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Generation of a Free Radical from Calphostin C by Microsomal Cytochrome P450 Reductase in Rat and Human Liver

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ABSTRACT. Calphostin C (cal C) is a potent and specific protein kinase C inhibitor that is thought to be photoactivated to generate a reactive perylenesemiquinone free radical intermediate. In this electron spin resonance spectrometry study, we show, for the first time, an enzymatic light-independent mechanism of cal C free radical formation. Such a free radical was generated in liver microsomes from rat and humans, and its formation was dependent on the presence of NADPH, functional microsomes, and protein and cal C concentrations. Inhibitor and purified enzyme studies showed that cytochrome P450 reductase is responsible for the cal C free radical observed, which is probably a perylenesemiquinone. BIOCHEM PHARMACOL 51;5:599–603, 1996.

KEY WORDS. calphostin C; free radical; cyt P450 reductase; human liver; esr

Cellular signals that regulate proliferation and differentiation are transduced by complicated cascades of events elicited via phosphorylation and dephosphorylation of key proteins. One important enzyme system that catalyses protein phosphorylations is protein kinase C (PKC). The recent explosion in knowledge of the role of kinases in cellular signal transduction has engendered the search for new drugs that might be useful in the treatment of proliferative diseases *via* inhibition of selective steps in these pathways, such as phosphorylation catalysed by PKC [1]. In recent years, potent inhibitors of PKC have been identified; for example, the indolocarbazole staurosporine and the perylenequinone calphostin C (cal C) [2, 3]. Two PKC-specific analogues of staurosporine, UCN-01 and CGP 41251, are currently undergoing phase 1 clinical trials in the U.S.A., Japan, and Europe as potential antineoplastic agents. Cal C, a metabolite of the fungus *Cladosporium cladosporoides* is a particularly intriguing molecule. Like staurosporine and its analogues, cal C possesses cytostatic and cytotoxic properties [4]. Cal C inhibits PKC at the regulatory domain, and inactivates the enzyme potently and irreversibly in the presence of light [5]. Photoactivation of cal C results in the formation of singlet oxygen, semiquinone radicals, and superoxide anions [6, 7], all of which are considered important for its biological actions. The necessity of light activation is an obvious drawback for the potential therapeutic usefulness of cal C. Given the quinone structure of the molecule, we tested the hypothesis that cal C can undergo activation by liver enzymes *via* a process independent of the presence of light.

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MATERIALS AND METHODS

Source of Materials

Rat liver microsomes and human liver microsomes were prepared by standard methods [8]. Excess samples of healthy human liver were obtained after graft reduction of livers from 2 donors. Purified rat liver cytochrome P450 reductase was obtained from CR Wolf, Biomedical Research Centre, Ninewells Hospital, Dundee, U.K. Calphostin C was a generous gift from Kyowa Hakko Kogyo, Tokyo, Japan. All chemicals and reagents were obtained from Sigma Chemical, Poole, Dorset, U.K.

Incubations of Calphostin C

A typical incubation consisted of rat liver microsomes (1–3 mg), NADPH (5 mM), calphostin C (10–200 μM, from a 10-mM stock solution prepared with DMSO) and phosphate buffer (0.1 M, pH 7.4) to a final volume of 300 μl. Incubations were performed in air or after nitrogen purging of all solutions. In some studies, human liver microsomes or purified rat liver cytochrome P450 reductase (0.3 mg of protein with activity of 1.89 μmole cyt c reduced mg protein^{-1} min^{-1}) was used as a source of biological material. For cyt P450 reductase inhibition studies, samples were incubated in the presence of diphenyliodinium chloride (5 mM). All incubations were performed under protection from light.

Electron Spin Resonance Spectrometry

Electron spin resonance spectroscopy was performed at room temperature on aqueous incubates prepared as described above, using a Varian E3 x-band spectrometer operating at

9.44 GHz with field set at 3370 G and 2 G field modulation. Microwave power was 10 mW, time constant 1 sec, and receiver gain typically at 10^5–10^6. All esr spectra were quantitated by measuring the peak-to-peak height of the singlet observed.

