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Electron Leakage from the Adrenal Cortex Mitochondrial P450scc and P450c11 Systems: NADPH and Steroid Dependence[1]

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In the present study we examined the coupling of NADPH oxidation to substrate hydroxylation and the effects of steroids on this process in reconstituted P450scc and P450c11 systems. To determine the relative rates of substrate hydroxylation vs electron leakage we assayed both the steroid product and H_2O_2 in the same sample. For both P450 systems the rates of steroid product and superoxide formation increased as NADPH concentration was increased. However, P450c11 was found to be more leaky. The leakage from the P450scc system was not affected by pregnenolone, the product of cholesterol side chain cleavage. In contrast, corticosterone, the product of P450c11, increased the rate of futile NADPH oxidation by the P450c11 system. We also tested a series of steroids to analyze the stereospecificity of their effects. Relative to the control without steroid, both C-19 and C-21 steroids with 11α-hydroxy groups (11α-OH-testosterone and 11α-OH-cortisol) decreased leakage, and those with 11β-OH groups (11β-OH-testosterone and cortisol) stimulated both NADPH oxidation and electron leakage as measured by H_2O_2 formation. The results revealed a correlation between the effects previously observed in living cells and in our reconstituted systems. These findings provide further evidence that mitochondrial P450 systems indeed function as a significant source of oxygen radicals in steroidogenic cells.

Key Words: **cytochrome P450; adrenodoxin; electron transfer; mitochondria; oxygen radicals; steroidogenesis.**

Cytochromes P450scc and P450c11 are the two most abundant steroidogenic enzymes in the adrenal cortex. P450scc catalyzes conversion of cholesterol to pregnenolone, and P450c11 catalyzes 11β-hydroxylation of steroids (1–3). These monooxygenation reactions are dependent on the transport of electrons from NADPH through adrenodoxin reductase and adrenodoxin (1–4). In systems reconstituted with purified proteins this electron transport chain can also function as a futile NADPH oxidase, oxidizing NADPH and reducing O_2 to produce superoxide even in the absence of a steroid substrate (5–7). The leakage of electrons from P450scc to O_2 is reduced but not eliminated during the metabolism of the substrate, cholesterol (7).

Several lines of evidence indicate that P450 systems also generate oxygen radicals in living cells (8–11). In cultured adrenocortical cells P450c11 can undergo rapid degradation after addition of some steroids (11, 12). This destructive effect could be prevented by antioxidants, suggesting that the steroids exert their effects by enhancing formation of harmful oxygen radicals (12, 13). The very high concentrations of ascorbic acid in the adrenal cortex may serve as an antioxidant for the oxygen radicals produced by the P450 systems (11).

In the present study we examined the coupling of NADPH oxidation to substrate hydroxylation and the effects of steroids on this process in reconstituted P450scc and P450c11 systems. The results indicate that there is a correlation between the effects observed in living cells and those observed in reconstituted systems. These findings support the hypothesis that the destructive effects of steroids on P450c11 in cultured cells result from their stimulation of electron leakage directly from this P450.

MATERIALS AND METHODS

Reagents and enzymes were purchased from previously indicated sources (7). Steroids were obtained from Sigma or Ikapharm. Adreno-

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doxin reductase, adrenodoxin, P450scc, and P450c11 were purified from bovine adrenal cortex (14–16). Concentrations of NADPH, adrenodoxin reductase, adrenodoxin, and P450scc were calculated from their extinction coefficients (mM^{-1} cm^{-1}) at 340 nm (6.2), 450 nm (10.9), 414 nm (11), and 390 nm (110), respectively.

All reactions were carried out at 37°C (P450scc) or 30°C (P450c11), in 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.2, under aerobic conditions. The reactions were started by the addition of adrenodoxin reductase. The concentrations of adrenodoxin reductase and adrenodoxin in reactions were essentially not rate limiting as previously investigated (7).

