Oleic Acid Derived Metabolites in Mouse Neuroblastoma $N_{18}TG_2$ Cells†

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ABSTRACT: Oleamide is an endogenous sleep-inducing lipid that has been isolated from the cerebrospinal fluid of sleep-deprived mammals. Oleamide is the best-understood member of the primary fatty acid amide family. One key unanswered question regarding oleamide and all other primary acid amides is the pathway by which these molecules are produced. One proposed pathway involves oleoyl-CoA and *N*-oleoylglycine as intermediates: oleic acid → oleoyl-CoA → *N*-oleoylglycine → oleamide. The first and third reactions are known reactions, catalyzed by acyl-CoA synthetase and peptidylglycine α-amidating monooxygenase (PAM). Oleoyl-CoA formation from oleic acid has been demonstrated in vitro and in vivo while, to date, *N*-oleoylglycine cleavage to oleamide has been established only in vitro. PAM catalyzes the final step in α-amidated peptide biosynthesis, and its proposed role in primary fatty acid amide biosynthesis has been controversial. Mouse neuroblastoma $N_{18}TG_2$ cells are an excellent model system for the study of oleamide biosynthesis because these cells convert [^{14}C]-oleic acid to [^{14}C]-oleamide and express PAM in a regulated fashion. We report herein that growth of the $N_{18}TG_2$ cells in the presence of [^{14}C]-oleic acid under conditions known to stimulate PAM expression generates an increase in [^{14}C]-oleamide or in the presence of a PAM inhibitor generates [^{14}C]-*N*-oleoylglycine. This represents the first identification of *N*-oleoylglycine from a biological source. In addition, $N_{18}TG_2$ cell growth in the presence of *N*-oleoylglycine yields oleamide. These results strongly indicate that *N*-oleoylglycine is an intermediate in oleamide biosynthesis and provide further evidence that PAM does have a role in primary fatty acid amide production in vivo.

Fatty acid amides represent an important class of bioactive lipids in humans and other mammals. The members of this lipid class include the *N*-acylethanolamines (NAEs, also called the *N*-acylethanolamides and *N*-(2-hydroxyethyl)-acylamides, R−CO−NH−CH_2−CH_2−OH) and the primary fatty acid amides (PFAMs, R−CO−NH_2)[1]. The NAEs were first isolated from a biological source over 40 years ago (*1*) and have been widely studied. The functions served by specific NAEs are known (*2*−*4*), and the pathways for their biosynthesis and degradation have been established (*5*−*7*). The PFAMs are a relatively late addition to the fatty acid amide family, first identified in luteal phase plasma by Arafat et al. (*8*) in 1989. The discovery of PFAMs in human plasma went largely unnoticed until Cravatt et al. (*9*) reported that one PFAM, oleamide (*cis*-9,10-octadecenoamide), was an endogenous sleep-inducing lipid found in the cerebrospinal fluid of sleep-deprived mammals. Basile et al. (*10*) have also shown that endogenous oleamide levels were significantly increased in the CSF of rats after they had been deprived of sleep for 6 h or longer, and Stewart et al. (*11*) found that oleamide levels in the brain of the ground squirrel *Spermophilus richardsonii* were ∼2.5-fold higher in hibernating animals relative to nonhibernating animals.

Oleamide research has progressed rapidly since its identification as a sleep-inducing lipid. In addition to its role in regulating the sleep/wake cycle, oleamide has been shown to block gap junction communication in glial cells (*12*, *13*),

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[1] Abbreviations: ACGNAT, acyl-CoA:glycine *N*-acyltransferase; BAT, bile acid-CoA:amino acid *N*-acyltransferase; BSTFA, bis-trimethylsilyltrifluoroacetamide; DMEM, Dulbecco's Modified Eagle's Medium; ESI-MS, electrospray ionization mass spectrometry; FAAH, fatty acid amide hydrolase; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GC-MS, gas chromatography with mass spectrometric detection; NAE, *N*-acylethanolamine; NGF, nerve growth factor; OAc, oleic acid; OAm, oleamide; OG, *N*-oleoylglycine; PAM, peptidylglycine α-amidating monooxygenase; PBA, *trans*-4-phenyl-3-butenoic acid; PBS, phosphate buffered saline; PFAM, primary fatty acid amide.