RESULTS

Figure 1 shows that incubation of cal C with NADPH-supplemented rat liver microsomes in the presence of air generated an esr signal. This signal was not observed when cal C was incubated in buffer alone or when microsomes or NADPH were omitted, indicating that cal C free radicals were not generated nonenzymatically by light under the conditions used. Denatured (boiled) microsomes supplemented with NADPH also failed to yield an esr signal. Metabolism of cal C to a free radical occurred also in aerobic human liver microsomes (Fig. 2). During the first 10–15 min of aerobic incubation cal C esr signal intensity was dependent on both rat liver microsomal protein (Fig. 3a) and drug concentration (Fig. 3b). The loss of signal intensity with time during the course of these studies may be due to either formation of nonradical secondary products or rapid depletion of NADPH cofactor. Cal C free radical under aerobic conditions was observed, also using NADPH-supplemented purified rat liver cytochrome P450 reductase (Fig. 4). Incubation of either microsomes or purified cyt P450 reductase with diphenyliodinium chloride (5 mM) totally abolishes free radical generation (result not shown). Figure 5 demonstrates that a cal C free radical was observed during incubation not only in air, but also in nitrogen. However, the appearance of the esr signal caused by cal C in air was delayed by 5–10 min compared to that in nitrogen.

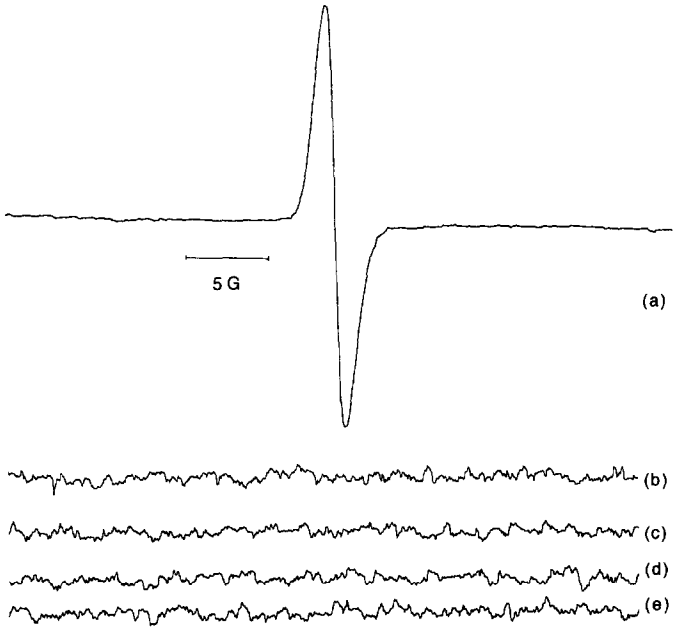

FIG. 1. Esr spectra of (a) calphostin C (200 μM) after 30 min incubation with NADPH-supplemented rat liver microsomes under aerobic conditions; (b) incubated without calphostin C; (c) incubated without NADPH; (d) incubated with denatured microsomes; (e) a mixture of calphostin C and NADPH alone. Incubation conditions and esr parameters were as described under Methods. Esr receiver gain for (a) was 2.5 × 10^5 and for (b)–(e) 8 × 10^5.

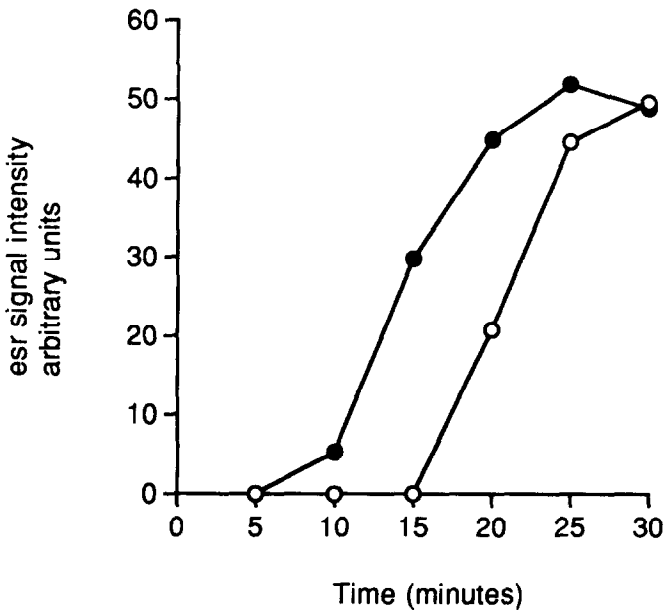

FIG. 2. Time dependency of formation of a calphostin C free radical esr signal in NADPH-supplemented human liver microsomes, either 1 mg (—○—) or 2 mg (—●—) per incubate, and calphostin C 100 μM. Results shown are representative of 3 separate experiments; see Methods for experimental detail.

