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OXIDATION OF 1,4-DIHYDROPYRIDINES BY PROSTAGLANDIN SYNTHASE AND THE PEROXIDIC FUNCTION OF CYTOCHROME P-450

DEMONSTRATION OF A FREE RADICAL INTERMEDIATE

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Abstract—Oxidation of 1,4-dihydropyridines by the hydroperoxidic function of cytochrome P-450 and prostaglandin synthase was investigated using felodipine as a model substance. Nifedipine and the 2,6-dichlorophenyl analogue of felodipine were used in some experiments with similar results. Felodipine was metabolized to a pyridine metabolite *in vitro* when incubated with liver microsomes and cumene hydroperoxide, as well as with ram seminal vesicle microsomes and arachidonic acid. The oxidation of 1,4-dihydropyridines is proposed to proceed via formation of a free radical intermediate, judging from EPR analysis with the spin trap POBN. When reduced glutathione was added the EPR signal was decreased as well as the formation of the pyridine metabolite while an oxidation of glutathione was observed. This effect was due to a reduction of the radical intermediate back to felodipine by glutathione.

Felodipine interacts with the hydroperoxidase activity of prostaglandin synthase, since the pyridine metabolite was formed also when 15-hydroperoxyeicosatetraenoic acid was used as a substitute for arachidonic acid. Indomethacin could only inhibit the metabolism of felodipine when arachidonic acid was used as substrate. The cooxidation of felodipine by prostaglandin synthase is associated with an increased metabolism of arachidonic acid. This was further supported by a stimulated oxygen consumption and an increased formation of prostaglandin E_2.

Prostaglandin synthase is an enzyme system with two different enzyme activities. The cyclooxygenase activity inserts two molecules of oxygen in the arachidonic acid molecule (AA), forming prostaglandin G_2 (PGG_2). This reaction is inhibited by nonsteroidal anti-inflammatory drugs like indomethacin. PGG_2 is reduced by the second enzyme activity, a hydroperoxidase, forming prostaglandin H_2 (PGH_2) and a reactive oxygen radical. PGH_2 can then be converted to different prostaglandins and thromboxanes, depending on the cell type and the occurrence of additional enzymes and co-factors. Several compounds, e.g. polycyclic hydrocarbons and aromatic amines, have been shown to be cooxidized by this system. The cooxidation of some drugs has been suggested to proceed via a free radical intermediate [1–5].

Cytochrome P-450 catalyse the metabolism of many drugs by acting as a monooxygenase using NADPH and oxygen as cofactors. However, cytochrome P-450 can also act as a peroxidase in the presence of an organic hydroperoxide [6–8]. Metyrapone inhibits both functions of cytochrome P-450.

Most 1,4-dihydropyridines are metabolized to products with a pyridine nucleus in their structure [9–11]. The 2,3-dichlorophenyl-1,4-dihydropyridine, felodipine, was metabolized primarily to the corresponding pyridine analogue by the NADPH- and oxygen-dependent action of cytochrome P-450 [10]. It was therefore of interest to determine whether the oxidation of 1,4-dihydropyridines could occur by the hydroperoxide-supported action of cytochrome P-450, as well as by interaction with a different peroxidase system like the prostaglandin synthase. As a model substance for 1,4-dihydropyridines, felodipine, a calcium antagonist with selective vasodilating activity, was used. Nifedipine and the 2,6-dichlorophenyl analogue of felodipine was also tested in some experiments. Paracetamol was used as a reference compound for the interaction with the prostaglandin synthase system.

MATERIALS AND METHODS

Materials. Felodipine (3,5-pyridinedicarboxylic acid, 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl, 5-ethyl 3-methyl ester), [^{3}H]felodipine, [4-$^2H_1$]felodipine, [$^2H_5$]felodipine, the pyridine analogue, the 3-isopropyl ester analogue of felodipine (internal standard), the 2,6-dichlorophenyl analogue of felodipine, and nifedipine (3,5-pyridinedicarboxylic acid, 4-(2-nitrophenyl)-1,4-dihydro-2,6-dimethyl, 3,5-dimethyl ester) were synthesized at the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). NADP and isocitrate dehydrogenase were obtained from Boehringer Mannheim GmbH (Mannheim, F.R.G.). Arachidonic acid (AA), butylated hydroxyanisole (BHA), cumene hydroperoxide, reduced glutathione (GSH), indomethacin, metyrapone, par-