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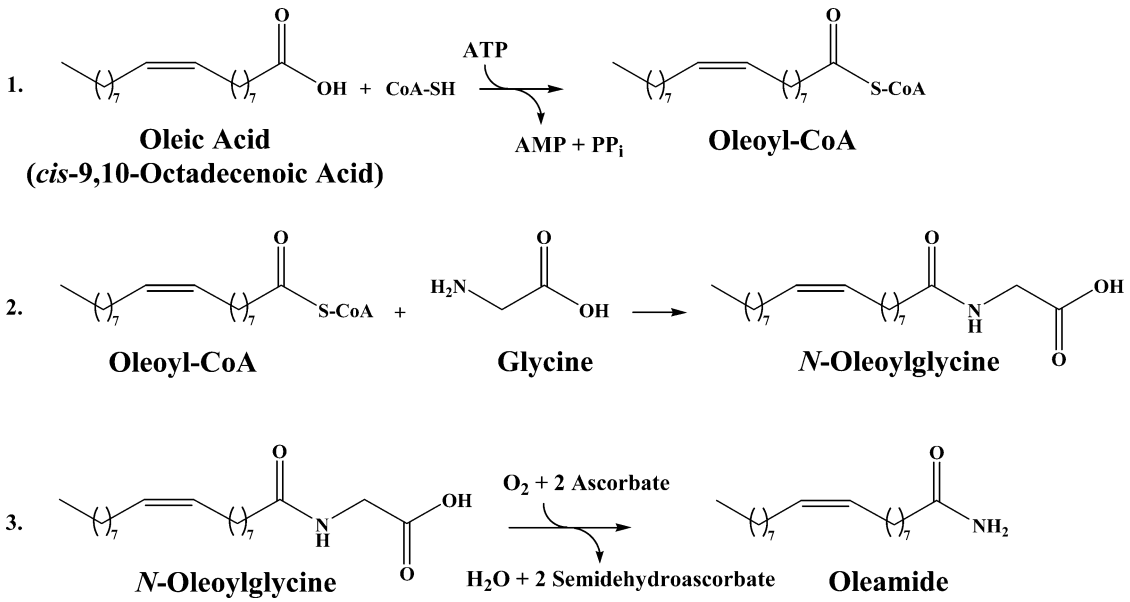

FIGURE 1: Proposed pathway for the biosynthesis of oleamide.

to modulate memory processes (*14*), to decrease body temperature and locomotor activity (*10*, *15*), to stimulate Ca^{2+} release (*16*), to modulate depressant drug receptors in the CNS (*17*, *18*), and to allosterically activate the $GABA_A$ receptor (*18*−*20*) and specific serotonin receptor subtypes (*21*, *22*). Oleamide may also inhibit cancer proliferation (*23*, *24*). PFAMs other than oleamide are bioactive. Linoleamide increases Ca^{2+} flux (*25*) and inhibits the erg current in pituitary cells (*26*). Erucamide is angiogenic (*27*) and regulates fluid imbalance (*28*). Last, Morisseau et al. (*29*) reported that elaidamide is an endogenous inhibitor of epoxide hydrolase (K_i = 70 nM), an enzyme implicated in emphysema, spontaneous abortion, and cancer. In sum, the PFAMs are an exciting class of newly emerging neuromodulatory lipids that may also have significant roles in regulating other mammalian processes.

The PFAMs are hydrolytically degraded by fatty acid amide hydrolase (FAAH): PFAM + H_2O → fatty acid + NH_3 (*7*, *30*). A pathway for the biosynthesis of the PFAMs is unknown. We proposed that the PFAMs were produced in three steps from fatty acids (*31*−*33*) by their initial conversion to acyl-CoA thioesters, reaction of the CoA thioesters with glycine to form the *N*-fatty acylglycines, and then oxidative cleavage of the *N*-acylglycines to the PFAMs and glyoxylate (*31*−*33*) (Figure 1). The ATP-dependent formation of the acyl-CoA thioesters (reaction 1 in Figure 1) is a well-established reaction that is catalyzed by acyl-CoA synthetase (*34*).

Peptidylglycine α-amidating monooxygenase, an enzyme involved in α-amidated neuropeptide hormone production in vi*vo* (*35*), catalyzes reaction 3 in Figure 1. There are data suggesting that *N*-fatty acylglycines are produced by the reaction of the acyl-CoA thioesters with glycine (reaction 2 in Figure 1). Huang et al. (*36*) identified *N*-arachidonoylglycine as an endogenous lipid conjugate found in the mammalian brain and showed that it was generated by the addition of arachidonate and glycine to rat brain membrane preparations. Subsequently, O'Byrne et al. (*37*) found that human bile acid-CoA:amino acid *N*-acyltransferase (BAT) will catalyze the formation of *N*-fatty acylglycines in vitro from the corresponding acyl-CoA thioesters and glycine, but with relatively low V/K values, the *V*/*K* for arachidonoyl-CoA being ∼5% of the value for the bile acid-CoA thioesters. However, human BAT is cytosolic (*37*), and Killenberg and Jordan (*38*) reported that rat BAT would not utilize either acetyl-CoA or palmitoyl-CoA as substrates. It remains to be determined if BAT is the enzyme responsible for *N*-fatty acylglycine synthesis in vivo. Nonetheless, the recent identification of *N*-arachidonoylglycine from mammals (*37*, *39*) and insects (*40*) indicates that long-chain *N*-fatty acylglycines are produced in biological systems and that there must be enzyme(s) responsible for their biosynthesis. The presence of *N*-arachidonoylglycine in the mammalian brain is consistent with our proposed PFAM biosynthetic pathway (Figure 1).