DISCUSSION

The importance of light in the reduction of perylenequinones, including cal C, and the role of the resulting perylenesemiquinones in reactive oxygen generation have been well described [5–7]. It is believed that such light activation is involved in the inactivation of PKC by perylenequinone pigments. Recently, a mechanism of PKC inactivation was described that involves both specific covalent binding of cal C to the regulatory domain of PKC and the production of reactive oxygen [6]. The importance of light activation of perylenequinones clearly compromises greatly the therapeutic potential of cal C as a PKC inhibitor under the conditions of darkness that would prevail in systemic and solid cancers. We considered the structural similarity between perylenequinones with the polycyclic quinones, which are known to undergo bioactivation mediated by flavoproteins [9]. This similarity renders it conceivable that cal C undergoes an analogous metabolic pathway. Using rat liver microsomes as a source of cytochrome P450 reductase, we show for the first time that cal C is, indeed, metabolised to a free radical intermediate. The broad singlet esr spectrum observed ($g = 2.0035$) is typical of a semiquinone generated in microsomal incubates; the lack of hyperfine detail is probably due to slow tumbling, on the esr time scale, of aggregated or protein-bound perylenesemiqui-

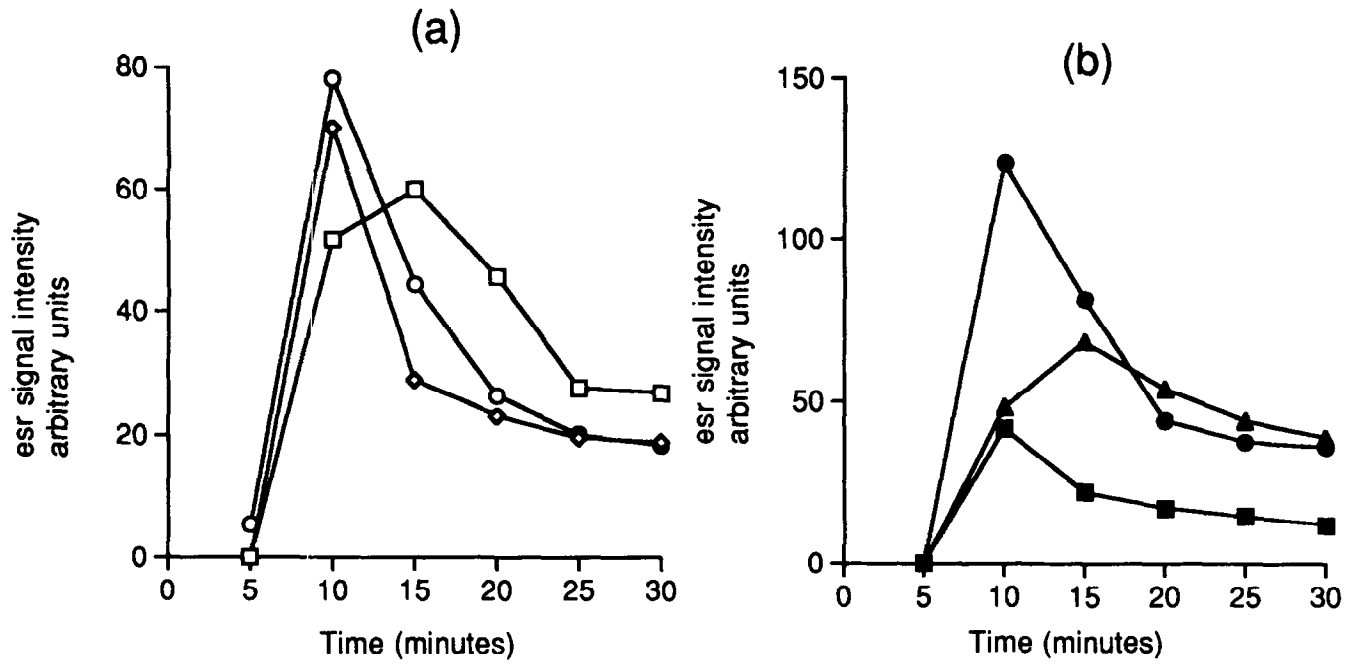

FIG. 3. Dependency of formation of a calphostin C free radical esr signal in rat liver microsomes on (a) microsomal protein at (-□- 1 mg; -◇- 2 mg; 3 -○- mg) with calphostin C 100 μM and (b) calphostin C concentration at (-■- 50 μM; -▲- 100 μM; -●- 150 μM) with 2 mg microsomal protein. Results are representative of 3 separate experiments; see Methods for experimental detail.

none molecules. Diminishing the microwave power or field modulation had little effect on the esr singlet spectrum observed. A cal C esr spectrum generated by light and with hyperfine structure identifying it as a semiquinone has been described previously [7]. The dependence of cal C free radical generation on functional enzyme was confirmed by the requirement for both NADPH and viable microsomes. We suggest that cyt P450reductase is responsible for the cal C free radical observed in microsomes. This contention is supported by the complete abolition of microsome-mediated formation of the cal C free radical in the presence of diphenyliodinium chloride, a potent inhibitor of cytochrome P450 reductase [10]. The activity of purified rat liver P450 reductase con-