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† Abbreviations: EPR, electron paramagnetic resonance; LC, liquid chromatography; TLC, thin-layer chromatography; AA, arachidonic acid; BHA, butylated hydroxyanisole; GSH, reduced glutathione; GSSG, oxidized glutathione; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; PG, prostaglandin; POBN, α-(4-pyridyl-1-oxide) *N*-tert-butylnitrone; RSVM, ram seminal vesicle microsomes.

acetamol, α-(4-pyridyl-1-oxide) *N*-tert-butylnitrone (POBN), soybean lipoxygenase type 1 and trisodium isocitrate were purchased from Sigma Chemical Company (St. Louis, MO). [1-^{14}C]Arachidonic acid, tritium-labelled prostaglandin standards and tritium-labelled glutathione were obtained from New England Nuclear (Boston, MA). The [^{3}H]GSH was purified immediately before use by reversed phase LC on Nucleosil C_{18}, 5 μm (Macherey-Nagel, Düren, F.R.G.) with 0.044 M dimethylethylamine acidified to pH 3.3 with formic acid. 15-Hydroperoxy-eicosatetraenoic acid (15-HPETE) was prepared by incubation of soybean lipoxygenase with arachidonic acid [12]. The products were extracted at pH 3 with diethyl ether and purified by straight phase LC on Polygosil 60-5 (Macherey-Nagel, Düren, F.R.G.) in hexane : isopropanol : acetic acid (99 : 1 : 0.1, by vol.). The identity of 15-HPETE was confirmed by $NaBH_4$ reduction for 15-hydroxy-eicosatetraenoic acid, which was chromatographed in the above-mentioned system and then identified by gas chromatography–mass spectrometry.

All other chemicals were standard commercial products of analytical grade.

Preparation of microsomes. The liver-microsomal fraction was isolated according to Ernster *et al.* [13] from starved male Sprague–Dawley rats (250 g) which were pretreated with phenobarbital sodium (80 mg/kg) once daily for four days to induce enzyme activities. The microsomes were stored as a suspension in 0.25 M sucrose–50 mM Tris HCl buffer, pH 7.5, at −70° and were thawed immediately before use.

Ram seminal vesicles were kindly provided by Ms I. Tollman-Hansson, Department of Physiological Chemistry II, Karolinska Institutet. RSV microsomes (RSVM) were prepared by the method of Egan *et al.* [14].

The protein concentration was measured according to Lowry *et al.* [15] and the concentration of cytochrome P-450 according to Omura and Sato [16].

Incubation conditions. The oxidation by the NADPH- and oxygen-dependent system was studied by incubating liver microsomes from phenobarbital-pretreated rats (1 mg protein/ml) in 50 mM Tris HCl-buffer, pH 7.5, and a NADPH-generating system. The peroxide-supported reactions were carried out using the same buffer system (except for the NADPH generating system). The reaction was initiated by adding cumene hydroperoxide 1 mM (final concentration). The reactions were terminated by the addition of an equal volume of toluene. The concentration of the substrate to be tested was 1 μM (final concentration).

Incubations with RSV microsomes (1 mg protein/ml) were performed in 0.1 M K-phosphate buffer, pH 8.0, with 1 mM EDTA in a total volume of 2.0 ml. The drug to be tested was preincubated for 5 min at 37° before the addition of arachidonic acid (10–100 μM). After different times (30 sec or 5 min) of incubation, the reaction was terminated with methanol, ethyl acetate or 15% trichloroacetic acid. Separate incubations were performed to study the metabolism of [^{3}H]labelled (20 μCi) and [^{2}H]labelled felodipine, [^{14}C]labelled AA (0.2 μCi) and [^{3}H]labelled GSH (1 μCi).

Oxygen consumption. Oxygen consumption in RSVM (1 mg protein/ml) was monitored at 30° with a Clark electrode in 0.1 M K-phosphate buffer, pH 8.0, with 1 mM EDTA, in a total volume of 3 ml. The reaction was started with 100 μM AA (final conc.). The oxygen consumption rate was calculated from the linear part of the recorder graph.

Analysis of felodipine and its pyridine metabolite (H 152/37). Assays of unlabelled felodipine and its pyridine metabolite from the incubations with liver microsomes were carried out by gas chromatography with electron capture detection as described earlier [10, 17].