Mouse neuroblastoma $N_{18}TG_2$ cells are a model system for studying PFAM biosynthesis. These cells are known to convert oleic acid to oleamide and thus must contain the enzymatic machinery required to produce PFAMs (*41*). Furthermore, the $N_{18}TG_2$ cells express PAM in a regulated fashion; differentiation increases PAM expression 10−30-fold (*33*). We report here important new data regarding PFAM biosynthesis in the $N_{18}TG_2$ cells. We demonstrate that $N_{18}TG_2$ cell differentiation increases the metabolic flux of oleic acid to oleamide, that addition of *N*-oleoylglycine to $N_{18}TG_2$ culture medium generates oleamide, and that N_{18}-TG_2 cell growth in the presence of oleic acid and a PAM inhibitor results in the accumulation of *N*-oleoylglycine. This result represents the first report of *N*-oleoylglycine from a biological source. The data presented here strongly support the third step in the biosynthetic pathway outlined in Figure 1.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled *N*-oleoylglycine and oleamide were from Cayman Chemical, [1-^{14}C]-oleic acid (50 mCi/mmol) was from New England Nuclear, FBS was from Atlanta Biologicals, DMEM was from Mediatech Cellgro, 2.5S NGF was from Boehringer Mannhein, *trans*-4-phenyl-3-butenoic acid (PBA, also known as *trans*-styrylacetic acid, C_6H_5—

CH═CH−CH_2−COOH) was from Aldrich, BSTFA was from Acros Organics, and the mouse neuroblastoma $N_{18}TG_2$ cells were from DSMZ (Deutsche Sammlung von Mikrooganism und Zellkuturen GmbH). [*oleoyl*-1-^{14}C]-*N*-Oleoylglycine was synthesized from [1-^{14}C]-oleic acid using a published protocol (*32*). All other reagents and cell culture supplies were of the highest quality available from commercial suppliers.

[1-^{14}C]-Oleamide. Radiolabeled oleamide was synthesized from [1-^{14}C]-oleoyl chloride. [1-^{14}C]-Oleoyl chloride was prepared by mixing together 1 μCi of [1-^{14}C]-oleic acid, 0.18 mol of unlabeled oleic acid (58 mL), and 0.2 mmol of thionyl chloride (15 mL) and incubating the mixture with gentle stirring at 55 °C. After a 24 h incubation, the resulting mixture was evacuated in situ at 120 °C to remove any unreacted thionyl chloride. An aliquot of the crude [1-^{14}C]-oleoyl chloride (5 g, ~15 mmol) was added dropwise to 50 mL of NH_4OH (0.74 mol) and stirred for 10 min at room temperature. The mixture was extracted thrice with diethyl ether. The ether extracts were combined, dried with $MgSO_4$, and taken to dryness in vacuo. The [1-^{14}C]-oleamide was recrystalized from $CHCl_3$/hexane (1:1) to yield a white waxy solid. Compound verification was accomplished via ^{1}H NMR. [1-^{14}C]-Oleamide and [*oleoyl*-1-^{14}C]-*N*-oleoylglycine were prepared as standards for the development of HPLC separations.

Metabolism of Unlabeled Oleic Acid and N-Oleoylglycine by the $N_{18}TG_2$ Cells. The $N_{18}TG_2$ cells were grown in 25 cm^2 culture dishes containing DMEM supplemented with 1000 I.U./mL penicillin, 1.0 mg/mL streptomycin, 10% FBS, and 100 μM 6-thioguanine at 37 °C and 5% CO_2. Cultures were grown to 60% confluency, the culture medium was removed, and the cells were washed with PBS. After washing, the culture medium was replaced with either DMEM containing 0.5% FBS and either 200 μM oleic acid or *N*-oleoylglycine (undifferentiated cells) or DMEM containing 0.5% FBS, 0.2 μg/mL NGF, 1.0 mM dibutyryl cAMP, and either 200 μM oleic acid or 200 μM *N*-oleoylglycine (differentiated cells). The cells were then grown for 2 days at 37 °C and 5% CO_2 and then detached from the culture flasks using a cell scraper. The cells were collected by centrifugation (5 min at 250*g*). The cell pellets were flash frozen in a dry ice/methanol bath and stored at −80 °C until analyzed for oleic acid-derived metabolites.

To extract the metabolites derived from oleic acid or *N*-oleoylglycine, $N_{18}TG_2$ cell pellets were first thawed, 1.0 mL of chloroform/methanol (3:1) was added to the thawed pellets, and the mixture was sonicated for 10 min. The mixture was centrifuged (5 min at 250*g*) to remove cellular debris, and the organic solvent was removed. The entire process was repeated twice, and the three organic extracts were combined and taken to dryness in vacuo. The extract was silylated in toluene with BSTFA as described (*42*) and analyzed by GC-MS.