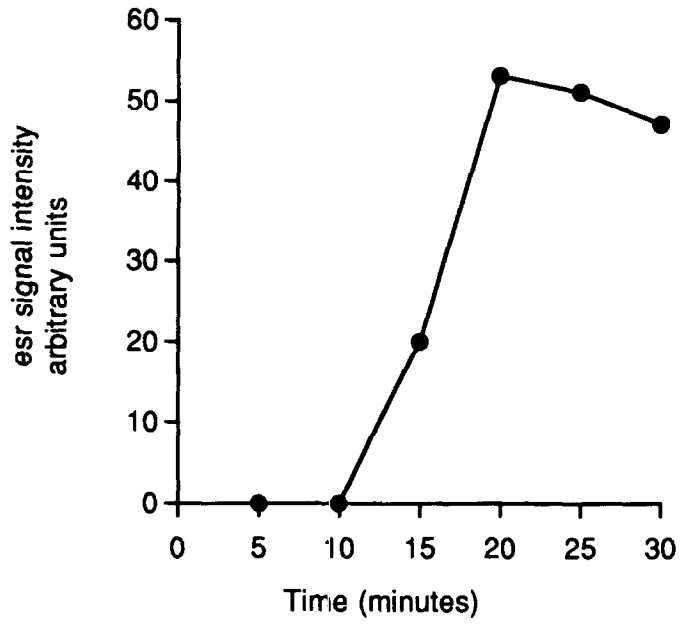

FIG. 4. Formation of calphostin C free radical by purified NADPH cytochrome P450 reductase incubated with calphostin C (100 μM) and NADPH (5 mM). Results are representative of 3 separate experiments; see Methods for experimental detail.

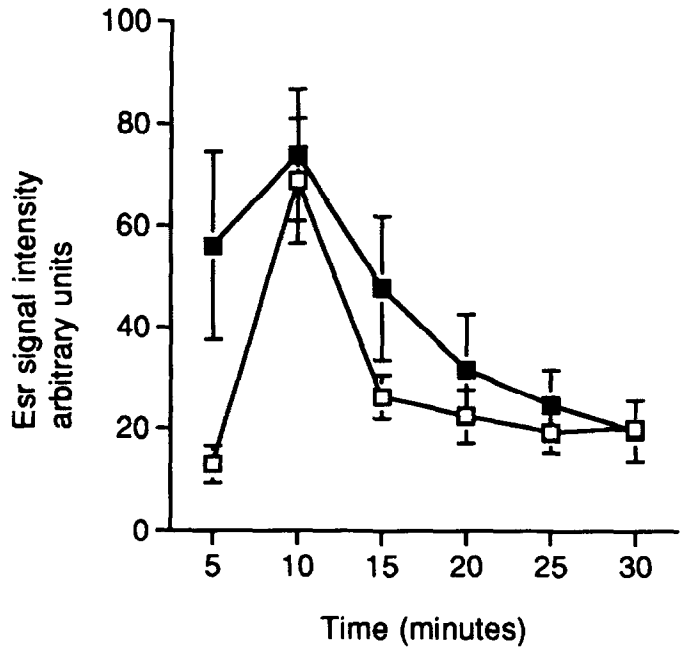

FIG. 5. Generation of calphostin C free radical by NADPH-supplemented rat liver microsomes (2 mg) and calphostin C (100 μM) under oxic (-□-) or anaerobic (-■-) conditions. Results are the mean ± SD of 3 separate experiments; see Methods for experimental detail.