The spectrophotometric assays were carried out in a final volume of 0.5 ml in a thermostatically controlled cuvette. NADPH oxidation rate was determined at 340 nm during the linear phase of the reaction.

The reactions for the measurement of electron leakage also included superoxide dismutase (SOD),[3] catalase, and 6% methanol to convert superoxide to H_2O_2 and to stochiometrically produce formaldehyde per molecule of H_2O_2 generated as described in our recent method (17). The method is based on peroxidation of methanol to formaldehyde and quantitation of formaldehyde by the fluorescence of its reaction product with the Nash reagent (17). This fluorimetric assay is more sensitive than the assay we used previously (7), and it can be used in enzymatic systems including NAD(P)H.

The reactions in Fig. 1 were carried out in 200 μl; the reaction was stopped by transferring 100 μl into the Nash mixture for H_2O_2 assay (17), and adding 100 μl ethanol to the remainder for further processing for steroid assays.

Cholesterol conversion to pregnenolone (side chain cleavage) was assayed by a radiometric method (7, 18). Deoxycorticosterone 11β-hydroxylation activity was measured by a fluorometric assay for corticosterone (19). The results were presented as e^- transfer rate calculated by multiplying by two the rate of NADPH oxidation, substrate hydroxylation, or hydrogen peroxide formation. The concentration of electrons consumed during cholesterol conversion to pregnenolone was calculated by multiplying by six the concentration of pregnenolone formed, because this reaction involves three consecutive hydroxylations (2).

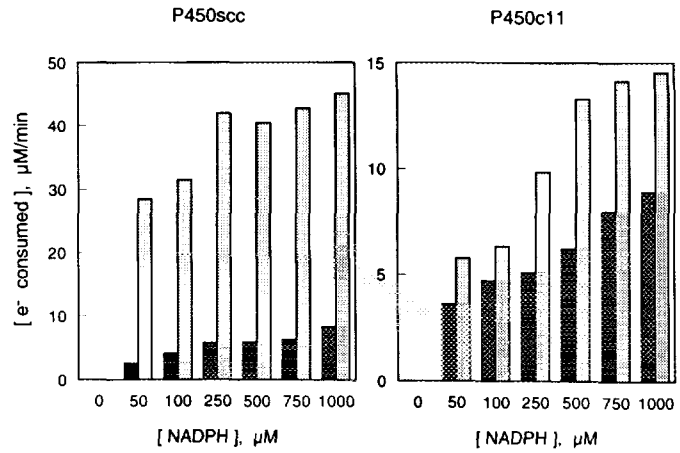

FIG. 1. Electron (e^-) consumption for substrate hydroxylation (light bars) and H_2O_2 formation (dark bars) in reconstituted mitochondrial P450 systems during substrate metabolism. P450scc and P450c11 reactions were assayed by the conversion of cholesterol to pregnenolone and 11β-hydroxylation of deoxycorticosterone to corticosterone, respectively. The reactions were carried out in 10 mM Hepes, pH 7.2, 100 mM KCl, with 0.1 μM adrenodoxin reductase, 9 μM adrenodoxin, and indicated concentrations of NADPH. Under these conditions adrenodoxin reductase and adrenodoxin are not rate limiting (7). All reactions also included 6% methanol, 200 U/ml catalase, and 10 U/ml SOD for H_2O_2 assay (17). P450scc reaction included 0.3 μM P450scc, 0.3% Tween 20, and 200 μM cholesterol (added from a 16 mM stock solution in dimethyl formamide) and were carried out at 37°C for 4 min. P450c11 reactions included 0.15 μM P450c11, 50 μM deoxycorticosterone (added from a 10 mM stock solution in methanol) and were carried out at 30°C for 3 min. Each bar represents the average of triplicate assays.

