A systematic analytical chemistry/cell assay approach to isolate activators of orphan nuclear receptors from biological extracts: characterization of peroxisome proliferator-activated receptor activators in plasma

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Abstract Using a novel combination of analytical chemical and molecular biological techniques, lipophilic components of human plasma separated according to their physico-chemical properties were screened for their ability to activate the rat peroxisome proliferator-activated receptor (rPPAR). Activation of an rPPAR/glucocorticoid receptor chimera stably expressed in CHO cells by fractions in the initial screening guided further subfractionation. Characterization of an active subfraction by gas chromatography alone and in combination with mass spectrometry (GC-MS), indicated the presence of free fatty acids. Individual active components in this mixture were isolated by a final fractionation using high performance liquid chromatography (HPLC). GC-MS analyses of HPLC fractions able to activate the chimeric receptor identified palmitic acid, oleic acid, linoleic acid, and arachidonic acid as endogenous activators of rPPAR. No other activators were identified. This approach is able to specifically extract and identify endogenous activators of PPAR from a complex biological extract and as such may be valuable in the identification of activators of other orphan receptors in the steroid hormone receptor superfamily.—**Banner, C. D., M. Göttlicher, E. Widmark, J. Sjövall, J. J. Rafter, and J-Å. Gustafsson.** A systematic analytical chemistry/cell assay approach to isolate activators of orphan nuclear receptors from biological extracts: characterization of peroxisome proliferator-activated receptor activators in plasma. *J. Lipid Res.* 1993. **34:** 1583–1591.

Supplementary key words nuclear receptor superfamily • fatty acids • chromatography • transactivation assay

A nuclear hormone receptor that can be activated by several different peroxisome proliferators (clofibric acid, nafenopin, and WY 14,643) has been identified in mouse liver and termed the mouse peroxisome proliferator-activated receptor (PPAR) (1). Together with other nuclear receptors that are activated by a diverse range of ligands such as steroid hormones, triiodothyronine, and retinoic acid, PPAR is a member of the steroid hormone receptor superfamily of ligand-dependent transcription factors (2–4).

We have earlier reported the establishment of a transcriptional transactivation assay using a chimera of rat PPAR and the human glucocorticoid receptor, stably expressed in CHO cells. Screening of compounds related to lipid metabolism or peroxisomal proliferation, using this assay, indicated that fatty acids such as arachidonic and linoleic acid, activated the receptor chimera and it was suggested that PPAR may play a role in lipid or fatty acid homeostasis (5).

As a continuation of this work, we considered it of interest to determine whether we could isolate endogenous activators of PPAR from a complex biological extract. While transactivation assays have been widely used to screen for activators/ligands for novel members of the steroid nuclear receptor superfamily, termed "orphan receptors" (6–8), we recently reported an alternative analytical chemical approach to this problem (9). The latter methodology involves extensive fractionation of the tissue extract into well-characterized subfractions, followed by enrichment of the endogenous ligand in the pertinent subfraction by exploiting the high affinity and specificity of

Abbreviations: rPPAR, rat peroxisome proliferator-activated receptor; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; GR, glucocorticoid receptor; PAP, placental alkaline phosphatase.

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the receptor for its ligand. In the present study, we have combined a tissue fractionation approach with the PPAR transactivation assay, referred to above, to screen for endogenous activators of PPAR in human plasma. It was also our intention to develop methodology that may be generally applicable to identifying endogenous activators/ligands to the growing number of orphan receptors.

MATERIALS AND METHODS

Initial plasma fractionation and screening for PPAR activators

The overall approach to plasma fractionation is illustrated in **Fig. 1.** Emphasis should be placed on the importance of avoiding incorporation of exogenous contaminants during sample handling. Glass and Teflon were the only materials used throughout the entire fractionation procedure and great care was taken to avoid contact of samples and solvents with plastics and latex gloves. When necessary, column flow rates were achieved by application of nitrogen pressure.

Glassware and solvents. After normal washing, all glassware was rinsed in chloroform–methanol 1:1 (v/v) followed by ethanol. All solvents were of reagent grade and were distilled twice before use. Water was obtained from a Milli-Q reagent-grade water system (Millipore Co., Milford, MA).

