measured by radioimmunoassay on triplicate samples. Pregnenolone (500 ng/ml) was undetectable in the testosterone radioimmunoassay. Values represent means $\pm$ S.D.. Results of inhibitor concentrations were compared with control values by Student's *t*-test: **P* < 0.05, ***P* < 0.001.

Inhibitor	Concentration (μ M)	Testosterone production (ng/10 ⁶ cells per 2h)
None	—	169.1 $\pm$ 3.0
NDGA	0.625	199.5 $\pm$ 4.9**
	1.25	238.4 $\pm$ 3.2**
	2.56	202.1 $\pm$ 3.3**
	5.0	173.3 $\pm$ 0.9*
	10.0	166.4 $\pm$ 2.8
	20.0	161.1 $\pm$ 0.9
ETYA	3.125	225.6 $\pm$ 2.8**
	6.25	241.1 $\pm$ 2.4**
	12.5	226.7 $\pm$ 4.6**
	25.0	198.4 $\pm$ 4.8**
	50.0	184.5 $\pm$ 0.9*
	100.0	169.7 $\pm$ 1.6

reduced the amount of radioactive products in fractions 2, 3 and 4 (Fig. 3, lane 5) and in three comparable experiments the percentage reduction of these products was statistically significant (Table 6). BW755C and benoxaprofen also produced a similar pattern of inhibition of the arachidonic acid metabolites in these fractions (Table 6) while aspirin had similar inconsistent effects to indomethacin (Table 6).

Fig. 3. *Autoradiograph of ¹⁴C-labelled metabolites of arachidonic acid produced by rat tumour Leydig cells* Rat tumour Leydig cells were incubated with either [¹⁴C]arachidonic acid (0.5 $\times$ 10⁶ d.p.m.) (lane 1) or [¹⁴C]arachidonic acid + LH (1.0 μ g/ml, lane 2), + A23187 (1.9 μ M, lane 3), + A23187 and indomethacin (50 μ M, lane 4) or A23187 and NDGA (10 μ M, lane 5), under the conditions described in the legend to Table 4, and the subsequent t.l.c. of the products was autoradiographed as described in the Materials and methods section. The arrows represent the positions of radiolabelled markers run in separate lanes on the t.l.c. plate. Fractions 1–6 are the areas of each lane for this and subsequent experiments in which the radioactivity was determined, summated and represented as a percentage of the total radioactivity as seen in Table 4.

Table 4. Effect of various inhibitors of arachidonic acid metabolism on LH-stimulated pregnenolone production by purified rat tumour Leydig cells

Rat tumour Leydig cells were preincubated in minimum essential medium + 0.1% bovine serum albumin for 2h and then for 15 min with various concentrations of aspirin, indomethacin, BW 755C or benoxaprofen followed by a 1 h incubation in the presence of LH (1 µg/ml) at 32°C under an atmosphere of air/CO₂ (9:1). Cyanoketone (5 × 10⁻⁹M) and SU-10603 (2 × 10⁻⁸M) were added 30min before LH. Pregnenolone was determined by specific radioimmunoassay as described in the Materials and methods section. Values represent means ± s.d. of duplicate determinations on triplicate samples. Basal pregnenolone production was 11.6 ± 2.4 ng/10⁶ cells per h (mean ± s.d., n = 3). Results of various inhibitors were compared with control values by Student's *t*-test: **P* < 0.05, ***P* < 0.001, all other values *P* > 0.05.

Inhibitor	[Inhibitor] (µM) ...	Pregnenolone production (ng/10 ⁶ cells per h)				
		0	10	20	40	80
None		38.7 ± 2.4	—	—	—	—
Aspirin		—	32.5 ± 2.7	35.2 ± 1.9	34.8 ± 1.7	35.3 ± 3.1
Indomethacin		—	34.3 ± 3.5	35.3 ± 2.9	36.5 ± 7.1	34.3 ± 4.2
BW 755C		—	32.4 ± 3.8	30.1 ± 3.6*	25.1 ± 2.5**	11.4 ± 1.9**
Benoxaprofen		—	31.3 ± 4.1	28.7 ± 2.98*	21.4 ± 3.7**	13.3 ± 2.3**

Table 5. Effect of NDGA on dibutyryl cyclic AMP-stimulated pregnenolone production by purified rat tumour Leydig cells

