The arachidonic acid cascade is involved in the masculinizing action of testosterone on embryonic external genitalia in mice

(teratogens/anogenital distance)

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ABSTRACT We have evaluated whether the arachidonic acid cascade may be involved in the folding and fusion of the penis and scrotum in masculine differentiation, a possibility raised by recent observations of the involvement of the arachidonic acid cascade in the analogous embryonic processes of elevation and fusion of the palatal shelves and of folding and fusion of the neural tube. To test this hypothesis, during embryonic masculine differentiation in mice of the B10.A strain, we administered certain agents that produce blockade of masculinization. We report that arachidonic acid can reverse the inhibition of masculine development in male embryos produced by estradiol-17β or by cyproterone acetate, an androgen receptor-site blocker, and that such reversal can be prevented by an inhibitor of cyclooxygenase, such as indomethacin. We have also found that agents that block the arachidonic acid cascade at the level of phospholipase A_2 (cortisone, phenytoin) or at the level of cyclooxygenase (indomethacin, aspirin) also block masculine differentiation and that such antimasculinization is reversed by arachidonic acid. The masculinization of male embryos is inhibited by indomethacin and aspirin, and the masculinization of female embryos produced by exogenous testosterone is prevented by indomethacin. These findings provide evidence that the mechanism by which testosterone organizes the genitalia involves a role of the arachidonic acid cascade leading to prostaglandins at a critical period of development and that interference with testosterone synthesis or action leads to a teratogenic deficiency of arachidonic acid during this time in the genital anlagen.

Mammalian embryos of either genetic sex have initially an indifferent genital tubercle, which develops into a penis and scrotum, if there is a functioning embryonic testis or if there is not, by administration of testosterone to the mother; otherwise, the inborn female program is expressed (1). Agents that interfere with the synthesis (e.g., estradiol-17β), circulation, or action of testosterone (e.g., cyproterone acetate) block masculine differentiation of the genitalia of genetically male embryos, as manifested by the production of hypospadias—i.e., a displacement of the urethral orifice down the shaft of the penis from the tip with a shortening of the anogenital distance (2–10). The means by which testosterone organizes masculine differentiation of the genitalia in the embryo depends on classical androgen hormonal receptor mechanisms (5, 9, 11). However, the precise steps involved in this organizing action of testosterone in the embryo are not known.

A clue to the possible nature of these steps has been given by evidence (12, 13) suggesting that the arachidonic acid cascade leading to prostaglandins may be involved in the elevation and fusion of the embryonic shelves in differentiation of the palate. This suggestion is based on the findings that (*i*) arachidonic acid reverses both the incidence of cleft palate induced in mice and rats by glucocorticoids *in vivo* and the teratogenic action of glucocorticoids in an *in vitro* palate-culture model (12, 13); (*ii*) indomethacin, an inhibitor of prostaglandin and thromboxane synthesis at the level of cyclooxygenase, inhibits this corrective effect of arachidonic acid (12, 13); (*iii*) little or no thromboxanes are present or produced by the mouse embryo (14); and (*iv*) both aspirin, another inhibitor of cyclooxygenase, and indomethacin inhibit cyclooxygenase in the whole rat embryo *in vivo* during the period when palatal and masculine differentiation occur (15). These observations have been supported by recent data obtained from testing whether arachidonic acid may be involved in defects of neural tube folding and fusion in two animal models of diabetic embryopathy (16). Exogenous arachidonic acid injected into pregnant rats made diabetic by streptozotocin (17) significantly lowered the incidence of neural tube defects, cleft palate, and micrognathia occurring in their offspring (16). Moreover, the addition of arachidonic acid to B10.A mouse embryos in culture also resulted in a significant reversal of hyperglycemia-induced failure of neural tube fusion (16).

In this paper we present evidence that the arachidonic acid cascade leading to prostaglandins may be involved in the mechanism by which testosterone produces masculinization of the embryonic genitalia.