Sorbents and gels. Beds of octadecylsilane-bonded silica (ODS silica, Preparative C18, Millipore Waters, 15 × 8

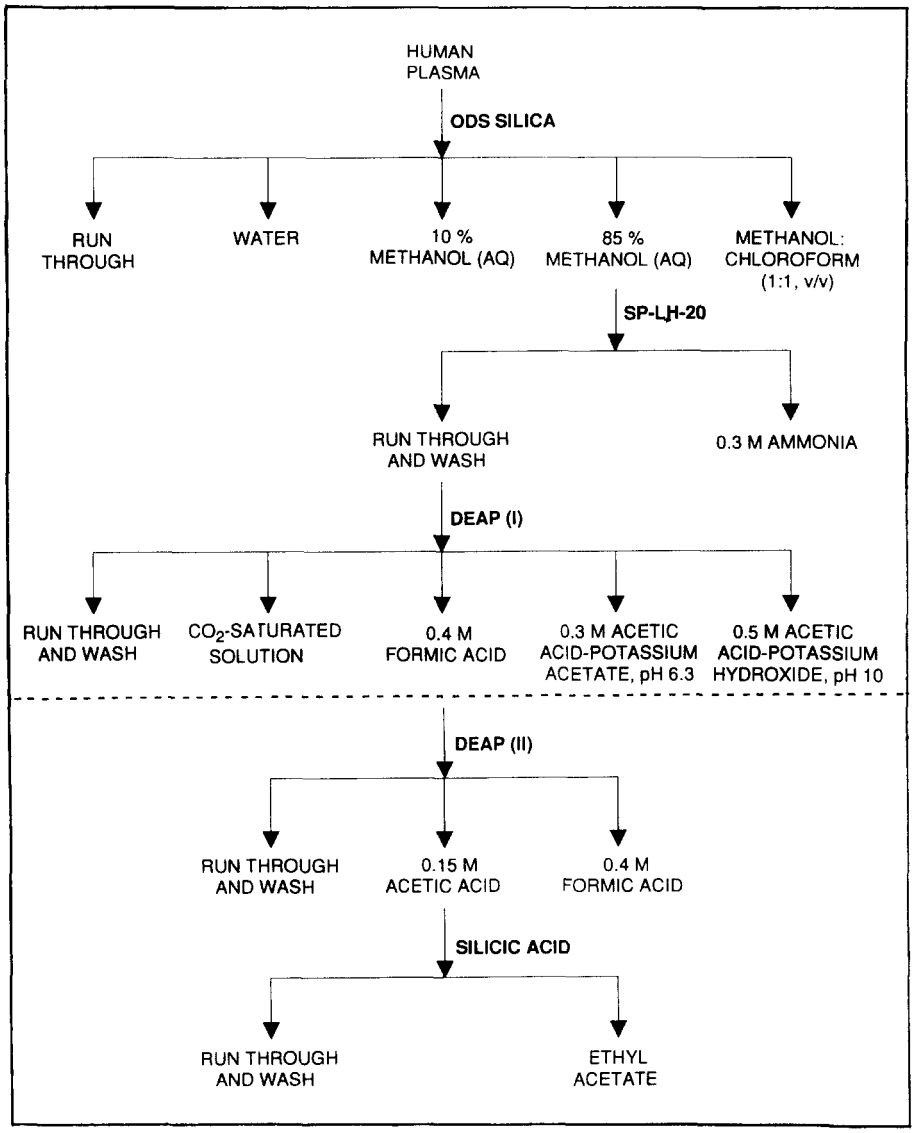

Fig. 1. Flow diagram illustrating plasma fractionation procedure. Initial fractionation is illustrated above the dotted line; further subfractionation, guided by response of each fraction in PPAR transactivation assay, is below the dotted line.

mm, approximately 300 mg) were prepared in a jacketed column and pre-washed with 5 ml each of methanol, chloroform–methanol 1:1 (v/v), and methanol at ambient temperature and then with 5 ml water at 64°C.

Sulfohydroxypropyl Sephadex LH-20 (SP-LH-20) (10) was stored in Na^+ form at 4°C in methanol. Prior to use, a few grams were converted to H^+ form by washing in a Büchner funnel with 100 ml each of 0.3 M HCl in 25% aqueous ethanol, water (until neutral), 50% aqueous ethanol, and 70% aqueous methanol. A 40 × 4 mm bed was packed in 70% aqueous methanol under gravity flow and washed with a further 10 ml of this solvent.

