In Vitro Synthesis of Oleoylglycine by Cytochrome c Points to a Novel Pathway for the Production of Lipid Signaling Molecules*

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Long chain fatty acyl glycines represent a new class of signaling molecules whose biosynthetic pathway is unknown. Here we report that cytochrome c catalyzes the formation of oleoylglycine from oleoyl-CoA and glycine, in the presence of hydrogen peroxide. The identity of oleoylglycine product was confirmed by isotope labeling and fragmentation mass spectrometry. Synthesis of oleoylglycine by cytochrome c was dependent upon substrate concentration and time. Other heme-containing proteins, myoglobin and hemoglobin, did not catalyze oleoylglycine synthesis. The functional properties of the reaction closely resemble those observed for the ability of cytochrome c to mediate the synthesis of oleamide from oleoyl-CoA and ammonia, in the presence of hydrogen peroxide (Driscoll, W. J., Chaturvedi, S., and Mueller, G. P. (2007) J. Biol. Chem. 282). The ability of cytochrome c to catalyze the formation of oleoylglycine experimentally indicates the potential importance of cytochrome c as a novel mechanism for the generation of long chain fatty acyl glycine messengers in vivo.

Oleamide is the prototype member of the long chain primary fatty acid amides (7). It was first detected in human serum with four other related amides in 1989 (8) and was subsequently shown to accumulate in the cerebrospinal fluid of sleep-deprived cats (9, 10). The ability of oleamide to produce a profound sleep-like state when administered to rodents (9–11) has led to the proposal that oleamide contributes to the biochemical drive to sleep (9–11). Possible cellular mechanisms by which oleamide might mediate the drive to sleep and other physiologic responses include modulation of serotonin receptor action (12) and inhibition of gap junctions (7, 13, 14). While arachidonamide has yet to be identified in vivo, a variety of other long chain primary fatty acid amides have been found in cultured cells and tissue (15).

Despite considerable interest in the biology and pharmacology of long chain fatty acyl glycines and amides, pathways for their biosynthesis have not been defined. While glycination is essential for the excretion of short chain fatty acids (16), detoxification of small molecule xenobiotics (17, 18) and synthesis of bile acids (19, 20), the enzymes mediating these conjugations are quite specific and essentially unreactive with long chain fatty acyl-CoAs (coenzyme A). We recently identified cytochrome c as a cellular activity that catalyzes the formation of oleamide from oleoyl-CoA and ammonium ion, in the presence of hydrogen peroxide (21). In the course of characterizing this activity, we examined glycine as a potential nitrogen donor. We report here that cytochrome c catalyzes the formation of N-oleoylglycine from oleoyl-CoA and glycine. This discovery highlights the potential importance of cytochrome c as a mechanism for the generation of both long chain fatty acyl glycines and long chain primary fatty acid amides. This possibility is relevant to understanding the biochemistry of sleep, thermal regulation, nociception, and also apoptosis, where cytochrome c exhibits unique catalytic activities that mediate programmed cell death (22).

EXPERIMENTAL PROCEDURES

Radiochemicals and Chemicals—[glycine-1-14C]Oleoylglycine (4.1 mCi/mol) was prepared as a custom synthesis by PerkinElmer Life Sciences. [1-14C]Oleoyl-CoA (53 mCi/mmol) was purchased from GE Healthcare, Piscataway, NJ. Rat and yeast cytochrome c, equine myoglobin, H2O2, glycine, fatty acyl-CoAs, and other laboratory reagents were purchased from

*2 The abbreviations used are: PAM, peptidylglycine α-amidating monooxygenase; CoA, coenzyme A; ESI, electrospray ionization; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TLC, thin layer chromatography.

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3 M. Johnson, personal communication.
Sigma-Aldrich. Oleoylglycine was purchased from Cayman Chemical, Ann Arbor, MI. The rat cytochrome c was determined to be homogeneous as described in Ref. 21.

Assay for Oleoylglycine-synthesizing Activity and Thin Layer Chromatography (TLC)—Assays for oleoylglycine synthesis were performed at 37 °C in 100 μl 50 mM Tris, pH 7.4, containing the desired amounts of cytochrome c (or myoglobin or hemoglobin), glycine, H₂O₂, and [¹⁴C]oleoyl-CoA (see Ref. 21). Reactions were terminated by the addition of 5 μl of 1 N HCl and extracted with 4 volumes of ethyl acetate. The organic layer was collected, dried under nitrogen, solubilized in 25 μl of ethanol and spotted to heat-desiccated (45 min at 95 °C) Silica Gel G Uniplates™ (Analtech, Newark, DE). TLC plates were developed (1 h) using chloroform/methanol/glacial acetic acid, 90:10:1. Data were visualized using a FAL-5100 phosphorimager (FUJIFILM, Stamford, CT), and quantitative image analysis was performed with Multi Gauge V3.0 software (FUJIFILM). Data are expressed as percent conversion of oleoyl-CoA substrate to oleoylglycine product calculated on the basis of a defined amount of radiolabeled oleoylglycine standard run on each plate.