Metabolism of [1-^{14}C]-Oleic Acid by the $N_{18}TG_2$ Cells in the Presence and Absence of 4-Phenyl-3-butenoic Acid (PAM Inactivator). The $N_{18}TG_2$ cells were grown to 75% confluency at 37 °C and 5% CO_2 in 25 cm^2 culture dishes containing DMEM supplemented with 10% FBS and 100 μM 6-thioguanine. After reaching 75% confluency, the spent culture medium was removed, the cells were washed with PBS, the medium was replaced with DMEM containing 10% FBS, 100 μM 6-thioguanine, 0.04 μCi of [1-^{14}C]-oleic acid, and either 0 or 1.0 mM PBA, and the cells were grown for 16 h at 37 °C and 5% CO_2. After the 16 h incubation in the presence of [1-^{14}C]-oleic acid with and without PBA, 100 μM PMSF was added to the culture medium to inhibit any proteolytic or FAAH-mediated hydrolysis of the [^{14}C]-*N*-oleoylglycine. After an additional 1 h incubation post-PMSF addition, the cells were removed from the incubator and prepared for extraction. The bioavailability of PBA is low, and the concentration of PBA used for these experiments (1.0 mM) is based on the work of Bradbury et al. (*43*).

The culture medium was removed, the cells were washed with PBS, and the wash was discarded. Fresh PBS was added to the washed cells (25 mL/25 cm^2 dish), and the cells were detached from the culture flasks using a cell scraper. The cell suspensions were collected, an equal volume of ethyl acetate/hexane (1:1) was added, the mixture was sonicated for 20 min, and the organic layer was removed. The remaining aqueous layer was reextracted twice with ethyl acetate/hexane (1:1), and the three organic extracts were combined. The collected organic extracts were taken to a cloudy oil in vacuo at room temperature. The resultant oily product was dissolved in acetonitrile (5 mL), filtered through a 0.25 μm glass filter, and analyzed by HPLC or ESI-MS.

HPLC Separation of Oleic Acid, N-Oleoylglycine, and Oleamide. HPLC assays were performed on a Hewlett-Packard 1090L liquid chromatography system with a Phenomenex Luna C_8(2) column (250 × 4.6 mm, 5 μm particles, 100 Å pore size). HPLC method A consisted of a column temperature of 32 °C and the following binary solvent elution parameters (A = acetonitrile and B = H_2O, flow rate = 1.0 mL/min): a linear gradient of A/B of 70:30 to A/B of 95:5 over 10.0 min followed by isocratic elution for 10.0 min. HPLC method B was the same except that the column temperature was 40 °C, the flow rate was 1.25 mL/min, and the gradient was A/B of 75:25 to A/B of 95:5 over 5 min followed by isocratic elution for 5 min. Analytes were detected by absorbance at 205 nm. Peaks were identified by comparison to the retention times of authentic standards.

[^{14}C]-Labeled analytes were detected using a Bertold LB 509 flow-through, dual-photomultiplier radioflow detector. All column eluent from the HPLC was directed to a mixing cell (T-mixer with a 1.0 mL volume), where it was mixed with Fisher Scintiverse II scintillation fluid. The scintillation fluid was delivered to the mixing cell at 2.7 mL/min using a separate pump. The column eluant/scintillation mixture was then pumped through the flow-through counting cell of the detector.

GC-MS. All separations were performed using a Varian 340 gas chromatograph coupled to a Varian Saturn II ion trap mass spectrometer (GC-MS). Separations were achieved on a Supelco Simplicity-5 column (0.25 mm × 30 m, PDMS 5% phenyl) that was situated between a deactivated capillary guard column and a deactivated capillary transfer line (both 0.25 mm × 5 m). The GC temperature program was 55−150 °C at 40 °C/min, hold at 150 °C for 3.6 min, ramp at 10 °C/min to 300 °C, and hold for 6 min. The transfer line was held at 280 °C and the injection port at 250 °C throughout the separation. Helium with a head pressure of 12.5 psi was the carrier gas. The injection volume was 1 μL unsplit. The ion trap was operated using an electron impact ionization energy of 70 eV. The ion trap was run at four scans per

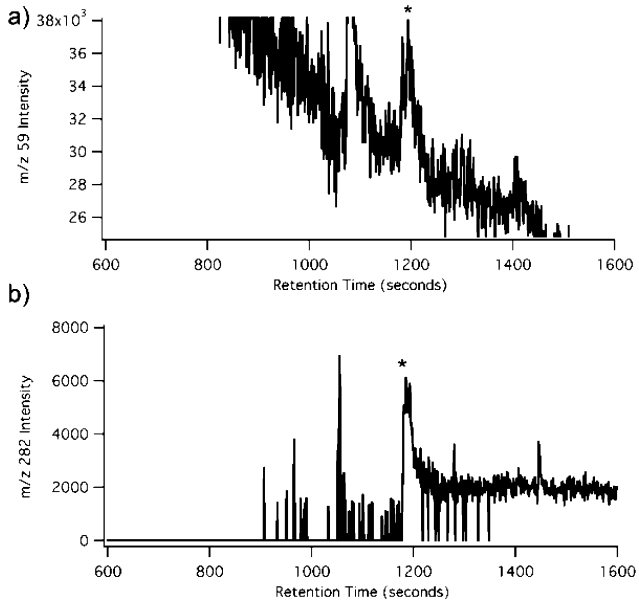

FIGURE 2: Production of oleamide from oleic acid in undifferentiated $N_{18}TG_2$ cells. Selected ion gas chromatograms at $m/z = 59$ (A) and at $m/z = 282$ (B) of chloroform/methanol extracts of undifferentiated $N_{18}TG_2$ cells grown in the presence of exogenously added oleic acid. The m/z 59 fragment resulted from McLafferty-like rearrangements of trimethylsilyated oleamide ($\bullet CH_2{-}C(=OH^+){-}NH_2$) (*42*). The m/z 282 fragment represents the M-71 charged-remote fragmentation radical (loss of pentyl fragment; FW of trimethylsilylated oleamide = 353.7 g/mol, $\bullet CH_2{-}(CH_2)_2{-}CH{=}CH{-}(CH_2)_7{-}C(=O^+){-}NH{-}Si{-}(CH_3)_3$). The asterisk (*) indicates mass spectral signature ions for oleamide.

