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REASONS FOR REDUCED ACTIVITIES OF 17α-HYDROXYLASE AND C_{17}–C_{20} LYASE IN SPITE OF INCREASED CONTENTS OF CYTOCHROME *P*-450 IN MATURE RAT TESTIS FETALLY IRRADIATED WITH ^{60}Co

HIROSHI INANO,* HIROKO ISHII-OHBA, KEIKO SUZUKI and KIYOMI IKEDA
National Institute of Radiological Sciences, 9-1, Anagawa-4-chome, Chiba-shi 260, Japan

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Summary—Pregnant rats received whole body irradiation with 2.6 Gy gamma-ray from a ^{60}Co source at Day 20 of gestation. When pups were 4 months old, activities of electron transport system and steroid monooxygenase in testes were assayed. The content of total cytochrome *P*-450 in the irradiated testes had increased to 170% of that in non-irradiated rats, but NADPH-cytochrome *P*-450 reductase activity had reduced to 36% of the control. Also, amounts of cytochrome b_5 in testicular microsomal fraction were decreased markedly after irradiation, but no significant change of NADH-cytochrome b_5 reductase activity was observed in the treated pups. Because both 17α-hydroxylase and C_{17}–C_{20} lyase activities tended to be decreased by fetal irradiation, testosterone production from progesterone and 17α-hydroxyprogesterone was reduced to about 30% of the control. From these results, it has been suggested that the testicular cytochrome *P*-450 is radioresistant but steroid monooxygenase activities are reduced after the fetal irradiation. We propose that the discrepancy arises from the marked decrement of NADPH-cytochrome *P*-450 reductase activity.

INTRODUCTION

Though testis is now recognized as one of the most radio-sensitive organs, Leydig cells and Sertoli cells in the mammalian testis are thought histologically to be radio-resistant. However, our previous data suggest that some of those cells may be biochemically radio-sensitive [1]. When pregnant rats at Day 20 of gestation were irradiated with gamma-ray, it was observed that male offspring had no germinal epithelium and contained essentially only Sertoli cells [2–4]. Fetal irradiation with 2.6 Gy gamma-ray produced male offspring with lower *in vivo* testosterone production than the control judging from atrophied sex accessory organs [5]. On the other hand, the effect of the irradiation on electron transport system in the testes has not been investigated. This study was undertaken to examine the effect of fetal ^{60}Co-irradiation on the electron transport system related to cytochrome *P*-450 and b_5.

*To whom correspondence should be addressed.

Enzymes: 17α-hydroxylase (EC 1.14.99.9); function of 17α-hydroxylation by cytochrome $P\text{-}450_{17\alpha,\,lyase}$, C_{17}–C_{20} lyase (EC 4.1.2.30); function of C_{17}–C_{20} cleavage by cytochrome $P\text{-}450_{17\alpha,\,lyase}$, 5-ene-3β-hydroxysteroid dehydrogenase (EC 1.1.1.145), 5-ene-4-ene-isomerase (EC 5.3.3.1), 17β-hydroxysteroid dehydrogenase (EC 1.1.1.64), NADPH-cytochrome *P*-450 reductase (EC 1.6.2.4.) and NADH-cytochrome b_5 reductase (EC 1.6.2.2.).

EXPERIMENTAL

Chemicals

[4-^{14}C]Progesterone (2.1 GBq/mmol) and [4-^{14}C]-17α-hydroxyprogesterone (1.9 GBq/mmol) were purchased from New England Nuclear (Boston, Mass) and non-radiolabeled steroids were obtained from Steraloids (Wilton, N.H.). NADH and NADPH were obtained from Boehringer (Mannheim, F.R.G.). Horse heart cytochrome *c* was obtained from Sigma (St Louis, Mo.) and Emulgen 913 was supplied by Kao Atlas (Tokyo, Japan).

Animals

Pregnant Wistar strain rats received a whole body irradiation at Day 20 of gestation (presence of a vaginal plug denotes Day 1) with 2.6 Gy gamma-ray from a ^{60}Co source. The dose rate was 0.18 Gy/min. Parturition occurred 3 days later. Within 2 days of birth, 10 pups were randomly distributed among the lactating irradiated mothers. Controls were not exposed to fetal irradiation. When pups were 4 months old, they were sacrificed by CO_2 asphyxiation. For measurement of activities of electron transport system, testes obtained from both groups were employed.