Rat tumour Leydig cells were preincubated in minimum essential medium + 0.1% bovine serum albumin for 2h and then for 15 min with various concentrations of NDGA followed by a 2h incubation in the presence of dibutyryl cyclic AMP (2mM) at 32°C under an atmosphere of air/CO₂ (9:1). Cyanoketone (5 × 10⁻⁹M) and SU-10603 (2 × 10⁻⁸M) were added 30min before dibutyryl cyclic AMP. Pregnenolone was determined by specific radioimmunoassay as described in the Materials and methods section. Values represent means ± s.d. of duplicate determinations on triplicate samples. Basal pregnenolone production was 10.4 ± 1.7 ng/10⁶ cells per h (mean ± s.d., n = 3). Results of various NDGA concentrations were compared with control values by Student's *t*-test: **P* < 0.05, ***P* < 0.001, all other values *P* > 0.05.

[NDGA] (µM)	Pregnenolone production (ng/10 ⁶ cells per h)
0	43.4 ± 2.1
2.5	40.1 ± 3.8
5.0	35.1 ± 1.9*
10.0	17.4 ± 3.1**
20.0	9.7 ± 2.5**

Discussion

In the present study it has been clearly demonstrated that inhibitors of lipoxygenase but not cyclo-oxygenase activity inhibit LH- and dibutyryl cyclic AMP-stimulated steroidogenesis in purified testis and tumour Leydig cells. It is doubtful if these inhibitions can be the result of non-specific toxic effects of these compounds since indomethacin and aspirin up to concentrations much in

excess of their cyclo-oxygenase inhibitory activity have little effect upon steroidogenesis. Also, the site of inhibition of steroidogenesis by all four compounds studied, NDGA, ETYA, BW755C and benoxaprofen seems to be the same; after cyclic AMP production and before the conversion of cholesterol to pregnenolone. Furthermore, since NDGA and ETYA are not structurally related to BW755C and benoxaprofen it seems unlikely that a general non-specific effect could be operating. The potency of inhibition of LH-stimulated steroidogenesis by NDGA, ETYA, BW755C and benoxaprofen is very similar to the known potencies of these compounds for inhibition of lipoxygenase activity in other cell systems (Walker & Dawson, 1979; Higgs *et al.*, 1979). These findings, together with the observation that [¹⁴C]-arachidonic acid is metabolized to products whose formation are inhibited by NDGA, BW755C and benoxaprofen, but not by aspirin or indomethacin, and that the degree of metabolism of arachidonic acid to these products is further increased by the calcium ionophore A23187, strongly suggests that lipoxygenase products are involved in the stimulation of steroidogenesis.

It has been known for some time that calcium is required for hormone-stimulated steroidogenesis (Birmingham *et al.*, 1953; Janszen *et al.*, 1976) and that the requirement for calcium is primarily at a step before the conversion of cholesterol to pregnenolone (Hall *et al.*, 1981). The metabolism of arachidonic acid, particularly the release of arachidonic acid from membrane phospholipids by phospholipase A₂, is considered to be a calcium-dependent mechanism (Craven *et al.*, 1981). Results from the present study indicate a potentiation of arachidonic acid metabolism by the calcium ionophore A23187, and A23187 is

Table 6. *Effect of various inhibitors of arachidonic acid metabolism on the metabolism of [¹⁴C]arachidonic acid by rat tumour Leydig cells*

Rat tumour Leydig cells were incubated in minimum essential medium for 30 min at 32°C in the presence of various inhibitors of arachidonic acid metabolism. At the end of the incubation period products were extracted and run on t.l.c. plates as described in the Materials and methods section, after which each lane of the chromatogram was cut into 0.5 cm sections and counted for radioactivity. The radioactivity of six main regions of each lane as indicated in Fig. 3 was summated and represented as a percentage of the control lane containing no inhibitors. The results represent means $\pm$ s.d. from three separate experiments for indomethacin and NDGA (A) and means $\pm$ range for two separate experiments for BW 755C, benoxaprofen and aspirin (B). A: * $P < 0.05$, ** $P < 0.001$, all other values $P > 0.05$; B: values represent means $\pm$ range of means, $n = 2$.