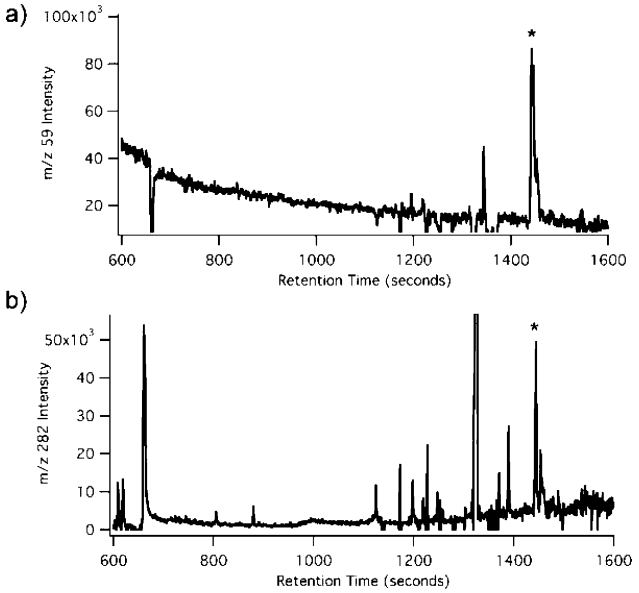

FIGURE 3: Production of oleamide from oleic scid in fifferentiated $N_{18}TG_2$ cells. Selected ion gas chromatograms at $m/z = 59$ (A) and at $m/z = 282$ (B) of chloroform/methanol extracts of differentiated $N_{18}TG_2$ cells grown in the presence of exogenously added oleic acid, NGF, and dibutyryl cAMP. The asterisk (*) indicates mass spectral signature ions for oleamide. Other details are as described in the legend to Figure 2. The differences in retention times for the ions shown in this figure relative to those of Figure 2 resulted from the use of a new GC column (same supplier and model, see Experimental Procedures). The ions correspond to those seen for an oleamide standard using the new Supelco Simplicity-5 column.

second (averaged to yield one spectrum per second) at a temperature of 220 °C. The mass scan range was 50−350 amu. Two different Supelco Simplicity-5 columns were used to generate the data reported here, and each gave slightly different retention times for the molecular ions generated from oleamide. Oleamide standards were run on each column to account for this difference.

ESI-MS. Mass spectra in the negative ion mode were generated using a Bruker Esquire spectrometer. The mass scan range was 100−600 amu. Samples were injected automatically from a 100 μL glass syringe at a rate of 60 μL/h.

RESULTS AND DISCUSSION

Conversion of Oleic Acid to Oleamide by Undifferentiated and Differentiated $N_{18}TG_2$ Cells. Overnight incubation of mouse neuroblastoma $N_{18}TG_2$ cells with [^{14}C]-oleic acid resulted in the production of [^{14}C]-oleamide (*41*). Consequently, the $N_{18}TG_2$ cells must contain the enzymes responsible for oleamide biosynthesis. We demonstrated that the $N_{18}TG_2$ cells express PAM and that differentiation of these cells by growth in low serum medium containing NGF and cAMP induces a 10−30-fold increase in both PAM activity and expression of the PAM protein (*33*). Consequently, the flux of oleic acid to oleamide would be expected to be greater in differentiated $N_{18}TG_2$ cells than in undifferentiated cells if our proposal that PAM has a role in PFAM biosynthesis is valid. As shown in Figure 2, oleamide levels were low when oleic acid was added to the culture medium for undifferentiated $N_{18}TG_2$ cells. The low percent conversion (∼0.05−0.10%) of [^{14}C]-oleic acid to [^{14}C]-oleamide using these undifferentiated cells is consistent with that of Bisogno et al. (*41*). In contrast, the oleamide levels were greater when oleic acid was added to the culture medium for differentiated $N_{18}TG_2$ cells (Figure 3).[2] The amount of oleamide produced from oleic acid in the differentiated cells relative to that produced in the undifferentiated cells (about an 8-fold increase; compare scales in Figures 2 and 3) was consistent with the presumed 10−30-fold increase in PAM upon differentiation (*33*), assuming that the oleamide extraction efficiency was the same for the two experiments. In the absence of added oleic acid, no oleamide was detectable in extracts from either undifferentiated or differentiated $N_{18}TG_2$ cells.