Reverse Phase HPLC—Separations were carried out at 45 °C on a 4.6 × 250 mm Luna 5 μC₈ column (Phenomenex, Torrance, CA) using a series 1100 HPLC (Agilent Technologies, Wilmington, DE) operated at 1 ml/min. The column was equilibrated with 50% solvent A (5% acetonitrile in water; 0.005% trifluoroacetic acid) and 50% solvent B (5% water in acetonitrile; 0.005% trifluoroacetic acid). Samples and standards were solubilized in 50 μl of ethanol for injection. Following sample injection, initial conditions were maintained for 5 min followed by a linear gradient to 100% B over 10 min. The system was maintained at 100% B for 10 min and returned to initial conditions over 5 min. Elution profiles were monitored at 205 nm. Fractions were collected and dried by lyophilization.

Mass Spectrometry—Mass fragmentation analysis was performed by electrospray ionization (ESI) mass spectrometry (MS) using a Q-Star®XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA). Oleoylglycine standard and lyophilized HPLC samples were first dissolved in 100% ethanol, adjusted to 50% acetonitrile containing 0.1% formic acid and infused into the ionization source (5,500 volts) at a rate of 10 μl per minute. Parent ions were identified and fragmented to produce product ions while preserving a small amount of parent ion. Confirmation of parent ion masses was established for each sample by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS using gentisic acid matrix and a Voyager™ MALDI-TOF DE STR instrument (Applied Biosystems) operated in positive ion reflector mode.

RESULTS

Synthesis of Oleoylglycine by Cytochrome c—Fig. 1 presents the time course for the formation of oleoylglycine by cytochrome c. Rat cytochrome c was incubated with oleoyl-CoA, glycine, and H₂O₂ for the times indicated. Panel A presents a representative TLC analysis of the reaction products observed. Radioactive bands that co-migrated with [¹⁴C]oleoylglycine standard (arrow, right lane) intensified overtime to a maximum signal by 10 min. Panel B is the graphic presentation of the oleoylglycine TLC data obtained through quantitative image analysis. Further investigations revealed that the loss of activity after 10 min was due to oxidative damage caused by exposure of the protein to H₂O₂. Specifically, preincubation of cytochrome c with H₂O₂ resulted in a loss of enzymatic activity, loss of antibody reactivity by Western analysis, change of mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis and loss of signature ion mass on MALDI-TOF MS (data not shown).

Oleoylglycine-synthesizing Activity of Cytochrome c: Comparison with Other Heme-containing Proteins—Fig. 2, panel A, demonstrates the dependence of oleoylglycine production on cytochrome c at submicellar (20 μM) and micellar (250 μM) concentrations of oleoyl-CoA. A threshold for oleoylglycine synthesis was observed at ~-40 nM cytochrome c per reaction at both substrate concentrations. Above this threshold the production of oleoylglycine by cytochrome c demonstrated essen-
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**Panel A**

A, synthesis of oleoylglycine by cytochrome c. Reactions were carried out for 10 min at 37 °C in 50 mM Tris, pH 7.4 (100 μl) containing 150 μM glycine, 2 mM H₂O₂, either 20 μM or 250 μM oleoyl-CoA, and rat cytochrome c as indicated. Reaction products were analyzed as described for Fig. 1. B, production of oleoylglycine by rat (CC-R) and yeast (CC-Y) cytochrome c, bovine hemoglobin (Hemo), and equine myoglobin (Myo) in the absence (−) or presence (+) of H₂O₂. Duplicate reactions were carried out for 10 min at 37 °C in 50 mM Tris, pH 7.4 (100 μl) containing 400 nM protein, 150 μM glycine, 50 μM oleoyl-CoA, and 2 mM H₂O₂. Data are presented as percent of maximum conversion of oleoyl-CoA substrate to oleoylglycine product for each experiment. These values were 7.1 nmol and 2.5 nmol for panels A and B, respectively.