Tissue preparation

After removal of the testes, the organs were decapsulated, rinsed with ice-cold 0.25 M sucrose–10 mM

Tris buffer (pH 7.4) and homogenized by a loose-fitting Teflon-glass homogenizer. The testicular homogenates were centrifuged at 10,000 *g* for 20 min at 4°C, and the supernatant was centrifuged at 105,000 *g* for 60 min. The precipitate was suspended in the sucrose buffer as microsomal fraction. Protein concentration was determined by Bradford's method [6] using bovine serum gamma-globulin as standard.

Assays

Microsomal fraction (0.75 mg protein) was suspended in 1 ml of 10 mM phosphate buffer (pH 7.4) containing 0.025% Emulgen 913, and then a few milligrams of solid $Na_2S_2O_4$ were added to the preparation. CO-difference spectra of cytochrome *P*-450 were measured by the methods of our previous paper [7]. Concentration of total cytochrome *P*-450 was estimated from difference of the absorption at 450 and 490 nm of the CO-induced difference spectrum, using a molecular extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ [8]. Microsomal fraction (0.75 mg protein) was suspended in 1 ml of 0.25 M sucrose solution containing 2 mM KCN. NADH (0.2 mM as final concentration) was added to the preparation and cytochrome b_5 was assayed as described previously [9]. Concentration of cytochrome b_5 was estimated from difference of the absorption at 423 and 409 nm, using a molecular extinction coefficient of 185 $mM^{-1} \cdot cm^{-1}$ [10]. NADPH-cytochrome *P*-450 reductase activity was followed as the change in absorbancy at 550 nm. The cuvette contained the following: 0.1 mM NADPH, 1 mM KCN, 30 μM cytochrome *c* and microsomal fraction in 1 ml of 100 mM phosphate buffer. The reaction was initiated by addition of NADPH [11]. The millimolar extinction differences between reduced and oxidized cytochrome *c* were 21.2 at 550 nm [12]. For assay of NADH-cytochrome b_5 reductase, the reaction mixture contained 0.1 mM NADH, 0.2 mM potassium ferricyanide and microsomal fraction in 1 ml of 100 mM phosphate buffer. A value of 1.02 $mM^{-1} \cdot cm^{-1}$ was used as the molecular extinction decrement of absorption at 420 nm [13]. In the measurement of steroid monooxygenase activities, the amounts of substrates used per incubation tube were: [4-^{14}C]progesterone (50 nmol, 3.5×10^4 cpm) for assay of 17α-hydroxylase and [4-^{14}C]17α-hydroxyprogesterone (50 nmol, 3.5×10^4 cpm) for C_{17}–C_{20} lyase. Volume of the incubation mixture per tube was adjusted to 2 ml by addition of 0.25 M sucrose–10 mM Tris buffer (pH 7.4).

Separation of steroid metabolites

After the incubation, methylene dichloride (3 ml) was added to the reaction flask and the mixture was vigorously shaken for extraction. The methylene dichloride layer was collected, and was dried with anhydrous sodium sulfate. The radioactive metabolites were separated by TLC on silica gel (E. Merck, $60F_{254}$) using benzene–acetone (4:1, v/v) as solvent system. After development, the radioactive spots were detected by exposing the thin-layer plate to X-ray film. Detected steroid spots were scraped off the glass plate. The radioactivity was measured with a liquid scintillation spectrometer (LS 1701, Beckman) for a sufficient time to reduce the counting error to below 2%. The efficiency of counting ^{14}C was about 90%. For identification of the metabolites, constant specific activities of crystals were observed by repeated crystallization of the radioactive metabolites with the corresponding authentic steroid standards.

RESULTS

Biological change induced by fetal irradiation

When pregnant rats were irradiated with 2.6 Gy gamma-ray at Day 20 of gestation, growth of the offspring, 4 months old, was markedly inhibited (Table 1). Also, testicular weight of the irradiated rats was 15% of that in the control group. In sex accessory organs, the weight of seminal vesicles in the irradiated animals was also significantly lower than that of the control, but no statistical difference was observed in the prostate glands.