The experiments leading to the data of Figures 2 and 3 provided no information regarding the formation of *N*-oleoylglycine, as they were designed only to examine oleamide biosynthesis from exogenously added oleic acid. Only oleic acid and oleamide were found upon HPLC analysis of a hexane/ethyl acetate (1:1) extract from undifferentiated $N_{18}TG_2$ cells grown in the presence of [^{14}C]-oleic acid (Figure 4). We chose the undifferentiated $N_{18}TG_2$ cells for these experiments in an attempt to increase the levels of [^{14}C]-*N*-oleoylglycine because these cells express lower levels of *N*-oleoylglycine-consuming PAM (*33*). No *N*-oleoylglycine was observed in separate experiments examined using different HPLC separation protocols (in the downward arrows

[2] The m/z 282 peak is diagnostic for the increase in amide production in this case, as the calibration curve is linear in this range of mass loads. The m/z 59 peak is nonlinear and saturates in regions where there is a substantial m/z 282 peak, which accounts for the lack of substantial increase in the m/z 59 peak for differentiated cells.

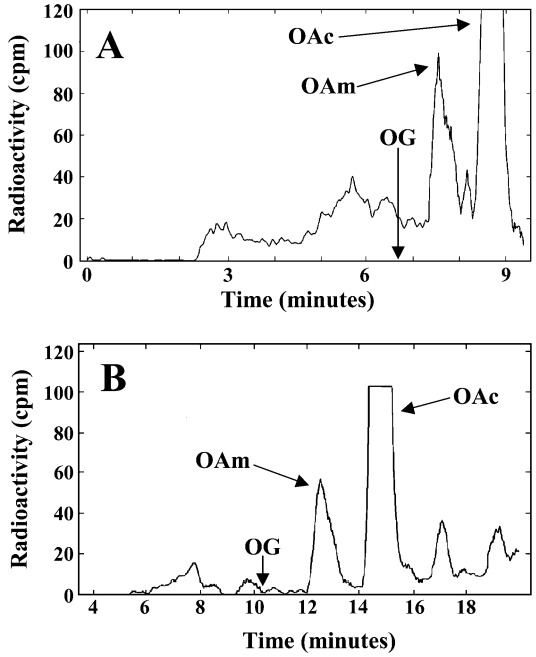

FIGURE 4: Production of [^{14}C]-oleamide from [^{14}C]-oleic acid in undifferentiated $N_{18}TG_2$ cells. HPLC chromatograms of ethyl acetate/hexane extracts of undifferentiated $N_{18}TG_2$ cells grown in the presence of exogenously added [1-^{14}C]-oleic acid. The organic extracts were analyzed for oleamide and *N*-oleoylglycine production using two different HPLC separation methods, HPLC method A in panel A and HPLC method B in panel B (see Experimental Procedures for details). Peaks were identified vs standards run immediately following this analysis, OG = *N*-oleoylglycine, OAm = oleamide, and OAc = oleic acid. The vertical arrows indicate the retention time for *N*-oleoylglycine, 6.7 min in A and 10.4 min in B.

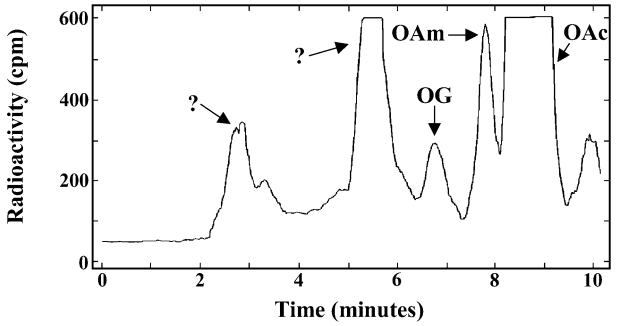

FIGURE 5: Production of [^{14}C]-*N*-oleoylglycine from [^{14}C]-oleic acid in undifferentiated $N_{18}TG_2$ cells grown in the presence of PBA (a PHM inactivator). HPLC chromatograms of ethyl acetate/hexane extracts of undifferentiated $N_{18}TG_2$ cells grown in the presence of exogenously added [1-^{14}C]-oleic acid and PBA. The organic extracts were analyzed for oleamide and *N*-oleoylglycine production using HPLC method A. Peaks were identified vs standards run immediately following this analysis, OG = *N*-oleoylglycine, OAm = oleamide, and OAc = oleic acid. Two unidentified peaks are indicated with the question marks (?).

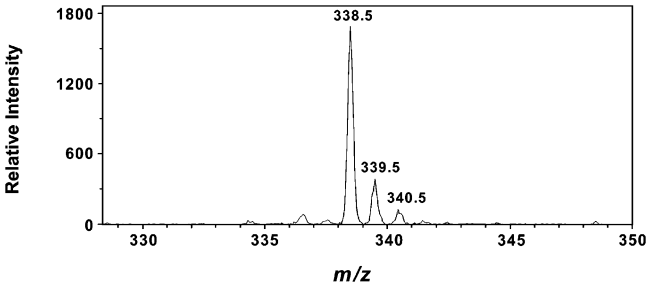

FIGURE 6: Mass spectrum of *N*-oleoylglycine isolated from undifferentiated $N_{18}TG_2$ cells grown in the presence of [1-^{14}C]-oleic acid. Ethyl acetate/hexane extract of undifferentiated $N_{18}TG_2$ cells grown in the presence of exogenously added [1-^{14}C]-oleic acid and PBA infused into a Bruker Esquire Electrospray-Ion Trap mass spectrometer. The mass spectrum represents a summation of 10 spectra collected in the negative ion mode.

in Figure 4A,B). Either *N*-oleoylglycine was not formed or the amounts produced were below the level of detection.