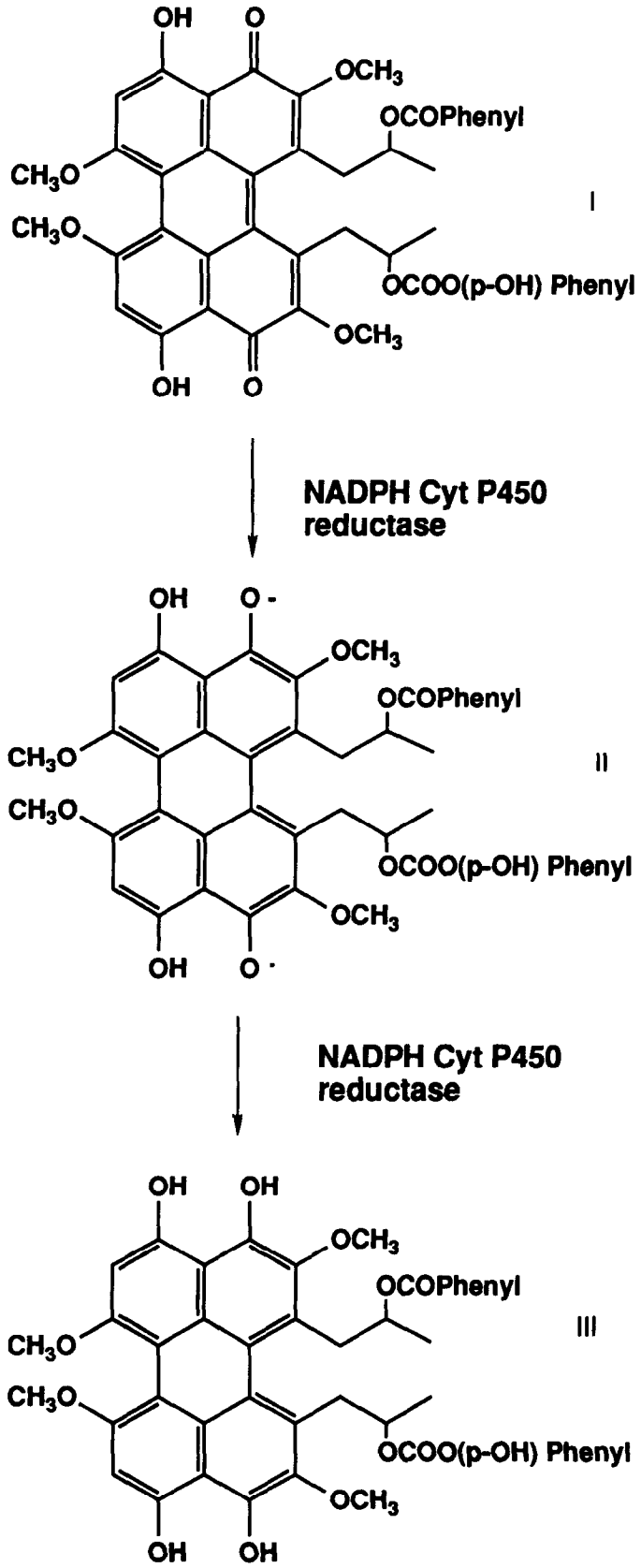

FIG. 6. Hypothetical scheme showing cyt P450 reductase-mediated one- and two-electron reduction of calphostin C (I) to a perylenesemiquinone (II) and perylenehydroquinone (III).

firmed that this flavoprotein *per se* can reduce cal C to a free radical intermediate. Because cytochrome P450 reductase facilitates, in principle, the transfer of one and two electrons from NADPH to quinone substrates [11], it might generate a cal C semiquinone (PQ-) directly *via* one electron reduction of the paraquinone moiety [Eqn 1] or *via* comproportionation of the quinone (PQ) and hydroquinone (PQH_2) [Eqn 2].

$$PQ + e \rightarrow PQ^{\cdot-} + e + 2H^+ \rightarrow PQH_2 \quad (1)$$

$$PQ + PQH_2 \rightleftharpoons 2PQ^{\cdot-} + 2H^+ \quad (2)$$

A possible sequence of reduction catalysed by cyt P450 reductase is shown in Fig. 6. The biological and chemical reactivity of the cal C semiquinone observed in this study is unknown. Generally, semiquinones are considered to be reactive intermediates in biological systems by facilitating (1) intermolecular electron transfer from NADPH to molecular oxygen to generate reactive oxygen *via* redox cycling; and/or (2) intramolecular electron transfer to generate an electrophilic species with covalent binding potential. Both these mechanisms have been suggested to occur in light-activated cal C [6]. It is not clear from the present study whether or not the enzymatically generated cal C semiquinone does undergo significant redox cycling because the presence of air did not cause abolition of the cal C esr spectrum. Its disappearance in air would have been expected if the perylenesemiquinone was reoxidised to an esr-silent perylenequinone. However, the possibility that cal C rapidly redox cycles consuming oxygen and, hence, creating anaerobic conditions in which the semiquinone is stable cannot be excluded. This interpretation is consistent with the time delay observed before the cal C esr signal was detected under aerobic conditions, as compared to the rapid onset of free radical formation in the absence of air.

In conclusion, this study demonstrates that cal C is metabolically converted to a free radical intermediate in both rat and human liver microsomes. This finding implies the existence of a light-independent mechanism of cal C activity. The possible role of cal C bioactivation in the formation of a selective PKC inhibitor is currently being explored.

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