Lipidex-DEAP (diethylaminohydroxypropyl Sephadex-LH-20, Packard Instruments Co., Inc., Downers Grove, IL) was washed in a Büchner funnel with 0.2 M HCl in 50% aqueous ethanol and water (until neutral), then stirred for 1 h at 70°C in each of 20, 50, and 99.5% aqueous ethanol followed by further washing in a Büchner funnel with each of 0.25 M acetic acid in 50% aqueous ethanol, water (until neutral), 20, 50, and 99.5% aqueous ethanol, and finally methanol in which it was stored in acetate form at 4°C. Prior to use, a few grams were converted to OH^- form by washing in a Büchner funnel with each of 0.25% sodium hydroxide in 25% aqueous ethanol, water (until neutral), 50% aqueous ethanol, and 70% aqueous methanol. A 40 × 4 mm bed was packed in 70% aqueous methanol under gravity flow and washed with 10 ml of the same solvent.

Activated silicic acid (Unisil, Clarkson Chemical Co., Inc., Williamsport, PA (11)) was packed dry to give a 29 × 4 mm bed and washed with 5 ml each of hexane and hexane–dichloromethane 1:4 (v/v).

Blood samples were taken from healthy human volunteers and plasma was stored at −20°C prior to fractionation. The initial fractionation procedure prior to the first PPAR transactivation screen (see below) was essentially as described previously (9). Five ml plasma was mixed with an equal volume of 0.5 M triethylamine sulfate, pH 7.2, prior to solid phase extraction on the ODS silica bed (12). With the tap closed, the sample was applied to the top of the bed, equilibrated at 64°C for 5 min, and passed at a flow rate of approximately 30 ml h^{-1} through the bed at the same temperature. After a wash of 5 ml water and 5 ml 10% aqueous methanol at 64°C, the column was cooled to room temperature and 8 ml 85% aqueous methanol, then 8 ml chloroform-methanol 1:1 (v/v) were applied to elute lipophilic compounds. One ml of water was added to the 85% aqueous methanol extract prior to its application to the cation-exchange column (SP-LH-20). The run-through plus an additional 5 ml 70% aqueous methanol were passed at a flow rate of approximately 7 ml h^{-1} and collected together. Six ml 0.3 M ammonia in 70% aqueous methanol was then applied to the SP-LH-20 column. The run-through and wash of the SP-LH-20 column was applied to the first anion exchange column (DEAP (I)). After a 3 ml methanol–chloroform 2:1 (v/v) wash, which was collected together with the run-through, the DEAP (I) column was eluted with four further solvents of increasing acidity and ionic strength. First, 6 ml CO_2-saturated solution, followed by 10 ml each of 0.4 M formic acid, 0.3 M acetic acid–potassium acetate, apparent pH 6.3, and 0.5 M acetic acid–potassium hydroxide, apparent pH 10, (all solutions in 70% aqueous methanol) were applied to the column.

Prior to PPAR transactivation screening, aliquots of each of the three final fractions from DEAP (I) were deconjugated as described by Axelson, Sahlberg, and Sjövall (13). The 0.4 M formic acid fraction was treated with *Helix pomatia,* and the acetic acid-based fractions were deconjugated by solvolysis.

The methanol–chloroform 1:1 (v/v) eluate from the ODS silica column and all fractions including deconjugated fractions from DEAP (I) were screened for activators of PPAR. All fractions were stored in a minimum volume of methanol at 4°C prior to screening.

Subfractionation of 0.4 M formic acid fraction from DEAP (I)

The 0.4 M formic acid fraction (untreated) was evaporated to dryness in vacuo at 40°C and reconstituted in 14 ml 95% aqueous methanol before application to the second anion exchange column (DEAP (II)). After collection of the run-through together with a 3-ml methanol–chloroform 2:1 (v/v) wash, 10 ml 0.15 M acetic acid, and then 10 ml 0.4 M formic acid, (both acids in 95% aqueous methanol) were applied to the column. Prior to silicic acid chromatography, the 0.15 M acetic acid fraction was evaporated to dryness in vacuo at 40°C, reconstituted in 2 ml methanol–diethyl ether 1:1 (v/v), and methylated at 0°C for 20 min with 2 ml freshly prepared diazomethane in diethyl ether. The methylated sample was evaporated to dryness under N_2 and reconstituted in 2 ml hexane–dichloromethane 1:4 (v/v), before application to the silicic acid bed. After collection of the run-through together with a 12-ml column wash with the same solvent, 13 ml ethyl acetate was applied to the column. Both fractions from the silicic acid column were, after evaporation to dryness in vacuo at 40°C, demethylated by incubation at 50°C for 1 h with 1 ml 5% sodium hydroxide in 50% aqueous methanol (alkaline hydrolysis). After acidification by addition of 100 μl glacial acetic acid, and reduction of methanol content to 20% by the addition of 1.5 ml water, the samples were purified by ODS silica chromatography. The column was washed with 5 ml each of water and 20% aqueous methanol; hydrolyzed products were eluted in 5 ml methanol. All fractions from DEAP (II) and the silicic acid column were stored in a minimum volume of methanol at 4°C prior to screening for PPAR activators.