RESULTS

NADPH Dependence of Electron Leakage

To determine the relative rates of substrate hydroxylation vs electron leakage we assayed both the steroid product and H_2O_2 in the same sample. For both P450 systems the rates of steroid product and H_2O_2 formation increased with NADPH concentration (Fig. 1). P450c11 showed stronger dependence on NADPH, as its activity increased 300%, while P450scc activity rose about 50%. In the P450scc system at most 15% of the total electron flow was directed to H_2O_2 formation, whereas in the P450c11 system 32–43% of the electrons were consumed in H_2O_2 generation (Fig. 1).

As compared to the leakage observed in the absence of a substrate, during substrate hydroxylation leakage decreased in the P450scc system and it increased in the P450c11 system (from 4.1 to 2.5 μM e^-/min for P450scc and from 2.1 to 3.1 μM e^-/min for P450c11 at 60 μM NADPH under the conditions of Fig. 1). The presence of SOD and catalase in the assay slightly enhanced the turnover rate of pregnenolone formation to 25 as compared to the usual rate of 15–20 mol/mol P450scc/min (16). The absolute rates are not directly comparable because P450scc reactions were carried out at 37°C and the P450c11 reactions at 30°C. At higher temperature the leakage increased and P450c11 was rapidly inactivated (P450c11 is much less stable than P450scc at 37°C; see Ref. 16). Tween 20, included only in the P450scc system to solubilize the substrate cholesterol, has a stimulatory effect on leakage (7). Despite this, the leakage from the P450c11 system without Tween was higher than that in the P450scc system.

After complete oxidation of NADPH, reduced adrenodoxin oxidizes spontaneously (autooxidation) (7). However, as NADPH concentration was increased, the proportion of adrenodoxin that reoxidized fully after complete oxidation of NADPH steeply decreased (Fig. 2). Apparently adrenodoxin was inactivated by oxygen radicals forming during its autooxidation. The leakage occurring at 100 μM NADPH prevented completely reoxidation of 9 μM adrenodoxin. However, when the reaction was carried out in the presence of SOD, 78% of the adrenodoxin reoxidized. Addition of catalase showed no significant protective effect (Fig. 2). These findings indicated that the superoxide radical generated during leakage from adrenodoxin attacked the protein directly before dismutation to H_2O_2. The lack of reoxidation of adrenodoxin could be due to destruction of the [2Fe2S]

[3] Abbreviation used: SOD, superoxide dismutase.

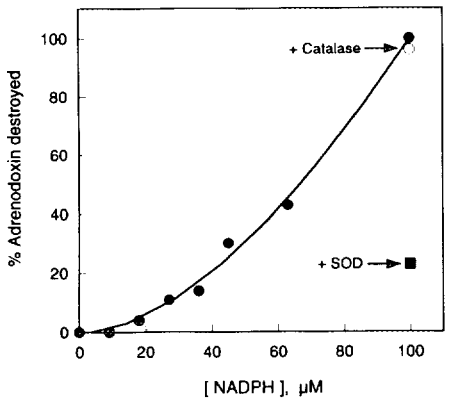

FIG. 2. NADPH dependence of adrenodoxin destruction. Percentage of adrenodoxin that reoxidized was determined by recovery of absorbance at 414 nm after NADPH depletion. Reaction conditions: 10 mM Hepes buffer, pH 7.2, 100 mM KCl, 0.1 μM adrenodoxin reductase, 9 μM adrenodoxin at 37°C. Catalase effect was tested at 50 U/ml, SOD effect at 20 U/ml.

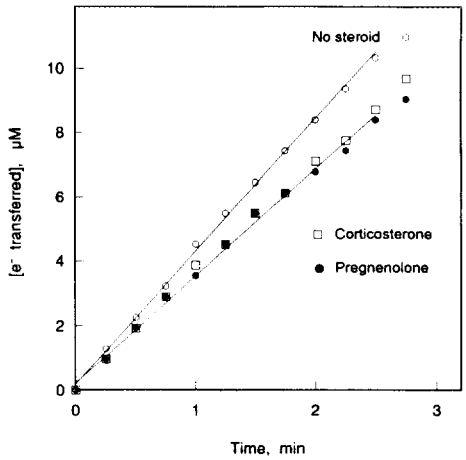

FIG. 3. Effect of cholesterol side chain cleavage product pregnenolone on NADPH oxidation by P450scc system. The reactions were carried out in 10 mM Hepes, pH 7.2, 100 mM KCl, 0.3% Tween 20, with 0.1 μM adrenodoxin reductase, 9 μM adrenodoxin, 0.3 μM P450scc, and 60 μM NADPH with (filled circles) or without (empty circles) 10 μM pregnenolone. The effect of corticosterone was tested at 50 μM.