MATERIALS AND METHODS

Pregnant B10.A mice from The Jackson Laboratory were injected subcutaneously with estradiol-17β, cyproterone acetate, cortisol, phenytoin, aspirin, or indomethacin, either alone or in combination with arachidonic acid (free acid, 99% pure; Sigma) or arachidonic acid and indomethacin on days 11–14 of gestation, the critical period for affecting the anogenital distance (5). The fetuses were removed by caesarean section on day 18 of gestation. They were weighed, the anogenital distance [the distance from the base of the genital papilla (of the penis or clitoris) to the anterior tip of the opening of the anus] was measured with micrometer calipers, and the gonadal sex was established by internal examination of the gonads (testes or ovaries) (2, 4).

RESULTS

The anogenital distance of B10.A male embryos is 1.70 ± 0.14 mm, whereas that of B10.A female embryos is 0.92 ± 0.17 mm (Table 1). The anogenital distance of genetically male embryos was significantly reduced by estradiol-17β or cyproterone acetate alone, to the point where the anogenital distance of treated males was not significantly different from that of untreated females. Arachidonic acid corrected the

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Table 1. Arachidonic acid blocks antimasculinization of anogenital distance by estradiol-17β or cyproterone acetate in B10.A embryos

Treatment (dose per day)*	No. of litters	Fetal weight, g	Anogenital distance, mm	
			Male (no.)	Female (no.)
Dimethyl sulfoxide	6	1.06 ± 0.12	1.70 ± 0.14 (19)	0.92 ± 0.17 (21)
Estradiol-17β (2 mg/kg)	5	1.01 ± 0.10	0.99 ± 0.19† (15)	0.82 ± 0.10 (21)
+ arachidonic acid (25 mg/kg)	6	1.02 ± 0.10	1.65 ± 0.16‡ (14)	0.84 ± 0.15 (12)
+ arachidonic acid (25 mg/kg) and indomethacin (1 mg/kg)	5	1.02 ± 0.14	0.97 ± 0.07§ (5)	0.85 ± 0.08 (3)
Cyproterone acetate (20 mg/kg)	5	1.04 ± 0.11	1.00 ± 0.12† (17)	0.85 ± 0.11 (15)
+ arachidonic acid (100 mg/kg)	5	1.04 ± 0.10	1.56 ± 0.14¶ (21)	1.32 ± 0.22¶ (16)
+ arachidonic acid (25 mg/kg)	5	1.06 ± 0.15	1.50 ± 0.14¶ (18)	0.90 ± 0.17 (17)
+ arachidonic acid (100 mg/kg) and indomethacin (1 mg/kg)	5	1.04 ± 0.12	0.97 ± 0.07‖ (17)	0.88 ± 0.06 (16)
Arachidonic acid (100 mg/kg)	5	1.02 ± 0.14	1.57 ± 0.09 (18)	1.22 ± 0.08† (19)
Arachidonic acid (25 mg/kg)	4	1.05 ± 0.10	1.60 ± 0.12 (14)	0.94 ± 0.11 (13)

Values are means ± SD.
*Given daily on days 11–14 of gestation. Dimethyl sulfoxide was vehicle for all test agents with the exception of arachidonic acid, which was injected by itself.
†$P < 0.01$ compared to dimethyl sulfoxide (Student's t test).
‡$P < 0.01$ compared to estradiol-17β.
§$P < 0.01$ compared to estradiol-17β + arachidonic acid.
¶$P < 0.01$ compared to cyproterone acetate.
‖$P < 0.01$ compared to cyproterone acetate + arachidonic acid.

antimasculinization induced by each of these compounds to normal (Table 1). Complete resorption of litters was observed with the combination of arachidonic acid at 100 mg/kg and estradiol-17β at 2 mg/kg. However, at a dose of 25 mg/kg, arachidonic acid produced nearly a complete correction of the inhibition of masculinization induced by estradiol-17β or cyproterone acetate. Indomethacin completely blocked the corrective effect of arachidonic acid on either estradiol-17β- or cyproterone acetate-induced antimasculinization (Table 1).

Cortisone or phenytoin completely blocks the masculinization of the external genitalia in genetically male embryos (Table 2). Arachidonic acid corrects the blockade of masculinization produced by cortisone or phenytoin (Table 2), to about the same degree as that produced in the corrective effect of arachidonic acid on cyproterone acetate-induced antimasculinization (Table 1). Aspirin (according to dose) and indomethacin block the masculinization of genetically male embryos (Table 2). Indomethacin also blocks both the virilization of genetically female genitalia produced by testosterone and of genetically male genitalia despite exogenous testosterone (Table 3).