**Panel B**

FIGURE 2. Oleoylglycine-synthesizing activity of cytochrome c and other heme-containing proteins. A, synthesis of oleoylglycine by cytochrome c. Reactions were carried out for 10 min at 37 °C in 50 mM Tris, pH 7.4 (100 μl) containing 150 μM glycine, 2 mM H₂O₂, either 20 μM or 250 μM oleoyl-CoA and rat cytochrome c as indicated. Reaction products were analyzed as described for Fig. 1. B, production of oleoylglycine by rat (CC-R) and yeast (CC-Y) cytochrome c, bovine hemoglobin (Hemo), and equine myoglobin (Myo) in the absence (−) or presence (+) of H₂O₂. Duplicate reactions were carried out for 10 min at 37 °C in 50 mM Tris, pH 7.4 (100 μl) containing 400 nM protein, 150 μM glycine, 50 μM oleoyl-CoA, and 2 mM H₂O₂. Data are presented as percent of maximum conversion of oleoyl-CoA substrate to oleoylglycine product for each experiment. These values were 7.1 nmol and 2.5 nmol for panels A and B, respectively.

**Panel C**

FIGURE 3. Dose response for oleoyl-CoA, glycine, and H₂O₂ on the formation of oleoylglycine by rat cytochrome c. Rat cytochrome c (400 nM) was incubated with varying amounts of oleoyl-CoA, glycine, or H₂O₂ for 10 min at 37 °C. Reaction products were analyzed as described for Fig. 1. A, reactions contained 5 mM H₂O₂, 150 mM glycine, and oleoyl-CoA at the concentrations indicated. B, reactions contained 100 μM oleoyl-CoA, 5 mM H₂O₂, and glycine at the concentrations indicated. C, reactions contained 100 μM oleoyl-CoA, 150 mM glycine, and H₂O₂ at the concentrations indicated. Data plotted are the mean values for duplicates expressed as percent of maximum conversion of oleoyl-CoA to oleoylglycine. These values were 1.2, 3.1, and 1.6 nmol for A, B, and C, respectively.

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Panel A shows the dose response for increasing concentrations of oleoyl-CoA. The reaction was maximal at ~100 μM oleoyl-CoA and half-maximal at ~30 μM. Additional analyses of similar experiments using saturating concentrations of oleoyl-CoA confirmed a Kₘ of ~30 μM for oleoyl-CoA (not shown). It should be noted that oleic acid does not serve as a substrate for oleoylglycine synthesis by cytochrome c. Panel B shows the results of a similar experiment in which concentrations of glycine were varied. Glycine became saturating at ~300 mM, and half-maximal activity was achieved at ~40 mM glycine. The effects of increasing concentrations of H₂O₂ on oleoylglycine formation by cytochrome c are pre-

Oleoylglycine Synthesis by Cytochrome c

Optimal Reactant Concentrations for Oleoylglycine Synthesis by Cytochrome c—Fig. 3 shows the concentration dependence for oleoyl-CoA, glycine, and H₂O₂ on the formation of oleoylglycine by cytochrome c. Reactions were performed at optimal, fixed concentrations of two of the reactants with varied concentrations of the third. Data are expressed as a percent of maximum conversion of oleoyl-CoA to oleoylglycine for each experiment. Untransformed data are given in the figure legend. Panel A shows the dose response for increasing concentrations of oleoyl-CoA. The reaction was maximal at ~100 μM oleoyl-CoA and half-maximal at ~30 μM. Additional analyses of similar experiments using saturating concentrations of oleoyl-CoA confirmed a Kₘ of ~30 μM for oleoyl-CoA (not shown). It should be noted that oleic acid does not serve as a substrate for oleoylglycine synthesis by cytochrome c. Panel B shows the results of a similar experiment in which concentrations of glycine were varied. Glycine became saturating at ~300 mM, and half-maximal activity was achieved at ~40 mM glycine. The effects of increasing concentrations of H₂O₂ on oleoylglycine formation by cytochrome c are pre-

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in panel C. In comparison to oleoyl-CoA or glycine, H$_2$O$_2$ exhibited a broad range of concentration effect. Half-maximal conversion was observed at $\sim$60 $\mu$M H$_2$O$_2$. At concentrations 10 mM and above, H$_2$O$_2$ reduced the formation of oleoylglycine as compared with maximum conversion observed over the range of 1–3 mM. As discussed above, the decrease in activity observed at higher H$_2$O$_2$ concentrations was caused by oxidative damage caused by H$_2$O$_2$ itself. The findings presented in panels A, B, and C are representative of results of at least three independent experiments.