[$^2H_1$]Felodipine was assayed using an HP 5890 gas-chromatograph with an HP 5970A mass selective detector and HP 59970A microcomputer workstation (Hewlett-Packard, Palo Alto, CA). [$^2H_5$] Felodipine was used as an internal standard. Selected ion monitoring for m/z 243, 239, 238 and 346 was used for analysis of [$^2H_5$]felodipine, [$^2H_1$]felodipine, felodipine and the pyridine metabolite, respectively.

[^{3}H]Felodipine and [^{3}H]labelled pyridine metabolite were assayed by liquid chromatography on Polygosil C_{18}, 5 μm, with an eluent of 70% methanol in 0.1 M perchlorate buffer, pH 1.7. The radioactivity was determined by liquid scintillation counting after addition of 10 ml Luma-gel®.

Analysis of arachidonic acid metabolites. The [^{14}C]-labelled arachidonate metabolites formed in the incubations were extracted into ethyl acetate after acidification to pH 3 with 1 M HCl. The ethyl acetate extract was then subjected to thin-layer chromatography (TLC) on Silica gel 60 (Merck, Darmstadt, F.R.G.) using the organic phase of ethyl acetate : isooctane : acetic acid : water (110 : 50 : 20 : 100, by vol.). Radioactive material was detected and quantified with a Berthold Linear Analyzer equipped with an Apple IIe microcomputer (Berthold, Wildbad, F.R.G.).

Analysis of GSH and its oxidized form (GSSG). For assay of [^{3}H]labelled GSH and GSSG, the incubation with RSVM was terminated by addition of an equal volume of 15% trichloroacetic acid. After centrifugation, the samples were concentrated to about 0.3 ml under vacuum in a Speed-Vac® concentrator and diluted with an equal volume of mobile phase. An aliquot was chromatographed on Nucleosil C_{18}, 5 μm, with 0.01 M dimethylethylamine acidified to pH 3.3 with formic acid.

EPR studies. The EPR spectra were recorded on a Varian E-9 spectrometer with a cylindrical cavity in the presence of the spin trap POBN (80 mM). The incubation mixtures contained either RSVM (1 mg protein/ml) and AA (100 μM), or liver microsomes (6 mg protein/ml) and cumene hydroperoxide (1 mM). Felodipine or its 2,6-dichlorophenyl analogue was added to a final concentration of 300 μM. EPR spectra were recorded at room temperature after 1 min incubation at 37°.

RESULTS

Oxidation of 1,4-*dihydropyridines by cytochrome* P-450

Oxidation of felodipine, to its corresponding pyri-

dine metabolite, was studied in liver microsomes from phenobarbital-pretreated rats. The incubations were performed with either a NADPH-generating system or an organic hydroperoxide, cumene hydroperoxide. As illustrated in Fig. 1, the initial rates of pyridine formation are about the same using either cofactors. However, the rate of the hydroperoxide-supported reaction decreased more rapidly than the NADPH-dependent reaction, probably due to destruction of cytochrome P-450 by cumene hydroperoxide [18]. In both reactions, the disappearance of felodipine corresponded to the amount of pyridine metabolite formed. Both pathways were inhibited to about 50% by metyrapone (125 μM). In the hydroperoxide-dependent system the formation of the pyridine metabolite was reduced in the presence of high concentrations of GSH (2.5 mM and 12.5 mM).

Oxidation of 1,4-dihydropyridines by prostaglandin synthase

Cooxidation of felodipine. Felodipine was oxidized to its pyridine metabolite when incubated with RSVM and AA (100 μM) (Fig. 2). The amount of felodipine eliminated was equal to the amount of pyridine metabolite formed. The metabolism of felodipine was inhibited by the cyclooxygenase inhibitor indomethacin (10 μM) and by the antioxidant BHA (500 μM). The metabolism of felodipine in this system was not as efficient as that catalysed by cytochrome P-450 in liver microsomes.

When the reaction was initiated with the hydroperoxide, 15-HPETE, instead of AA, felodipine was transformed to its pyridine metabolite to the same extent as with AA (Fig. 2). This reaction was not inhibited by indomethacin, indicating that felodipine interferes with the hydroperoxidase activity of prostaglandin synthase.