Cytochrome P-450 and its reductase

When fetal rats were irradiated *in utero*, the content of total cytochrome *P*-450 in the testicular microsomal fraction was increased to 170% of that in non-irradiated animals (Table 2). When amounts of cytochrome *P*-450 were expressed on the basis of 1 mg protein in the microsomal fraction, the specific content in the irradiated testes was 6.3 times that of the control. On the other hand, the NADPH-cytochrome *P*-450 reductase activity per organ, which catalyzes the transfer of electron of NADPH to cytochrome *P*-450, was reduced to 36% by fetal irradiation. However, specific activity of the reductase was observed as 150% of that in the non-irradiated animals.

Cytochrome b_5 and its reductase

After irradiation, amounts of cytochrome b_5 based on a pair of testes were significantly decreased, but the activity of NADH-cytochrome b_5 reductase was not different from the non-irradiated group. On the basis of mg protein in the microsomal fraction, cytochrome b_5 and NADH-cytochrome b_5 reductase were markedly higher in the irradiated group than those in the control.

Table 1. Biological change induced by fetal ^{60}Co-irradiation

Group	No. of rats	Body wt (g)	Testes wt (g)	Prostate wt (mg)	Seminal vesicle (mg)
Control	4	455 ± 11	3.02 ± 0.05	360 ± 14	1151 ± 167
Irradiation	4	354 ± 27	0.47 ± 0.04	287 ± 38	670 ± 97
Difference		$P < 0.05$	$P < 0.001$	NS	$P < 0.05$

Mean ± SE. NS, no significant difference ($P > 0.10$).

Table 2. Effect of fetal ^{60}Co-irradiation on electron transport system in testicular glands

Enzyme (unit)	Control ($n = 4$)	Irradiation ($n = 4$)	Difference
Cytochrome *P*-450			
(nmol/testes)	1.91 ± 0.09	3.28 ± 0.02	$P < 0.10$
(nmol/mg protein)	0.07 ± 0.03	0.45 ± 0.10	$P < 0.10$
NADPH-cytochrome *P*-450 reductase			
(nmol/testes)	180 ± 42	65 ± 7	$P < 0.10$
(nmol/min/mg protein)	5.69 ± 0.57	8.52 ± 0.71	$P < 0.10$
Cytochrome b_5			
(nmol/testes)	10.00 ± 0.37	5.55 ± 0.74	$P < 0.05$
(nmol/mg protein)	0.33 ± 0.05	0.74 ± 0.08	$P < 0.05$
NADH-cytochrome b_5 reductase			
(μmol/testes)	15.40 ± 1.47	11.6 ± 1.11	NS
(μmol/min/mg protein)	0.50 ± 0.02	1.53 ± 0.12	$P < 0.02$

Mean ± SE. NS, no significant difference ($P > 0.10$).

Metabolism of progesterone and 17α-hydroxyprogesterone in irradiated testes

The testicular microsomal fraction (equivalent to one-tenth of a pair of testes) obtained from the control and ^{60}Co-irradiated animals was incubated with the substrate in the presence of NADPH under aerobic conditions. When progesterone was used as substrate, 17α-hydroxyprogesterone, which was produced by cytochrome $P\text{-}450_{17\alpha,\,lyase}$, was obtained as the major metabolite in both groups (Fig. 1). However, the production rate was decreased by the irradiation, because the 17α-hydroxylase activity per organ of irradiated animals was significantly lower than that of the control group. Androstenedione was obtained as the direct metabolite by cytochrome $P\text{-}450_{17\alpha,\,lyase}$ following incubation with 17α-hydroxyprogesterone. A similar pattern of the metabolite was quantitatively observed in both groups. However, testosterone production from progesterone and 17α-hydroxyprogesterone in the irradiated group was reduced to about 30% of the control. Although the activity on the basis of organ of C_{17}–C_{20} lyase tended to decrease by the fetal irradiation, no statistical difference was observed (Table 3). On the other hand, the increased activities for 17α-hydroxylase and C_{17}–C_{20} lyase were demonstrated in the testes from fetally irradiated animals, when the enzyme activities were expressed on the basis of mg protein in microsomal fraction.