Characterization of run-through and wash from the silicic acid column

Gas chromatography. An aliquot of the methylated run-through and wash from the silicic acid column was subjected to gas chromatography (GC), using a Carlo Erba Strumentazione HRGC 5300 Mega series instrument (Farmitalia Carlo Erba, Milan, Italy) equipped with a DB-23 fused silica capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness, J&W Scientific, Folsom, CA). Nitrogen was used as the carrier gas at a flow rate of approximately 1 ml min^{-1}. The oven temperature was increased from 60°C (on-column injection) to 160°C at a rate of 25°C min^{-1}, then raised to 240°C at a rate of 2°C min^{-1}. Methylated fatty acids were identified by comparison of retention times with those of authentic standards, and quantification was achieved through use of an internal standard (n-dotriacontane).

Gas chromatography–mass spectrometry. An aliquot of the methylated run-through and wash from the silicic acid column was subjected to gas chromatography–mass spectrometry (GC–MS) using a VG 70-250 double-focussing instrument (VG Analytical Ltd., Manchester, UK) equipped with a Hewlett-Packard 5790A series gas chromatograph (Hewlett-Packard Co., Avondale, PA) and a VG 11-250 data system. The fused silica capillary column (methyl silicone, 25 m × 0.32 mm (internal diameter), 0.25-μm film thickness, Quadrex Co., New Haven, CT) in a temperature gradient of 60–240°C at 2°C min^{-1}, was connected directly to the ion source. An all-glass falling needle injection system was used. Spectra were recorded by repetitive scanning between *m/z* 500 and 100, at a scan rate of 2 s $decade^{-1}$, with an accelerating voltage of 6 kV, an electron energy of 70 eV, and a trap current of 200 μA. Methylated fatty acids were identified by comparison of retention times and mass spectra with those of authentic standards.

High performance liquid chromatography. An aliquot of the hydrolyzed run-through and wash from the silicic acid column was further fractionated by HPLC by injection onto a Zorbax C8 column (25 cm × 4.6 mm (internal diameter) 5 μm particle size, Rockland Technology Inc., Newport, DE) in a mobile phase comprising 50% acetonitrile, 28% tetrahydrofuran, and 22% H_3PO_4 (0.1%) at a flow rate of 1 ml min^{-1} at room temperature (Waters 600E multisolvent delivery system, Millipore Co.). Ultraviolet detection was at 215 nm using a photodiode array detector (Waters 991, Millipore Co.). After adjusting the aqueous content to 50%, each individual fraction was extracted in hexane and stored at −20°C prior to screening for PPAR activators. In order to prevent cytotoxicity in the transactivation assay, care was taken to avoid contamination of the hexane phase with the mobile phase.

PPAR transactivation assay

Cloning of the rPPAR and construction of the PPAR transactivation assay have been described previously (5). Briefly, a chimera consisting of the putative ligand-binding domain of rPPAR and the amino terminal and DNA-binding domains of the human glucocorticoid receptor (hGR) was stably expressed in CHO cells together with placental alkaline phosphatase (PAP) as a reporter gene. The reporter gene is under the control of the MMTV promoter which is normally inducible by glucocorticoids via the GR. The chimeric receptor is activated by activators of PPAR, e.g., WY 14, 643 and clofibric acid, but induces transcription from the GR-dependent promotor. Low levels of endogenous GR in CHO cells cause the reporter to be slightly induced by the addition of dexamethasone. This approach allows the search for activators of novel receptors without knowing their target genes.