Addition of GSH (0.1 mM and 2.5 mM) dose-

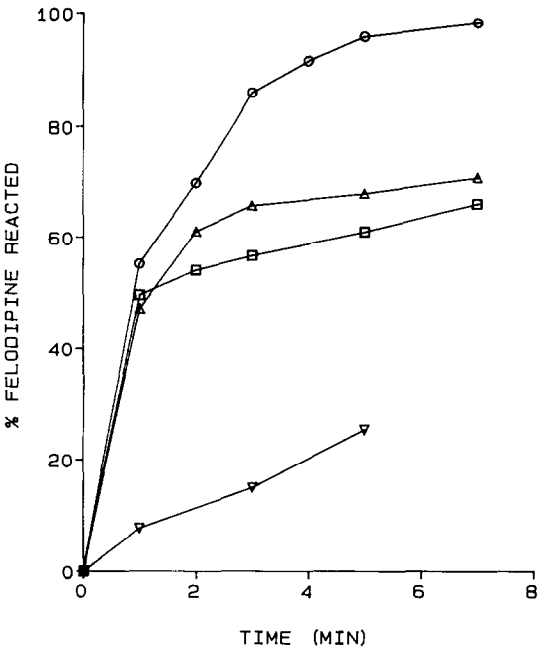

Fig. 1. The metabolism of felodipine (1 μM) in liver microsomes (1 mg protein/ml) from phenobarbital-pretreated rats. The reaction was supplied with an NADPH-generating system (○) or cumene hydroperoxide without GSH (△) and with GSH, 2.5 mM (□) or 12.5 mM (▽).

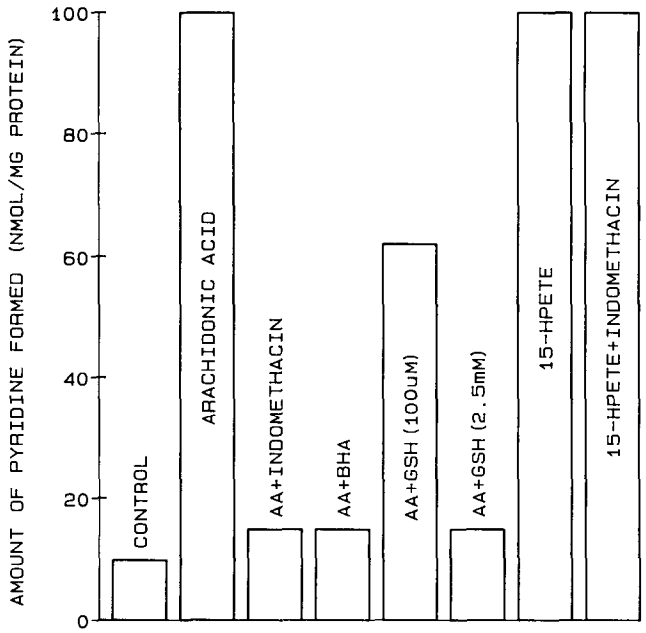

Fig. 2. The metabolism of [^{3}H]felodipine (1 μM) to the corresponding pyridine analogue in incubations with RSVM (1 mg protein/ml) and AA (100 μM) or 15-HPETE (100 μM). [^{3}H]Felodipine and the pyridine metabolite were assayed by LC (Materials and Methods). The amount of felodipine reacted is equal to the amount of pyridine formed.

dependently decreased the formation of the pyridine metabolite (Fig. 2). The interaction between GSH and felodipine was examined by determination of the amount of GSH and GSSG in RSVM incubations using tritium-labelled GSH. There was a twofold increase in the formation of GSSG with felodipine compared to a fivefold increase when paracetamol was added (Fig. 3). The oxidation of GSH, together with the decreased formation of the pyridine metabolite when GSH was added, might indicate that an intermediate in the formation of the pyridine metabolite is reduced to felodipine by GSH. A free radical intermediate is probably formed since an EPR spectrum was recorded when felodipine was incubated in RSVM in the presence of the spin trapper POBN. The found adduct was stable for at least 10 min. The EPR signal was reduced when GSH (2.5 mM) was added to the system (Fig. 4). Similar spectra were observed when the incubations were performed in rat liver microsomes together with cumene hydroperoxide. However, a higher GSH concentration (12.5 mM) was needed to reduce the EPR signal in this system compared to that used in RSVM.