The *N*-oleoylglycine concentration could be low and difficult to detect as an intermediate in the bioconversion of oleic acid to oleamide. For example, the rate of conversion of *N*-oleoylglycine to oleamide could be higher than the conversion rate of oleoyl-CoA to *N*-oleoylglycine, resulting in a low steady-state concentration of the glycinated intermediate. Detailed studies of substance P and gastrin biosynthesis, two α-amidated peptide hormones, showed that the cellular levels of the glycine-extended precursors were only 2−4% of that measured for the mature α-amidated hormones (*44*, *45*). Our in vitro data indicate that the *V*/*K* for the PAM-catalyzed amidation of *N*-oleoylglycine (22 M^{-1} s^{-1}) is comparable to that measured for the glycine-extended precursors of the α-amidated peptide hormones (*32*). Other, as yet undefined, metabolic fates for *N*-oleoylglycine (such as protease degradation to the oleic acid and glycine) would further decrease its steady-state concentration. The fact that *N*-arachidonoylglycine has only recently been identified as a metabolite in mammals (*36*, *39*) and insects (*40*) suggests that the cellular, steady-state concentrations of the long-chain *N*-fatty acylglycines are low.

Conversion of Oleic Acid to N-Oleoylglycine in Undifferentiated $N_{18}TG_2$ Cells Treated with a PAM Inhibitor. If the steady-state concentration of *N*-oleoylglycine is low and its major route of metabolic disappearance is the PAM-mediated conversion to oleamide, inhibition of PAM in the undifferentiated $N_{18}TG_2$ cells should result in the accumulation of *N*-oleoylglycine. A peak with the same retention time as *N*-oleoylglycine was present in the HPLC radiochromatogram of an organic extract from undifferentiated $N_{18}TG_2$ cells incubated with exogenously added [^{14}C]-oleic acid and PBA (Figure 5). PBA is a suicide substrate for PAM (*43*, *46*) known to decrease PAM activity in cultured cells (*43*, *47*, *48*). PMSF was added to inhibit the FAAH-mediated hydrolysis of oleamide but also may have been beneficial for the accumulation of [^{14}C]-*N*-oleoylglycine, as PMSF would also inhibit any serine protease-mediated cleavage of *N*-oleoylglycine to glycine and oleic acid. Two additional unidentified peaks (indicated with question marks) were present in the radiochromatogram of Figure 5. These represent either further metabolites of [^{14}C]-*N*-oleoylglycine that are not produced (or detectable) when the cellular levels of *N*-oleoylglycine are low or are other unknown metabolites of [^{14}C]-oleic acid that formed as a consequence of reactions stimulated by PBA. We have not explored this question further.

To provide more definitive data on the identification of *N*-oleoylglycine, we used ESI-MS to assay for the presence of [^{14}C]-*N*-oleoylglycine in an organic extract of undifferentiated $N_{18}TG_2$ cells grown in the presence of [^{14}C]-oleic acid and PBA. The negative ion mode ESI-MS spectrum is shown in Figure 6. The family of ions in Figure 6 is consistent with

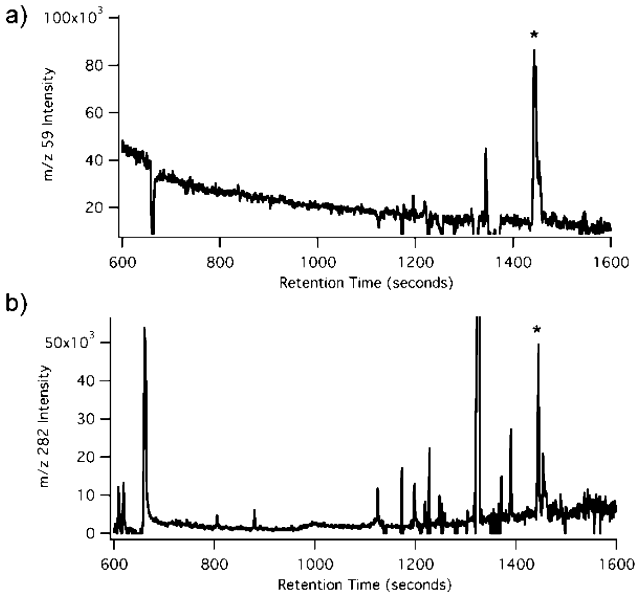

FIGURE 7: Production of oleamide from *N*-oleoylglycine in differentiated $N_{18}TG_2$ cells. Selected ion gas chromatograms at $m/z = 59$ (A) and at $m/z = 282$ (B) of chloroform/methanol extracts grown in the presence of exogenously added *N*-oleoylglycine, NGF, and dibutyryl cAMP. The asterisk (*) indicates mass spectral signature ions for oleamide. Other details are as described in the legend to Figure 2.