active center or degradation of the protein. Polyacrylamide gel electrophoresis of proteins isolated at the end of the reaction showed one major band corresponding to native adrenodoxin. Thus, the damage was not associated with fragmentation of adrenodoxin.

Steroid Dependence of Electron Leakage

The studies by Hornsby (10–12) showed that the incubation of cultured adrenocortical cells with the 11β-hydroxylated product of P450c11 and "pseudosubstrates" could lead to the degradation of this enzyme. Hence we examined whether the products of the mitochondrial P450s can increase the rate of electron leakage from these P450s.

P450scc system can oxidize NADPH in the absence of any substrate (7). The addition of pregnenolone, the product of P450scc, did not increase this basal rate (Fig. 3). The effect of corticosterone, the product of P450c11, was essentially identical to that of pregnenolone (Fig. 3).

In the P450c11 system we tested a series of steroids to analyze the stereospecificity of their effects as compared to the results in cultured cells (12). In the presence of each steroid tested, NADPH was oxidized at a linear rate within the first 2 to 3 min after initiation of the reaction (Fig. 4). In contrast to P450scc, the addition of the product of P450c11, corticosterone, increased the rate of futile NADPH oxidation by the P450c11 system (Fig. 5).

Relative to the control without a steroid, both C-19 and C-21 steroids with 11α-hydroxy groups (11α-OH-testosterone and 11α-OH-cortisol) decreased both NADPH oxidation and H_2O_2 formation, and those with 11β-hydroxy groups (11β-OH-testosterone and cortisol) stimulated NADPH oxidation and electron leakage as measured by H_2O_2 formation (Figs. 4 and 5).

The steroids we tested could be grouped into two categories based on their effects on electron transfer (Fig. 5). The total electron flow in the system was assayed by measuring NADPH oxidation. One group of steroids showed rates of H_2O_2 formation similar to NADPH oxidation (Fig. 5, top). These steroids apparently underwent little or no metabolism by the P450c11 as the electrons from NADPH were consumed in H_2O_2 formation with little or no electron flow directed to steroid hydroxylation.

A second group of steroids showed rates of NADPH oxidation higher than H_2O_2 formation (Fig. 5 bottom).

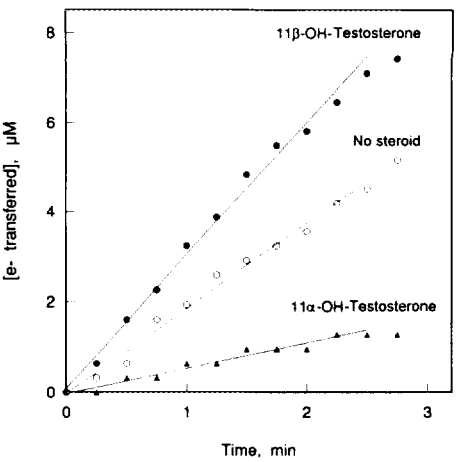

FIG. 4. Effect of 11α-OH and 11β-OH derivatives of testosterone on NADPH oxidation by P450c11 system. The reactions were carried out in 10 mM Hepes, pH 7.2, 100 mM KCl, with 0.1 μM adrenodoxin reductase, 9 μM adrenodoxin, 0.15 μM P450c11, and 60 μM NADPH. Other reaction conditions were as in Fig. 1. Steroids were tested at 50 μM.