About the same EPR spectra were recorded in incubation with the 2,6-dichlorophenyl analogue of felodipine in both RSVM and liver microsomes. An increased oxidation of GSH in RSVM was also seen for this analogue.

Incubations with RSVM, GSH, AA, and felodipine labelled with deuterium in position 4 were carried out to find out whether the hydrogen in position 4 of the dihydropyridine structure was lost during the formation of the intermediates. The ratio of felodipine to deuterium-labelled felodipine, as well as the amount of pyridine metabolite, was determined by mass spectrometry. Without GSH, oxidation of deuterium-labelled felodipine and loss of the deuterium label were seen. With GSH (2.5 mM), there was neither any formation of the pyridine metabolite nor any change in the ratio of felodipine to deuterium-labelled felodipine.

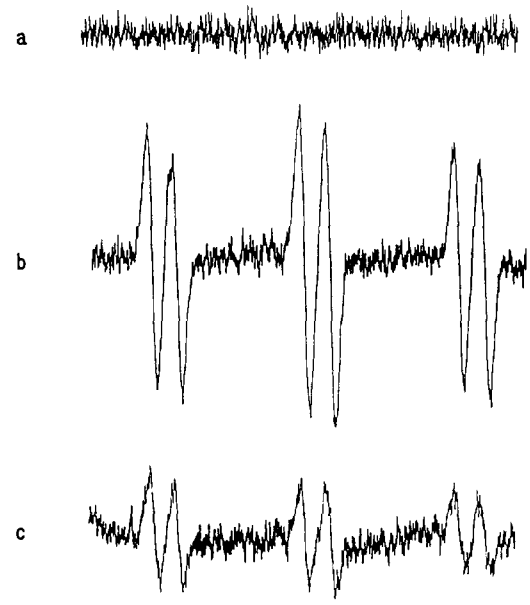

Fig. 4. EPR spectra of the POBN adduct obtained in RSVM incubations. (a) Control without felodipine, (b) with felodipine (300 μM), (c) with felodipine (300 μM) in the presence of GSH (2.5 mM). The incubations contained RSVM (1 mg protein/ml), AA (100 μM) and POBN (80 mM). EPR spectra were recorded after 1 min incubation at 37°. EPR spectrometer conditions: microwave power 10 mW; microwave frequency 9433 MHz; center field 3361 G; modulation amplitude 5.0 G; gain 5×10^4.

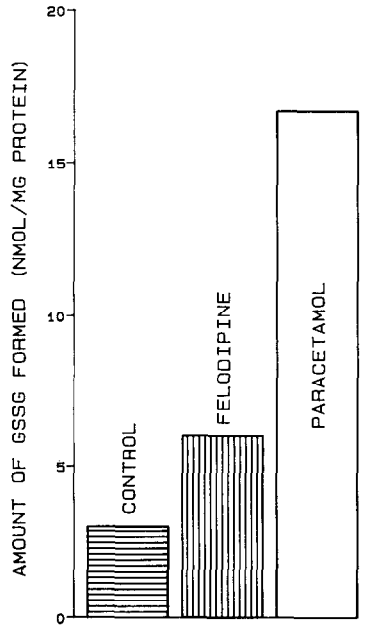

Fig. 3. The oxidation of [^{3}H]GSH (100 μM) to [^{3}H]GSSG after incubations with felodipine (300 μM) or paracetamol (100 μM) in RSVM (1 mg/ml) and AA (100 μM). [^{3}H]-GSSG was determined by LC (Materials and Methods).

Effect on the metabolism of arachidonic acid. Oxygen consumption in RSVM incubations was dependent on the addition of AA and was completely inhibited when the microsomes were preincubated with indomethacin (10 μM). This indicates that the oxygen consumption measured was related to cyclooxygenase activity. The initial velocity of the AA-stimulated oxygen consumption in the RSVM suspension was dose-dependently increased when felodipine or nifedipine was added (Fig. 5). The pyri-

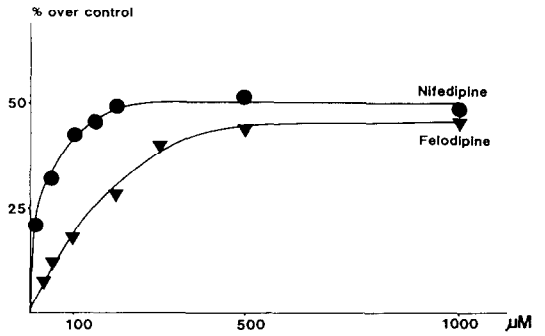

Fig. 5. The effect of felodipine and nifedipine on the oxygenation of AA (100 μM) in RSVM (1 mg protein/ml), 30°. The oxygen consumption was monitored with a Clark electrode.