the formation of [oleoyl-1-^{14}C]-*N*-oleoylglycine. The intensity of the $[M + 2 - H]^-$ ion ($m/z = 340.5$) should be 3.0% of that for the $[M - H]^-$ ion ($m/z = 338.5$) based on the natural abundance of the carbon, nitrogen, hydrogen, and oxygen isotopes in *N*-oleoylglycine. In contrast, the intensity of the $[M + 2 - H]^-$ ion is enriched to ~7% of that for the $[M - H]^-$ ion for the [^{14}C]-*N*-oleoylglycine produced by the $N_{18}TG_2$ cells (Figure 6). The specific radioactivity of the [^{14}C]-oleic acid added to the culture medium was 50 mCi/mmol, meaning that 74% of the oleic acid molecules contained a ^{14}C atom. Incorporation of ~5% of the exogenously added [^{14}C]-oleic acid into the [^{14}C]-*N*-oleoylglycine by the $N_{18}TG_2$ cells would yield the observed enrichment in $[M + 2 - H]^-$ ion observed in Figure 6. Two controls indicate that the enrichment in $[M + 2 - H]^-$ ion is genuine. Incorporation of ^{14}C into the *N*-oleoylglycine should not significantly enrich the $[M + 1 - H]^-$ ion. The intensity of the $[M + 1 - H]^-$ ion in Figure 6 versus that for the $[M - H]^-$ ion (22.6%) is in good agreement with the expected value based on natural isotopic abundances, 22.5%.[3] In addition, the $[M + 2 - H]^-$ ion for the [^{14}C]-*N*-oleoylglycine synthesized as described in the Experimental Procedures (containing only ~2×10^{-5}% ^{14}C atoms) was 3.5%, in reasonable agreement with expected 3.0% value based on natural isotopic abundances and the mass spectrum of standard (commercially) available *N*-oleoylglycine (data not shown)[3]. Our characterization of *N*-oleoylglycine from the $N_{18}TG_2$ cells treated with a PAM inhibitor is the first report of this molecule being produced in a biological system and is consistent with our proposal that *N*-oleoylglycine is an intermediate in the biosynthesis of oleamide from oleic acid (*31*, *32*).

Conversion of N-Oleoylglycine to Oleamide in Differentiated $N_{18}TG_2$ Cells. We have previously demonstrated in vitro that *N*-oleoylglycine is a substrate for purified PAM, being converted to oleamide and glyoxylate (*32*). The work reported here, coupled with earlier work, indicates that the $N_{18}TG_2$ cells metabolize exogenously added oleic acid to *N*-oleoylglycine or oleamide (*41*) and express PAM in a regulated fashion (*33*). However, there is no direct evidence that these cells will convert *N*-oleoylglycine to oleamide. Because PAM expression is higher in the differentiated $N_{18}TG_2$ cells, we incubated *N*-oleoylglycine with these cells to assay for oleamide production. Incubation of $N_{18}TG_2$ cells in culture medium containing *N*-oleoylglycine resulted in oleamide production (Figure 7). The level of oleamide production from *N*-oleoylglycine is ~2-fold higher than that produced from oleic acid (compare Figure 3 to Figure 7). This difference could simply be a consequence of higher bioavailability of the *N*-oleoylglycine relative to oleic acid or may indicate that the rate of oleamide formation from *N*-oleoylglycine is faster than the rate of *N*-oleoylglycine formation from oleic acid.

CONCLUSIONS

Merkler et al. (*31*) first proposed that oleamide and the other fatty acid primary amides were produced from the corresponding fatty acids in three steps with the acyl-CoA thioesters and the *N*-fatty acylglycines as intermediates (Figure 1). Herein, we provide strong evidence for the third step in the proposed pathway by showing that an increased expression of PAM in differentiated $N_{18}TG_2$ cells results in an increased flux of oleic acid to oleamide, that inhibition of PAM in the $N_{18}TG_2$ cells results in the accumulation of *N*-oleoylglycine, and that these cells metabolize *N*-oleoylglycine to oleamide. Our report of *N*-oleoylglycine from the $N_{18}TG_2$ cells is the first report of this fatty acid glycine conjugate from a biological system. An understanding of the enzymes responsible for PFAM biosynthesis are likely to lead to innovative strategies for the diagnosis and treatment of neurological dysfunction (sleep disorders, anxiety, depression, and suicidal behavior) and for other human diseases.

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[3] Natural abundance for ^{14}C is 1.12×10^{-10}%. While our synthesis of [^{14}C]-*N*-oleoylglycine containing ~2×10^{-5}% ^{14}C atoms is a ~2×10^5-fold enrichment over background, the low abundance of ^{14}C atoms in the synthetic [^{14}C]-*N*-oleoylglycine will not significantly alter the intensity of $[M + 2 - H]^-$ ion.

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