To determine the capacity of fractions from the scheme outlined in Fig. 1 to activate the PPAR/GR chimera, stably transfected cells were treated with aliquots of each fraction. Fractions were evaporated to dryness under N_2, reconstituted in 2 μl dimethylsulfoxide, and diluted in 0.5 ml cell medium prior to addition to the cells which had been seeded at a density of 20,000/2 cm^2 culture and grown in 0.5 ml medium for 24 h. After 48 h treatment, cell culture supernatants were assayed for PAP induction as described previously (5).

In order to determine the appropriate amounts for assay, cells were initially treated with approximately 1 ml plasma-equivalent of each fraction. If this was toxic to the cells, the plasma-equivalent volume was reduced until no toxicity was observed. Alternatively, if no response was observed with 1 ml plasma-equivalent, the volume was increased until either a response or toxicity was indicated. Responses equal to or greater than those induced by dexamethasone are expressed as a percentage of the induction by WY 14,643. As a negative control, cells stably expressing full length GR instead of the PPAR chimera were also treated (5).

RESULTS AND DISCUSSION

Initial plasma fractionation and screening for PPAR activators

Plasma was fractionated according to the scheme in Fig. 1. Interactions between plasma proteins and lipophilic compounds are reduced by the addition of triethylamine sulfate, permitting sorption of the latter on ODS silica (12). Hydrophilic material is eluted in the run-through and the washes with water and 10% aqueous methanol. Compounds of intermediate and low polarity

are eluted with 85% aqueous methanol and chloroform–methanol 1:1 (v/v), respectively. Components of the 85% aqueous methanol fraction were fractionated on the basis of charge using two lipophilic ion-exchange columns. The first, a cation exchanger (SP-LH-20), retains positively charged compounds at neutral pH, while uncharged and acidic compounds appear in the column run-through and wash. The positively charged compounds are eluted with 0.3 M ammonium hydroxide. Acidic compounds in the run-through and wash are retained by the anion exchanger, DEAP (I), while components with no net charge are again unretained. Solvents of increasing acidity and ionic strength are used to elute compounds of graded acidity. Contact of organic solvents in all the above fractions with polypropylene microcentrifuge tubes in the final step before screening was found to have toxic effects on the CHO cells. For this reason and to avoid contamination of fractions with plastic and rubber-related compounds (14), only glass equipment was used for the final evaporation/reconstitution stages.

The results of screening for PPAR activators in the initial plasma fractions are given in **Table 1.** The most potent PPAR activation within the lipophilic spectrum was observed with the 0.4 M formic acid fraction from the DEAP (I) column. This fraction contains compounds with properties like those of free fatty acids and bile acids, bile acids conjugated with glycine, and steroids conjugated with glucuronic acid (15). A low level of induction was observed on one occasion by the CO_2-saturated solvent fraction. This may be attributable to leakage from the neighboring highly active fraction. Activity after deconjugation of the 0.4 M formic acid fraction from DEAP (I) indicates the presence of activators in the free form. However, we interpret the decrease in activity after deconjugation as a loss of free acids during the hydrolysis procedure rather than the hydrolysis of active conjugates.

TABLE 1. Activation of PPAR by initial plasma fractions

Column	Fraction	n[a]	Plasma Volume Equivalent[b]	Induction[c]	Mean Induction/ml Plasma[d]
			ml	%	
ODS silica	methanol–chloroform 1:1 (v/v)	2	0.56	NR[e]	NR
			0.19	NR	
SP-LH-20	0.3 M ammonia	2	8.00	NR	NR
			4.00	NR	
DEAP(1)	run-through and wash	2	0.56	NR	NR
			0.19	NR	
	CO_2-saturated solvent	3	4.20	16	3.8
			4.20	NR	
			1.67	NR	
	0.4 M formic acid (untreated)	7	1.70	68	70
			0.57	24	
			0.56	104	
			0.56	NR	
			0.30	17	
			0.19	19	
			0.19	NR	
	0.4 M formic acid (deconjugated)	4	1.70	51	35
			1.19	NR	
			1.10	49	
			0.56	19	
	0.3 M acetic acid-potassium acetate, pH 6.3 (untreated)	2	0.56	NR	NR
			0.19	NR	
	0.3 M acetic acid-potassium acetate, pH 6.3 (deconjugated)	2	1.67	NR	NR
			0.42	NR	
	0.5 M acetic acid-potassium hydroxide, pH 10 (untreated)	2	0.28	NR	NR
			0.09	NR	
	0.5 M acetic acid-potassium hydroxide, pH 10 (deconjugated)	2	1.67	NR	NR
			0.28	NR	

[a]Number of assays that yielded a response or no response (NR). Assays resulting in toxic effects are not reported.
[b]Volume of each fraction reported as equivalent-plasma volume.
[c]The percent induction of the positive control Wy 14,643 (100 μM) is reported.
[d]Mean induction per ml plasma equivalent is reported for comparison of activation capacity between fractions.
[e]Inductions less than or equal to that of dexamethasone are reported as no response (NR).