Verification of Oleoylglycine as a Cytochrome c Reaction Product—The identity of oleoylglycine as an enzymatic product was confirmed by HPLC and mass spectral fragmentation analysis. Reaction products were isolated by solvent extraction and reverse phase HPLC from large scale syntheses using non-radioactive oleoyl-CoA and either glycine or isotopically labeled glycine-$^{13}$C$_2$. The peak eluting at the retention time of oleoylglycine standard (18.6 min; Fig. 4, panel A) was analyzed by ESI MS/MS and MALDI-TOF MS as detailed under “Experimental Procedures.” Both ESI and MALDI-TOF analyses revealed that the most prominent peaks in the spectra exhibited the correct masses for oleoylglycine and $^{13}$C$_2$-labeled oleoylglycine at $m/z$ 340.53 and 342.54, respectively (confirmatory MALDI-TOF results not shown). These peaks were absent from the HPLC effluents (18.6 min) of negative control reactions. Panel B presents the fragmentation spectrum for commercially obtained oleoylglycine standard. Signature ions in the spectrum include oleoylglycine parent ion ($m/z$ 340.53), glycine daughter ion ($m/z$ 76.07), and glycine immonium ion ($m/z$ 30.04). Additionally, the series of ions differing by 14 amu reflects products of varying lengths of the alkane chain. Panel C shows the fragmentation spectrum for cytochrome c-synthesized oleoylglycine. It is qualitatively identical to that of standard oleoylglycine (panel B). Substitution of glycine-$^{13}$C$_2$ for glycine in the synthesis reaction (panel D) produced oleoylglycine parent and glycine daughter ions that were two mass units heavier ($m/z$ 342.54 and $m/z$ 78.08, respectively) than those observed for unlabeled oleoylglycine. The glycine immonium ion was one mass unit heavier ($m/z$ 31.04) than its $^{12}$C counterpart ($m/z$ 30.03). These data provide unequivocal evidence for the synthesis of oleoylglycine by cytochrome c from oleoyl-CoA and glycine.

Oleoylglycine Synthesis by Cytochrome c Demonstrates Specificity for Long Chain Fatty Acyl-CoA Substrates—A substrate specificity study was conducted using varying chain length acyl-CoAs as competing substrates against [14C]oleoyl-CoA. The data presented in Fig. 5 show that acyl-CoAs with chain lengths of 16 carbons or more were the most effective competitors. Interestingly, octanoyl-CoA, while not as potent as the longer chain acyl-CoAs, was able to inhibit oleoylglycine synthesis.

150 $\mu$M of either glycine or glycine-$^{13}$C$_2$. Reaction products were purified by HPLC and analyzed by ESI MS/MS, A, HPLC profile (205 nM) of reaction products. The peak at 18.6 min, labeled O-Gly, eluted precisely at the retention time of oleoylglycine standard. Elution profiles for the oleoylglycine reaction products generated with glycine (shown) and glycine-$^{13}$C$_2$ were identical. B, fragmentation of oleoylglycine standard (parent ion $m/z$ 340.53). C, fragmentation of the cytochrome c-synthesized oleoylglycine (parent ion $m/z$ 340.53). D, fragmentation of the cytochrome c-synthesized oleoylglycine-$^{13}$C$_2$ (parent ion $m/z$ 342.54).
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FIGURE 5. Substrate specificity for oleoylglycine-synthesizing activity of cytochrome c by competitive analysis. Rat cytochrome c (200 nM) was incubated in 50 mM Tris, pH 7.4 (100 μl) containing 150 μM glycine, 2 mM H2O2, 10 μM [14C]oleoyl-CoA, 200 μM myristic acid, and 250 μM competing non-radioactive acyl-CoA as indicated in the figure. Duplicate reactions were carried out for 5 min at 37 °C. Reaction products were extracted into ethyl acetate, separated by TLC and quantitated by phosphorimaging. Data are presented as pmol of oleoylglycine product formed.

MALDI-TOF MS was used to confirm that palmitoyl glycine, stearoyl glycine, and arachidonyl glycine were, in fact, generated from their respective CoA substrates by cytochrome c (data not shown).

DISCUSSION

The diverse chemical nature and biologic importance of lipid signaling molecules became appreciated with the discovery of the prostaglandins, leukotrienes, and thromboxanes. Recently, long chain fatty acyl glycines have been recognized as a potential new class of lipid messengers. Prototype members of this class include oleoylglycine and arachidonoyl glycine, which occur naturally (1, 2) and elicit pronounced physiologic effects in vivo (3, 4). While long chain fatty acyl glycines exhibit distinct pharmacologic properties, their in vivo functions and regulation remain to be defined. An understanding of the biosynthetic pathway for long chain fatty acyl glycines will help to advance knowledge in these areas. Toward this goal, the present report documents the ability of cytochrome c to catalyze the formation of oleoylglycine utilizing oleoyl-CoA, glycine, and H2O2 as reactants.