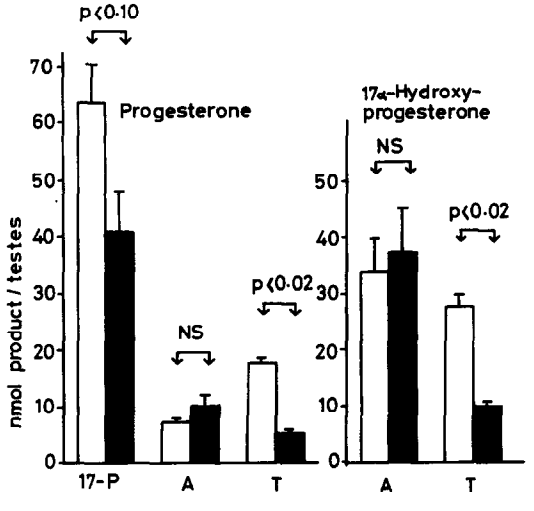

Fig. 1. Metabolism of progesterone and 17α-hydroxyprogesterone by fetally irradiated testes. Open column; control, closed column; irradiation, 17-P; 17α-hydroxyprogesterone, A; androstenedione, T; testosterone, NS; no significance difference ($P > 0.10$). Bars represent SE ($n = 4$).

DISCUSSION

After *in vitro* gamma-ray irradiation in the range of 50–1000 Gy to hepatic microsomal fraction, total cytochrome *P*-450 content was greatly reduced and radiation-induced lipid peroxidation was linearly increased with increasing radiation dose [14]. On the other hand, no change in the content of cytochrome *P*-450 was observed when irradiated purified cytochrome *P*-450 was combined with non-irradiated liposomes, but non-irradiated cytochrome *P*-450 was markedly destroyed after reconstitution with irradiated liposomes, suggesting that the radiation-induced lipid peroxidation causes the inactivation of hepatic cytochrome *P*-450 [15].

In the microsomal fraction of rat testes, pregnenolone was converted to progesterone by 5-ene-3β-hydroxysteroid dehydrogenase coupled with isomerase, and NADH was generated simultaneously during the reaction. Progesterone was metabolized to 17α-hydroxyprogesterone and androstenedione by cytochrome $P\text{-}450_{17\alpha,\,lyase}$. Cytochrome b_5 and NADH-cytochrome b_5 reductase in the testes catalyzed the transfer of an electron from NADH produced by 5-ene-3β-hydroxysteroid dehydrogenase to cytochrome $P\text{-}450_{17\alpha,\,lyase}$ [16, 17]. Therefore, two different systems for electron transport are involved in the production of androstenedione from progesterone in testicular microsomal fraction. Nevertheless

Table 3. Effect of fetal ^{60}Co-irradiation on steroid monooxygenase activities in testicular microsomal fraction

Enzyme (unit)	Control ($n = 4$)	Irradiation ($n = 4$)	Difference
17α-Hydroxylase			
(nmol/testes)	89.1 ± 6.2	57.4 ± 7.7	$P < 0.10$
(nmol/mg protein)	2.9 ± 0.2	7.6 ± 0.9	$P < 0.05$
C_{17}–C_{20} Lyase			
(nmol/testes)	62.0 ± 7.8	47.6 ± 7.4	NS
(nmol/mg protein)	2.0 ± 0.02	6.3 ± 0.9	$P < 0.05$

Mean ± SE. NS, no significant difference ($P > 0.10$).

the total contents of cytochrome *P*-450 were increased by the fetal ^{60}Co-irradiation, and *in vivo* production of androgen in the irradiated animals appeared to be relatively lower than the control, judging from the weight of sex accessory organs. Also, *in vitro* formation of testosterone from progesterone was significantly decreased by the irradiation. One of the reasons for both the *in vivo* and *in vitro* phenomena may be that the activity of NADPH-cytochrome *P*-450 reductase and content of cytochrome b_5 were significantly reduced by the irradiation. For another reasons, it has been suggested that 17β-hydroxysteroid dehydrogenase which catalyzes testosterone formation from androstenedione was also sensitive to radiation [5].

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