Fraction no.	Percentage of control [¹⁴ C]arachidonic acid metabolism				
	A		B		
	Indomethacin (50 μ M)	NDGA (10 μ M)	BW755C (50 μ M)	Benoxaprofen (50 μ M)	Aspirin (50 μ M)
1	74.9 $\pm$ 11.69	61.0 $\pm$ 38.62	46.7 $\pm$ 3.60	67.3 $\pm$ 1.65	48.0 $\pm$ 4.3
2	108.5 $\pm$ 6.05	36.2 $\pm$ 17.65*	68.0 $\pm$ 6.20	35.2 $\pm$ 14.7	97.4 $\pm$ 12.79
3	105.8 $\pm$ 32.2	36.8 $\pm$ 2.86**	27.0 $\pm$ 4.45	61.3 $\pm$ 4.8	89.5 $\pm$ 13.65
4	93.1 $\pm$ 15.61	45.1 $\pm$ 16.19**	53.5 $\pm$ 7.45	59.0 $\pm$ 23.1	113.6 $\pm$ 0.10
5	122.7 $\pm$ 15.05	109.4 $\pm$ 14.67	169.6 $\pm$ 22.20	146.4 $\pm$ 18.05	123.7 $\pm$ 3.46
6	105.0 $\pm$ 6.97	103.4 $\pm$ 30.67	49.8 $\pm$ 3.15	228.3 $\pm$ 68.9	192.4 $\pm$ 57.4

known to have stimulatory activity on steroidogenesis (Veldhuis & Klase, 1982). Phosphatidylinositol metabolism/turnover has been implicated in steroidogenesis (Farese *et al.*, 1980) and this turnover is the possible mechanism of calcium entry into the cell (Michell, 1975). It is interesting to speculate that the activation of arachidonic acid metabolism and the production of lipoxigenase products may be the link between phosphatidylinositol metabolism, calcium and steroidogenesis, and evidence from the present study would suggest that a lipoxigenase product of arachidonic acid metabolism stimulates the transport of cholesterol or cholesterol ester to the mitochondria.

A role for lipoxigenase products in stimulus-secretion coupling has also been implicated in the pancreatic β cell for the stimulation of insulin release by glucose and other agents (Metz *et al.*, 1982) and in the release of LH by LH releasing hormone in pituitary gonadotrophins (Naor & Catt, 1981). Lipoxigenase products may play a pivotal role in many stimulus-secretion coupling events in endocrine cells. However, in the present study we were unable to show a consistent or convincing effect of LH on arachidonic acid metabolism, which contrasts with the potent effects of the inhibitors on steroidogenesis. This may be because LH mobilizes large amounts of endogenous arachidonic acid from membrane stores, thus diluting the specific radioactivity of the exogenously applied arachidonic acid. Indeed, endogenous arachidonic acid may be metabolized in preference to the exogenous label, indicating that pre-incorporation of [¹⁴C]arachidonic acid may be necessary before

conclusions can be drawn on the role of LH in these events.

An interesting result is the potentiation of the conversion of both pregnenolone and (22R)-hydroxycholesterol to testosterone by low concentrations of NDGA and ETYA. The reason for this is unclear. It is unlikely that selective inhibition of the lipoxigenase pathway leading to an increased production of products via the cyclo-oxygenase pathway could explain this potentiation, since indomethacin did not inhibit the potentiation seen with ETYA or NDGA. A selective inhibition of cyclo-oxygenase at low concentrations of these compounds may result in an increased production of lipoxigenase products and this may have a stimulatory action on one of the enzymes involved in the conversion of pregnenolone to testosterone. It is also possible that both ETYA and NDGA could have direct effects on one of the enzymes in the steroidogenic pathway. However, since the enzymes involved in the conversion of pregnenolone to testosterone are not rate-limiting for LH-stimulated steroidogenesis this potentiation is of minor importance compared with the inhibiting actions of these compounds at early stages in the steroidogenic pathway.

In conclusion, we have demonstrated for the first time that inhibitors of lipoxigenase activity, but not of cyclo-oxygenase activity, cause a potent inhibition of LH- and dibutyryl cyclic AMP-stimulated steroidogenesis in rat Leydig cells. Preliminary evidence indicates that these inhibitors also inhibit products of arachidonic acid metabolism that have similar relative mobilities to

those of reported lipoxygenase products of other systems. Our results indicate that lipoxygenase products may be essential for one of the steps before the conversion of cholesterol or cholesterol esters to pregnenolone. Since these steps are common to all steroidogenic pathways, such findings may be important in the control of steroidogenesis not only in the testis but also in the ovary and the adrenal gland.

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