Variation in batches of plasma, fractionation procedure, and fraction storage conditions may account for the variation noted in the percent induction values.

Subfractionation of 0.4 M formic acid fraction from DEAP (I)

The activity of the 0.4 M formic acid fraction from DEAP (I) was subfractionated on a second DEAP column (DEAP (II)). Elution with 0.15 M acetic acid would be expected to yield a fraction containing free fatty acids and bile acids while conjugated steroids and bile acids would be retained and eluted with 0.4 M formic acid. Screening for PPAR activators indicated that the active compound(s) eluted with 0.15 M acetic acid (**Table 2**). No activity was observed with equivalent plasma volumes of the other two fractions. The observation that the 0.4 M formic acid fraction from DEAP (II), which contained conjugated material, did not activate PPAR supports the interpretation of previous data from the deconjugated 0.4 M formic acid fraction from DEAP (I).

To determine whether the PPAR activation capacity of the 0.15 M acetic acid fraction was associated with nonpolar or hydroxylated acids, the material was methylated and subfractionated on silicic acid (Fig. 1). Two fractions were collected, one of low polarity (run-through and wash) and another expected to contain hydroxylated acids and free bile acids (ethyl acetate fraction). Although both fractions activated the PPAR (after hydrolysis of the methyl esters, Table 2), the run-through and wash displayed a 10-fold more potent induction than the ethyl acetate fraction. The low activity induced by the ethyl acetate fraction may be associated with a spill-over of active components present in the column run-through and wash.

The chromatographic behavior of the activator(s) provides evidence to support a role of endogenous free fatty acids in the activation of PPAR. Additional evidence is supplied by comparison of the activation capacities of methylated and hydrolyzed run-through and wash of the silicic acid bed (Table 2); the methylated fraction is unable to induce PPAR activity until it is hydrolyzed. Previous studies with standard compounds have indicated that free fatty acids can activate PPAR in a transactivation assay, whereas the methyl esters, e.g., methyl stearate, are inactive (M. Göttlicher, unpublished observations).

The poor activation capacity of the ethyl acetate fraction argues against a role of cholestenoic acids as activators of PPAR. These are present in plasma at levels of 0.1–0.2 μM (16) and undergo β-oxidation catalyzed by peroxisomal enzymes.

The recovery of fatty acids in the extraction and subfractionation was studied by addition of [U-^{14}C]linoleic acid to plasma. Ninety-two percent of the radioactivity was recovered in the 0.15 M acetic acid fraction of the DEAP (II) column. After losses in the methylation proce-

TABLE 2. Activation of PPAR by subfractions of 0.4 M formic acid fraction from DEAP (I)

Column	Fraction	n[a]	Plasma Volume Equivalent[b]	Induction[c]	Mean Induction/ml Plasma[d]
			ml	%	
DEAP (II)	run-through and wash	2	1.00	NR[e]	NR
			0.56	NR	
	0.15 M acetic acid	4	1.00	65	49
			0.56	13	
			0.50	23	
			0.19	NR	
	0.4 M formic acid	2	1.00	NR	NR
			0.56	NR	
Silicic acid	run-through and wash (methylated)	2	1.50	NR	NR
			1.00	NR	
	run-through and wash (hydrolyzed)	5	1.50	61	35
			1.00	30	
			1.00	27	
			0.50	22	
			0.50	NR	
	ethyl acetate (hydrolyzed)	2	8.00	28	3.5
			4.00	NR	

[a] Number of assays that yielded a response or no response (NR). Assays resulting in toxic effects are not reported.
[b] Volume of each fraction reported as equivalent-plasma volume.
[c] The percent induction of the positive control Wy 14,643 (100 μM) is reported.
[d] Mean induction per ml plasma equivalent is reported for comparison of activation capacity between fractions.
[e] Inductions less than or equal to that of dexamethasone are reported as no response (NR).

dure, most of the radioactivity (68% of that added to plasma) appeared in the nonpolar fraction from the silicic acid column. Two percent was detected in the ethyl acetate fraction, indicating that the low levels of PPAR activation observed with this fraction are perhaps attributable to fatty acids. Depletion of activation capacity throughout the column fractionation procedure (Tables 1 and 2) is partly explained by batch-to-batch variation in plasma and losses of fatty acids. Thus, 28% of added radioactive linoleic acid and 50% of activation capacity are lost during methylation, silicic acid chromatography, and hydrolysis of the material in the 0.4 M formic acid fraction. However, this does not detract from the elution pattern of the activators being consistent with that of free fatty acids.