dine metabolite *per se* did not cause any increase in oxygen consumption. No oxygenated felodipine metabolite could be found in these experiments, indicating that the increased oxygen consumption was entirely due to an increased rate of oxygenation of AA to prostaglandin endoperoxides. The increase in oxygen consumption after addition of 300 μM felodipine corresponded to that of 100 μM paracetamol (without GSH). Stimulated oxygen consumption in RSVM by felodipine was also seen when 2.5 mM GSH was added to the system, while GSH alone did not affect the rate of oxygenation of AA.

Measurements of the oxygen consumption showed that the 1,4-dihydropyridines, felodipine and nifedipine, increased the rate of prostaglandin biosynthesis. This was further studied in RSVM using [^{14}C]-labelled AA (10 or 100 μM). The overall metabolism of AA was increased when felodipine was added to the system, as shown in Fig. 6. But there was also a quantitative change in the formation of various prostaglandins. Figure 7 shows a dose-dependent increase in the PGE_2 to 6-keto-$PGF_{1\alpha}$ ratio after addition of felodipine or nifedipine. There was also an increased formation of PGD_2 and some other unidentified metabolites (Fig. 6).

The effects of felodipine (300 μM), its 2,6-dichlorophenyl analogue (300 μM), and nifedipine (300 μM) on the metabolism of AA (100 μM) were also compared with those of paracetamol (100 μM). Paracetamol and the 2,6-dichlorophenyl analogue of felodipine produced a greater increase in the overall metabolism of AA than felodipine and nifedipine. The metabolic pattern of AA after paracetamol differed from that of the 1,4-dihydropyridines by a much more pronounced increase in PGE_2 formation after paracetamol addition.

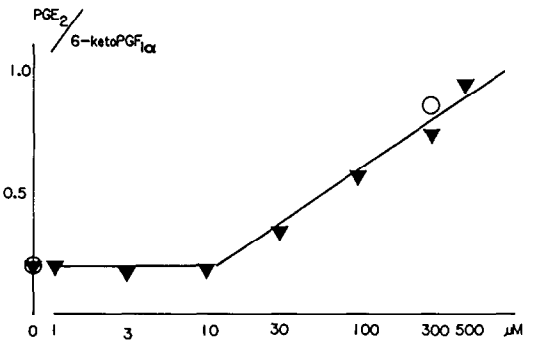

Fig. 7. The effect of felodipine (▼) and nifedipine (○) on the formation of PGE_2 and 6-keto-$PGF_{1\alpha}$ from [^{14}C]AA (10 μM) incubated with RSVM (1 mg protein/ml), 5 min, 37°. The prostaglandins were assayed by TLC.