Previous investigations revealed cytochrome c as a cellular activity capable of catalyzing the synthesis of oleamide from oleoyl-CoA and ammonia, in the presence of H2O2 (21). This finding suggested that a primary amine might substitute for ammonia. In exploring this possibility we discovered that glycine effectively supports the formation of oleoylglycine by cytochrome c. The reaction exhibits a similar time course, reactant concentration optima, preference for long chain acyl-CoAs and Michaelis-Menten kinetics with respect to oleoyl-CoA, as was observed for the generation oleamide (21; Table 1). The ability of cytochrome c to effectively catalyze the formation of oleoylglycine product at concentrations of fatty acyl-CoA substrate both below and well above the critical micellar concentration (25) indicates that the present findings involving varying concentrations of fatty acyl-CoAs (Figs. 2, 3, and 5) were not influenced by either the absence or presence of micelle formation.

While incomplete, our working model predicts that oleoyl-CoA binds to cytochrome c with a geometry that places the thioester adjacent to the heme center. In this position the iron can accept an electron liberated by the nucleophile attack of glycine at the thioester bond. In this mechanism, Fe3+ is reduced to Fe2+ and subsequently reoxidized to Fe3+ by H2O2. This model is supported by evidence that cytochrome c exhibits: (i) high binding affinity for long chain fatty acyl-CoA molecules (26), (ii) peroxidase enzymatic activity (27–30), and (iii) the ability to catalyze the formation of oleamide from oleoyl-CoA and ammonia in the presence of H2O2, a reaction that is sensitive to the iron chelator deferoxamine (21).

Glycination is essential in the synthesis of bile acids (19, 20) and metabolism of short chain fatty acids (16) and small molecules xenobiotics (17, 18); however, the enzymes that mediate these conjugations are highly specific and are essentially unreactive with unsaturated long chain fatty acyl-CoAs (see Ref. 31). The present findings indicate that cytochrome c provides a possible mechanism for the glycination of unsaturated long chain fatty acyl-CoAs in vivo. Cytochrome c may therefore have a central role in the generation of oleoylglycine, which either can act independently (3), or serve as a substrate for the generation of oleamide (1).

The ability of cytochrome c to catalyze the formation of bioactive lipid messengers may be relevant to the process of apoptosis where cytochrome c is liberated from the mitochondria and becomes catalytically active (see Refs. 22 and 23). In this regard the formation of oleamide, either directly by cytochrome c or via the conversion of oleoylglycine by PAM (1) could play an important role in defining the extent of an apoptotic event. Under pathologic conditions including myocardial infarction and stroke, apoptotic molecules can be inappropriately transmitted from cell-to-cell (14, 33, 34). This process of bystander killing appears to result from a failure of gap junctions to close.

| Parameter                  | Oleoylglycine synthesis | Oleamide synthesis |
|----------------------------|-------------------------|--------------------|
| Kc for oleoyl-CoA          | 30 μM                   | 21 μM              |
| [H2O2] concentration optimum | 2 mM                   | 2 mM               |
| [Amine] concentration optimum | 300 mM                 | 125 mM             |
| pH optimum                 | 7.5                     | 7.5                |
| Temperature optimum        | 35–50 °C                | 35–50 °C           |
| Chain length specificity for fatty acyl-CoAs | Yes | Yes |
Because oleamide normally inhibits gap junction communication (7, 13, 14) and presumably, bystander killing, it is possible that the spread of apoptosis across healthy networks of cells results, at least in part, from a failure in the synthesis or actions of oleamide. Preliminary experiments conducted in our laboratory using cultured HeLa cells and staurosporine to induce apoptosis have revealed that oleamide synthesis is indeed up-regulated in this paradigm.4

In addition to apoptosis, lipid messengers generated by cytochrome c may serve in more conventional roles of intercellular communication. Immunoreactive cytochrome c is concentrated in secretory granules (35), structures also known to contain PAM (5). This subcellular localization raises the possibility that cytochrome c may generate oleoylglycine for regulated secretion or subsequent conversion to oleamide by PAM. Finally, the possibility that the generation of oleoylglycine occurs within mitochondria remains to be established.

We have also verified that, in addition to glycine, cytochrome c will catalyze the conjugation of other selected, but not all, amino acids with oleoyl-CoA. Further, cytochrome c effectively utilizes other primary amines and other long chain fatty acyl-CoAs as substrates (Ref. 21).4 Accordingly, cytochrome c exhibits the potential to generate a diverse spectrum of lipid signaling molecules as well as precursor substrates for other bioactivating enzymes such as PAM.

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