All fractions that activated PPAR were unable to induce a response in control cells without the PPAR ligand-binding domain (full length GR), indicating that the ligand-binding domain of PPAR is pivotal to transactivation. As an additional control to ensure that induction of transactivation was mediated by an endogenous factor, a reagent blank was performed of the silicic acid subfractionation. None of the fractions collected were able to induce a response.

Characterization of nonpolar fraction from silicic acid column

To define the nature of the components of the active nonpolar fraction from the silicic acid column, gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), and high performance liquid chromatography (HPLC) were used, the latter in conjunction with the PPAR transactivation assay.

Gas chromatography. Preliminary qualitative and quantitative data from GC analysis of the active fraction indicated the presence of nine fatty acids. After correction for losses as indicated above, the total concentration was about 0.2 mmol l^{-1} plasma which is slightly low in comparison to levels reported previously (17). The major components were oleic, palmitic, and linoleic acids, contributing 34, 32, and 26%, respectively, to the total amount of fatty acids.

Gas chromatography–mass spectrometry. The increased sensitivity of a GC–MS analysis permitted qualitative characterization of a further 12 fatty acids, in lower amounts in this subfraction (**Fig. 2**). Most of the 32 compounds detected were fatty acids. Of the remaining compounds, one was likely to be a hydrocarbon, perhaps a contaminant of the internal standard and one was bis(2-ethylhexyl) phthalate, a common contaminant. It can be mentioned that it is a metabolite of this compound, mono-ethylhexyl phthalate, that has been previously reported to activate mPPAR (1). These data suggest that the active final subfraction is composed mainly of free fatty acids and it is therefore most likely that these compounds contribute to the activity of this fraction.

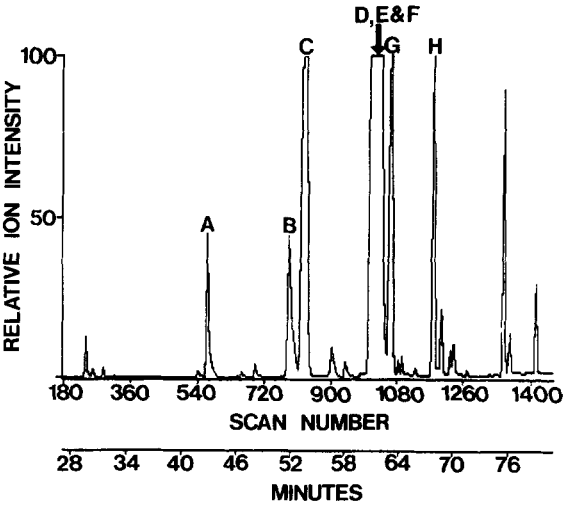

Fig. 2. GC–MS analysis of the nonpolar fraction from the silicic acid column. The principal fatty acids indicated in letters on the total ion current chromatogram are A, myristic; B, palmitoleic; C, palmitic; D, linoleic; E, linolenic; F, oleic; G, stearic; and H, arachidonic acids.

High performance liquid chromatography. In order to determine which of the components in this subfraction activate PPAR, the hydrolyzed sample was subjected to HPLC fractionation. Transactivation assay of each HPLC fraction indicated that hexane extracts from four peaks were able to activate PPAR (**Fig. 3**). After methylation, active HPLC fractions were analyzed by GC–MS as above. On the basis of these data and HPLC retention times compared with authentic standards, the four active components were indicated to be palmitic, oleic, linoleic, and arachidonic acids. The concentrations of endogenous fatty acids that activate PPAR in the transactivation assay are in the range 10–100 nmol ml^{-1}, and are comparable with active concentrations reported by Göttlicher et al. (5).