Arachidonic acid, when given alone at 100 or 200 mg/kg, virilizes (increases) the anogenital distance of genetically

female embryos; the effect is greater with the 200-mg/kg dose than with the 100-mg/kg dose (Fig. 1). Virilization of genetically female embryos is exhibited when this dose of arachidonic acid is administered along with cyproterone acetate (Table 1) or with cortisone or phenytoin (Table 2), but no effect on female genitalia appears with arachidonic acid at 25 mg/kg, either alone (Fig. 1) or with estradiol-17β or cyproterone acetate (Table 1).

DISCUSSION

The present report shows that exogenous arachidonic acid reverses the antimasculinization of the external genitalia of male embryos (as manifested by hypospadias and shortening of the anogenital distance) produced by estradiol-17β or cyproterone acetate. Estradiol-17β is thought to interfere with embryonic synthesis of testosterone (5), and cyproterone acetate, an androgen receptor-site blocker, is thought to interfere with the action of testosterone (9). We have also observed the same degree of reversal by exogenous arachidonic acid at 25 mg/kg on days 11–14 in CD-1 mice treated with estradiol-17β at 2 mg/kg (data not shown), so that the effect does not appear to be strain-specific. The degree of this protective effect is dependent on the dose of arachidonic

Table 2. Effect of inhibitors of the arachidonic acid cascade and arachidonic acid supplementation on anogenital distance in B10.A embryos

Treatment (dose per day)	No. of litters	Fetal weight,* g	Anogenital distance,* mm	
			Male (no.)	Female (no.)
Dimethyl sulfoxide	6	1.06 ± 0.12	1.70 ± 0.14 (19)	0.92 ± 0.17 (21)
Cortisone (100 mg/kg)	5	1.05 ± 0.10	0.84 ± 0.11† (15)	0.80 ± 0.09 (14)
+ arachidonic acid (100 mg/kg)	5	1.05 ± 0.09	1.54 ± 0.14‡ (19)	1.14 ± 0.10†‡ (18)
Phenytoin (50 mg/kg)	5	1.05 ± 0.14	0.95 ± 0.14† (14)	0.83 ± 0.16 (15)
+ arachidonic acid (100 mg/kg)	5	1.04 ± 0.10	1.52 ± 0.16‡ (16)	1.22 ± 0.13†‡ (17)
Aspirin (150 mg/kg)	5	1.05 ± 0.12	1.40 ± 0.26† (25)	0.83 ± 0.08 (20)
Aspirin (400 mg/kg)	5	1.05 ± 0.14	1.05 ± 0.20† (17)	0.88 ± 0.12 (16)
Indomethacin (1 mg/kg)	5	1.05 ± 0.10	1.43 ± 0.14† (20)	0.87 ± 0.11 (19)

Dosage schedule was as described for Table 1. Dimethyl sulfoxide was vehicle for all test agents with the exception of phenytoin, which was injected in a commercially supplied vehicle (Parke-Davis), and aspirin, which was injected as a suspension in 0.9% NaCl.
*Mean ± SD.
†$P < 0.01$ compared to dimethyl sulfoxide (Student's t test).
‡$P < 0.01$ compared to cortisone or phenytoin.

Table 3.   Indomethacin blocks masculinization of anogenital distance of female embryos by testosterone in B10.A mice

Treatment (dose per day)	No. of litters	Anogenital distance,* mm	
		Male (no.)	Female (no.)
Dimethyl sulfoxide	6	1.70 ± 0.14 (19)	0.92 ± 0.17 (21)
Testosterone (10 mg/kg)	5	1.66 ± 0.05 (21)	1.52 ± 0.11† (17)
Testosterone (10 mg/kg) + indomethacin (1 mg/kg)	5	1.05 ± 0.17‡ (20)	0.88 ± 0.12‡ (20)

Treatment, dosage schedule, and vehicles were as described for Tables 1 and 2.
*Mean ± SD.
†$P < 0.001$ compared to dimethyl sulfoxide (Student's t test).
‡$P < 0.001$ compared to testosterone.