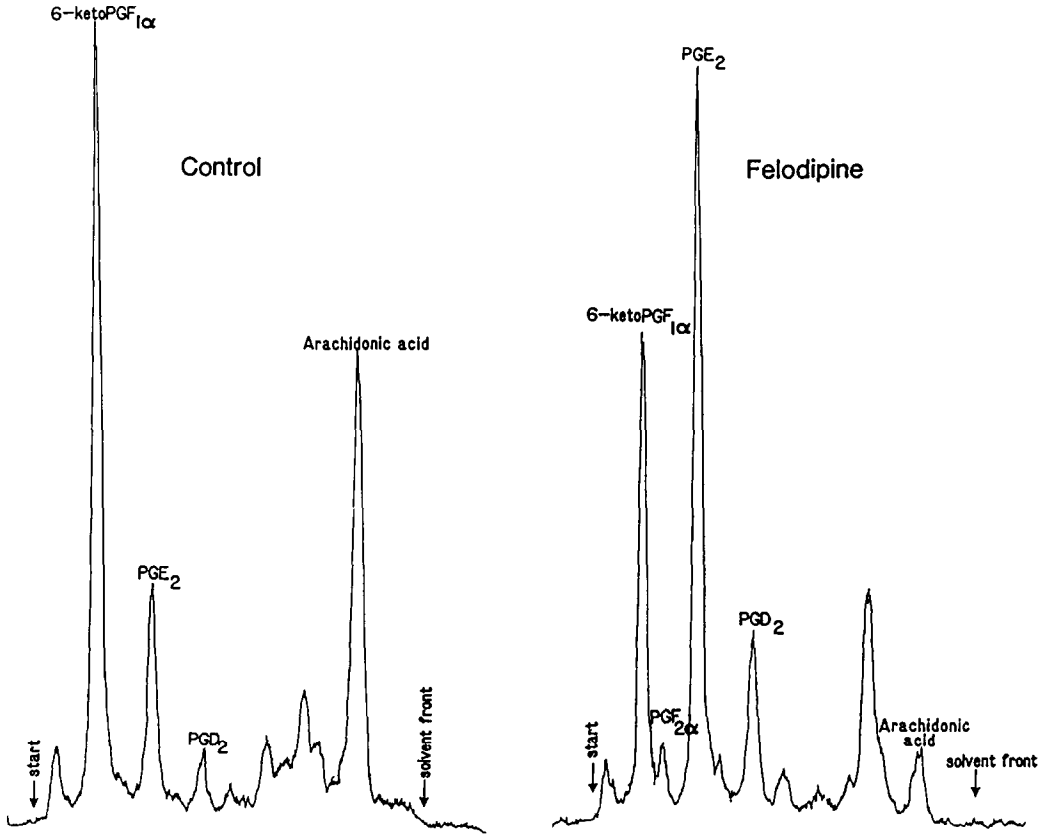

Fig. 6. Thin-layer chromatogram of labelled metabolites of [^{14}C]AA (10 μM) obtained after incubation with RSVM (1 mg protein/ml) and felodipine (300 μM), 5 min, 37°. Arachidonic acid and its metabolites were extracted from the incubation mixture at pH 3.0 with ethyl acetate (Materials and Methods).

DISCUSSION

As shown earlier [10], 1,4-dihydropyridines like felodipine can be oxidized by the NADPH-supported action of cytochrome P-450. The results presented in this report show that 1,4-dihydropyridines can also be oxidized by the peroxidic function of cytochrome P-450 as well as by other peroxidases such as prostaglandin synthase. Irrespective of the action of cytochrome P-450 the primary metabolic step in the metabolism of felodipine seems to be the formation of the pyridine metabolite.

The interaction of the 1,4-dihydropyridines with the prostaglandin synthase is via the hydroperoxidase activity of the enzyme since felodipine was oxidized even when AA was replaced by the peroxide 15-HPETE. In this case, the oxidation was not inhibited by indomethacin. These results are in accordance with earlier findings for cooxidation of various compounds, including paracetamol, by prostaglandin synthase [1, 2]. For several substrates, the cooxidation has been proposed to occur by a free radical mechanism [1–5]. The EPR spectra recorded for felodipine and its 2,6-dichlorophenyl analogue using spin trapping with POBN suggest a free radical intermediate in the metabolism of dihydropyridines by peroxidases. Furthermore, in the presence of GSH, the EPR signal was diminished and a decreased formation of the pyridine metabolite was observed in spite of a stimulated oxygen consumption. This might be explained by reduction of a possible free radical intermediate of felodipine by GSH, resulting in an apparent decreased metabolism of felodipine. An increased oxidation of GSH to GSSG was also seen. A proposed reaction mechanism for this process including one-electron oxidation of felodipine is shown in Fig. 8. The suggested intermediate has not lost the hydrogen in position 4, judging from the results with deuterium-labelled felodipine. *N*-demethylation via the peroxidase function of cytochrome P-450 has also been suggested to proceed by one-electron oxidation, resulting in the formation of a free radical [18, 19]. Augusto *et al.* [20] have shown evidence for a one-electron oxidation of 4-alkyl substituted 1,4-dihydropyridines by the NADPH-supported action of cytochrome P-450.