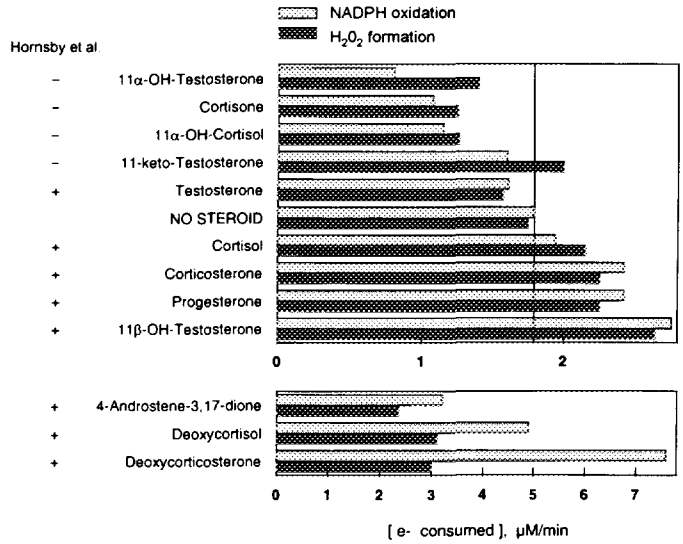

FIG. 5. Steroid dependence of NADPH oxidation (light bars) and H_2O_2 formation (dark bars) in reconstituted mitochondrial P450c11 system. Reaction conditions were as in Fig. 3. Each bar represents the mean of two independent determinations. The leftmost column titled "Hornsby *et al.*" indicates whether the specified steroid enhanced (+) or was without effect (−) on P450c11 degradation in cultured adrenocortical cells (10, 12).

These steroids are substrates of P450c11 (20–22). Thus, for these steroids the following equation should hold true:

$$[\text{NADPH oxidized}] = [H_2O_2 \text{ formed}] + [\text{substrate hydroxylated}].$$

Under the conditions of Fig. 5 the rate of 11β-hydroxylation of deoxycorticosterone (corticosterone formation) averaged 2.5 μM steroid hydroxylated/min, i.e., an electron flow of 5 μM e^-/min. This turnover indeed could account for the rest of NADPH oxidation in the presence of deoxycorticosterone (Fig. 5, bottom). To enable measurement of NADPH oxidation under the conditions of Fig. 5, NADPH was added at a low rate-limiting concentration (cf. Fig. 1). Thus, the turnover rate was about threefold lower than that observed in the presence of nonlimiting 1 mM NADPH (16).

The substrates of P450c11 also stimulated electron leakage as compared to the no-steroid control (Fig. 5). The large amount of H_2O_2 formed in their presence further showed that P450c11-catalyzed 11β-hydroxylation is poorly coupled relative to the P450scc activity. However, the leakage observed in our system was much less than that observed by Martsev *et al.* (6).

In addition to the steroids listed in Fig. 5, we also examined the effects of 5α-androstane-3β,17β-diol, 4-androstene-3β,17β-diol, and 5α-androstanedione. These three steroids showed no significant effect either on NADPH oxidation or H_2O_2 formation relative to the control without steroid. Thus, apparently the 3-keto group and 4-ene double bond are essential for interaction with the P450c11.

DISCUSSION

This study revealed major differences in the electron transfer reactions of P450scc and P450c11: During substrate metabolism, leakage from the P450scc system is reduced, whereas leakage from the P450c11 is increased. The product of P450scc, pregnenolone, does not affect futile NADPH oxidation by the P450scc system. In contrast, 11β-hydroxylated products of P450c11, and other steroids with a strict stereospecificity, strongly stimulate electron leakage from P450c11 but not from P450scc.

Studies of complex P450 electron transport systems in purified form raise questions about the relevance of results in reconstituted models to their normal function in the natural environment of the mitochondria of intact cells. The present findings revealed the following similarities between the behavior of the mitochondrial P450s in reconstituted systems and in living cells in culture.