acid. This corrective action of arachidonic acid is blocked by indomethacin, an inhibitor of cyclooxygenase, the enzyme converting arachidonic acid to prostaglandins and thromboxanes (18). Since the embryo at this time synthesizes little or no thromboxanes from arachidonic acid (14), these data suggest that the action of testosterone on the embryonic genitalia involves the arachidonic acid cascade leading to prostaglandins. This suggestion is supported by the present observations that aspirin [also an inhibitor of cyclooxygenase (18)], as well as indomethacin, inhibits masculinization of male genitalia and that virilization of female genitalia produced by testosterone is prevented by indomethacin. It is interesting that aspirin *in vivo* also produces cleft palate and neural tube defects (19).

Cortisone and phenytoin, at doses previously reported to produce cleft palate in these mice (20, 21), also completely block masculinization of the external genitalia in male embryos. This block is reversed by arachidonic acid, and this reversal is reblocked by indomethacin. That blockade of testosterone synthesis or action causes failure of masculine anogenital development by affecting availability of arachidonic acid makes this embryonic malformation similar to what has been described for experimentally produced cleft palate and for hyperglycemia-induced failure of neural tube fusion in experimental diabetic embryopathy. Penile and scrotal fusion may be analogous to palatal shelf and neural tube fusion, in that both latter embryonic processes require embryonic cell movements and apposition of the two advancing tissues (22, 23). In the case of palatal and neural tube fusion, both processes involve cell death at the point leading to fusion and closure (24, 25). However, it is not known whether cell death is involved in genital differentiation. In the malformation model of glucocorticoid- and phenytoin-induced cleft palate, this sequence of events has been delineated experimentally (26, 27). Glucocorticoids (28–30)

or phenytoin interact with a cytosolic receptor, and the receptor complex induces phospholipase A_2-inhibiting proteins (PLIP) (31, 32). Phospholipase A_2 catalyzes the release of arachidonic acid from its position on the number 2 carbon of phospholipids; and an increase in PLIP therefore sharply decreases the availability of arachidonic acid. This sequence has been verified by experiments that demonstrate that either specific blockade of the cytosolic glucocorticoid receptor or provision of exogenous arachidonic acid will act to prevent the malformation (12, 13, 33). In this system, arachidonic acid availability for prostaglandin synthesis appears to be of major importance, since indomethacin will reverse the protective effects of exogenous arachidonic acid (12, 13). In this connection, it is of interest that neural tube defects are also produced by glucocorticoids (34) and phenytoin (35); that neural tube defects, as well as cleft palate, are produced by cyproterone acetate (36) and estradiol-17β (37); and that children with the fetal diphenylhydantoin (phenytoin) syndrome occasionally have hypospadias (38).

Arachidonic acid prevents neural tube defects, cleft palate, and micrognathia in the rat models of diabetic embryopathy and neural tube defects in the mouse embryo culture model (16). Indomethacin prevents this arachidonic acid-induced reversal of diabetic embryopathy.† The present data, added to the previous findings for the palate and neural tube, raise the possibility that this biochemical system may be a major mechanism mediating many embryonic events that involve cellular movements and fusion.

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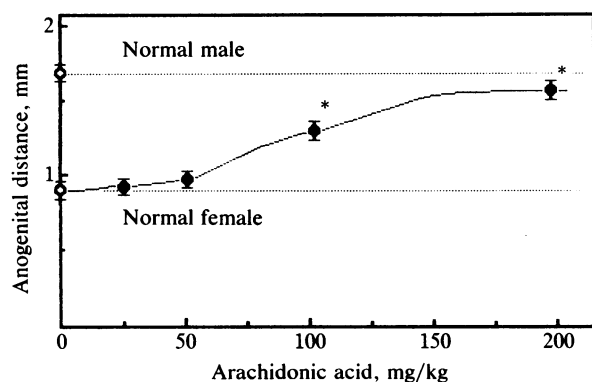
FIG. 1.   Arachidonic acid increases anogenital distance in female fetal mice. Number of fetuses examined: 10 (control); 10 (25 mg/kg); 12 (50 mg/kg); 20 (100 mg/kg); 25 (200 mg/kg). Asterisks indicate $P < 0.001$ compared to control.

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