The interaction of 1,4-dihydropyridines with the prostaglandin synthase system is further supported by the increased oxygen consumption and increased metabolism of AA to various prostaglandins, especially PGE_2. The effect on the rate of elimination of AA is correlated to how easily oxidized the cosubstrate is. Since the 2,6-dichlorophenyl analogue of felodipine is more easily oxidized in liver microsomes

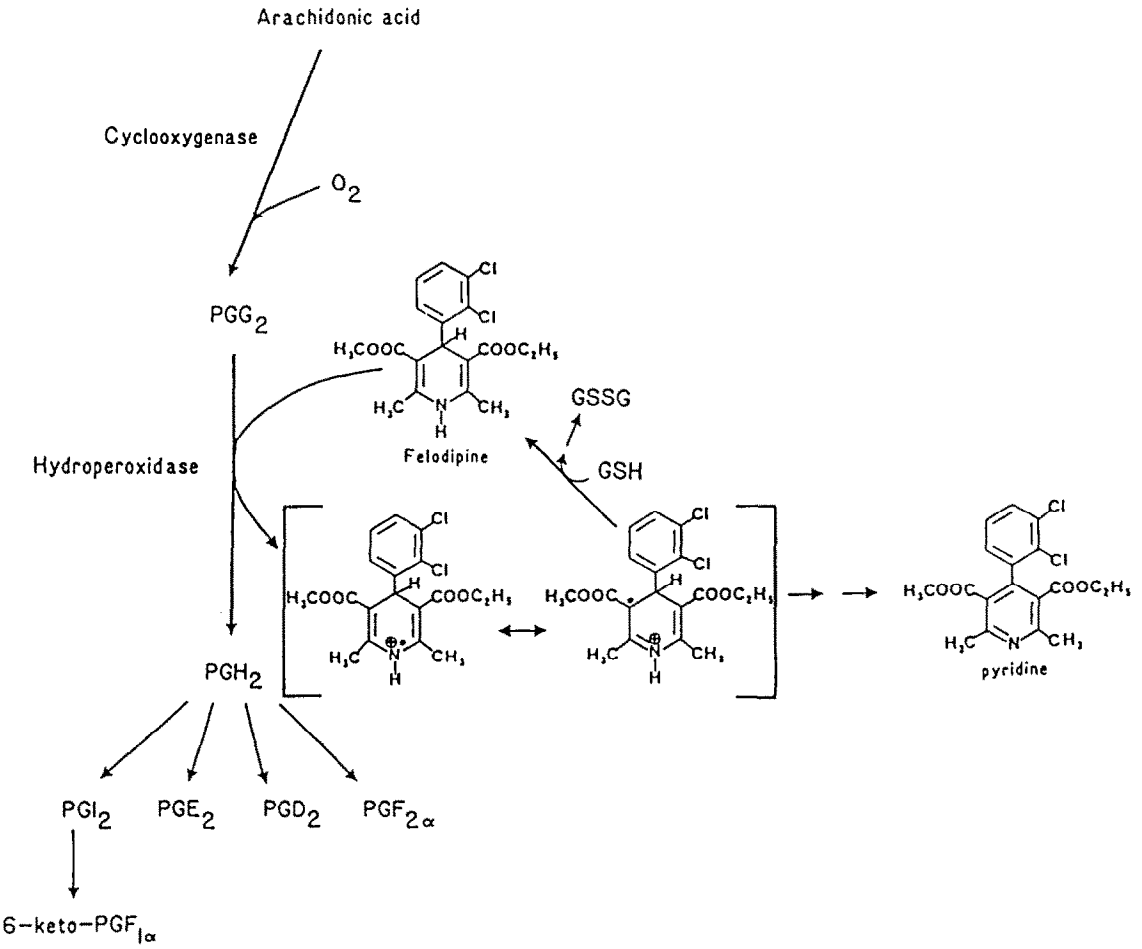

Fig. 8. A proposed mechanism for oxidation of felodipine by the prostaglandin synthase system.

than felodipine,* probably due to the position of the chloro atoms, a more pronounced stimulation of the metabolism of AA was observed. The *in vitro* effects of the 1,4-dihydropyridines felodipine and nifedipine on the prostaglandin synthase system are, however, not as pronounced as that of paracetamol. Even though both felodipine and its analogues interact with the biosynthesis of different prostaglandins to varying extent *in vitro*, extrapolation of the results in this study to *in vivo* situations is not possible.

In conclusion, 1,4-dihydropyridines can be oxidized to the corresponding pyridine analogues by peroxidases such as prostaglandin synthase and cytochrome P-450 peroxidase. This reaction is suggested to proceed through one-electron oxidation via formation of a free radical intermediate. Metabolism of 1,4-dihydropyridines by prostaglandin synthase may be of importance in tissues containing low mixed-function oxidase activity.

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