(i) Sensitivity to oxidative damage of P450c11 vs P450scc: In cultured adrenocortical cells the activity of P450c11 rapidly decreases apparently as a result of oxidative damage (10–12). The loss of the activity of P450c11 is not accompanied by loss of steroid synthesis, indicating that P450scc continues to function with little or no damage under the same conditions. The present findings revealed that P450c11 is much more leaky than P450scc. Thus, the inactivation of P450c11 in cultured cells may directly result from its much greater propensity to generate harmful oxygen radicals.

(ii) Effect of steroid products on P450c11 vs P450scc: The addition of the products of P450c11 to cultured cells was observed to strongly stimulate inactivation of P450c11 (12). Similarly, in our reconstituted systems the 11β-hydroxylated products of P450c11 enhanced electron leakage from P450c11 but not from P450scc. In contrast, the product of P450scc had no effect on P450scc.

(iii) Stereospecificity of steroid effects on P450c11: The stereospecificity of steroids that stimulate leakage from P450c11 is essentially identical to that of steroids that stimulate P450c11 degradation in cultured cells (Fig. 5). This correspondence was qualitative but not quantitative. For example, while in cultured cells testosterone was observed to suppress 11β-hydroxylase activity more than its 11β-hydroxylated derivative (12), in our system 11β-OH-testosterone enhanced leakage more than testosterone (Fig. 5). These quantitative differences may reflect the influence of additional factors, such as lipids, that are present in the natural environment. Cortisol showed only a small but consistent enhancement of leakage, but it stimulates strongly P450c11 degradation in cultured cells (12). Thus, even a small enhancement of leakage from the enzyme may be associated with a major detrimental effect in cells.

Our systematic examination of the effects of different isomers, particularly at C-11 and C-17 supported the stereospecificity rules based on results in cultured cells (11): 3-keto, 4-ene, C-19 or C-21 steroids with an 11β-H or 11β-OH group were associated with enhanced leakage, whereas an 11α-OH group rendered the steroids inhibitory. Steroids with a 3β-OH or 5α group showed no significant effect.

Steroids affect coupling of the P450 system probably by modifying the interaction of O_2 with the heme iron of P450. Apparently, cholesterol stabilizes the P450scc–O_2 complex, saving it for reduction by the second electron (2, 7, 23). In contrast, the substrates of P450c11 enhance the autooxidation of this complex, releasing superoxide. The reduction of leakage by 11α-OH steroids may result from inhibition of P450c11–O_2 complex formation or inhibition of the autooxidation of this complex. The steroids that have no effect on leakage may not enter into the substrate pocket of P450, or simply not affect the heme–O_2 interaction in the active site.

Overall, the above observations provide further evidence that the leakage reactions of the mitochondrial P450 systems in reconstituted systems reflect their capacity to function as a source of oxygen radicals in cells. The conditions that minimize the production of oxygen radicals include coupling of NADPH supply to P450scc during cholesterol side chain cleavage, and rapid clearance of the steroid products to minimize their accumulation and hence their stimulatory effect on leakage from P450c11 and possibly other P450s.

Currently, the regulation of NADPH supply during steroid biosynthesis by the mitochondrial P450 systems is not well understood (23). The expression of some enzymes that are involved in NADPH synthesis is regulated by ACTH and this may provide one long-term mechanism to modulate NADPH levels (24). Under normal conditions the products of the steroidogenic pathways do not accumulate in the cell as steroidogenesis is coupled to steroid secretion (1). Yet, when these products accumulate in cells in culture or in some pathological conditions, these would be expected to enhance oxygen radical formation (11). The deleterious effects of the reactive oxygen radicals may be exerted by a direct attack on the proteins producing them (as shown for adrenodoxin in Fig. 2) or by the formation of reactive lipid peroxides (10, 12, 25). These radicals may also have a functional role in the regression of the highly steroidogenic corpus luteum (26, 27). The high concentrations of antioxidants in steroidogenic cells may serve as scavengers for the radicals produced by the P450 systems (10, 26, 27).

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