In conclusion, using a novel combination of analytical chemical and molecular biology techniques, we were able to demonstrate that extracts of human plasma activate the rPPAR in CHO cells. Previous studies to identify molecular activators of orphan receptors have generally been limited to the screening of randomly chosen reference compounds in transactivation assays. The outlined methodology presents a more logical approach, as all components of the tissue of interest can be screened and the chromatographic procedure described is suitable for any biological extract.

The activity present in the lipophilic spectrum of plasma components was associated with the free fatty acids palmitic, oleic, linoleic, and arachidonic acid. The fact that fatty acids activate the rPPAR has been indicated by previous studies in which a large number of chemicals was screened using the transactivation assay and data concerning linearity and thresholds of response have been reported (5). Although the results of the present study ap-

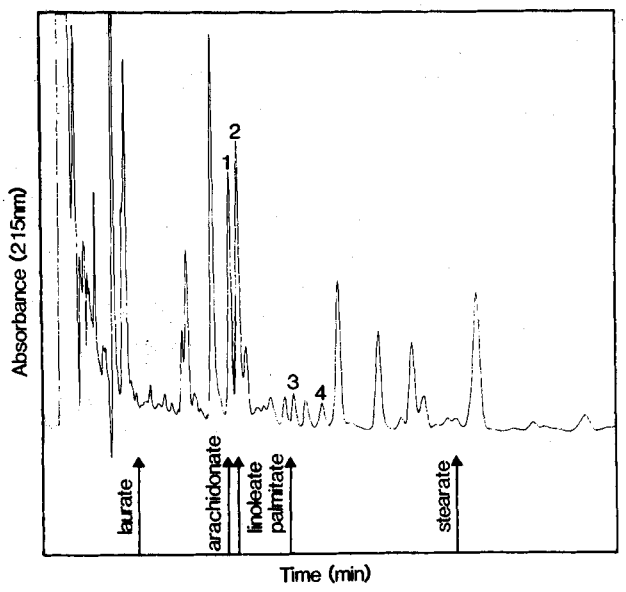

Fig. 3. HPLC profile of hydrolyzed nonpolar fraction from the silicic acid column. Absorbance at 215 nm is plotted versus absolute retention time up to 1 h. Arrows indicate retention of authentic free fatty acid standards. Fractions were collected up to 2 h retention time. Where possible, single peaks were collected in individual fractions; in the absence of peaks, fractions of 5–60 min retention time were taken. Peak areas do not reflect relative amounts of each free fatty acid as UV absorbance varies according to degree of saturation. Annotated fractions (numbers 1–4 inclusive) only were able to activate the receptor. GC-MS analyses together with HPLC retention time of these active fractions indicated that their major components were arachidonic acid (fraction 1), linoleic acid (fraction 2), palmitic acid (fraction 3), and oleic acid (fraction 4).

pear basically confirmatory, we now show that, using the logical approach illustrated, we are able to specifically extract and identify endogenous activators of PPAR from a complex biological extract. It can be pointed out that, had previous data concerning the activation of rPPAR not been available, the present approach alone would have identified free fatty acids in this role. In addition, it is of significant interest that, after thorough screening, no other active component was detected in the lipophilic spectrum of plasma constituents. It is possible that our approach may miss quantitatively minor, but physiologically relevant, activators. However, subfractionation should contribute to the enrichment of activators, if present, and remove other compounds that may cause toxicity in the cell assay.

The fact that we found the only activators to be fatty acids emphasizes their role in PPAR activation. It is still unclear whether these components are the ultimate ligands for PPAR and studies are underway to determine whether this is, in fact, the case, or whether fatty acid derivatives or other compounds activated by fatty acids represent the true intracellular ligands.

With regard to the general applicability of the approach, this logical screening of tissue components presents a potentially powerful tool for the identification of endogenous activators to the growing number of orphan nuclear receptors. It can be pointed out that the criteria for the identification of activators do not include high affinity/specificity binding and therefore the activators extracted do not necessarily represent specific ligands. However, results obtained with our approach could form the basis for such further receptor binding studies.

Tomas Cronholm is acknowledged for assistance with the gas chromatographic analyses and Dan Svensson for excellent technical assistance with the transactivation assays. This work was supported by grants from the Swedish Nutrition Foundation, the Axelson-Johnson Foundation, the Swedish Cancer Society, the Swedish Medical Research Council (O3X-219 and 13X-2819), and the European Molecular Biology Organization (fellowship to MG, ALTF 426-1990).

Manuscript received 30 December 1992 and in revised form 19 March 1993.

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