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Long-chain fatty acids increase cellular dopamine in an immortalized cell line (MN9D) derived from mouse mesencephalon

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Abstract

The lysate of an immortalized monoclonal cell line derived from the striatum (X61) contains a dopaminergic stimulatory activity that is capable of increasing the dopamine content of an immortalized mouse mesencephalic cell line (MN9D) which expresses a dopaminergic phenotype. Purification of an isoamyl alcohol extract of this lysate and subsequent identification by NMR spectroscopic analysis demonstrated that the dopaminergic stimulatory activity contained within the lysate was a mixture of 80–90% *cis*-9-octadecenoic acid (oleic acid) and 10–20% *cis*-11-octadecenoic acid (*cis*-vaccenic acid). The effect of oleic acid on MN9D dopamine is a prolonged event. MN9D dopamine increases linearly over a 48 h period suggesting the induction of an increased dopaminergic phenotype in these dividing cells. The ability to increase MN9D dopamine by oleic and *cis*-vaccenic acids is shared by a number of other long-chain fatty acids including arachidonic, linoleic, linolenic, palmitoleic, and *cis*-13-octadecenoic acid. The possibility that oleic or other relatively innocuous fatty acids might affect dopaminergic function in primary neurons is intriguing with respect to possible therapeutic approaches to the treatment of dopaminergic cell loss and the motor sequelae of Parkinson's disease.

Keywords: Dopamine; Fatty acids; MN9D; Nigrostriatal; Parkinson's disease; Trophic factor

Immortalized monoclonal cells of the mouse nigrostriatal projection have been developed as an approach to the identification of substances which could regulate dopaminergic function and cell survival [1,14]. The dopaminergic MN9D cell line of mesencephalic origin and the X61 cell line of striatal origin were obtained by somatic cell fusion with the N18TG2 neuroblastoma which is lacking the hypoxanthine phosphoribosyltransferase enzyme [1,14]. We have previously demonstrated that striatal-derived monoclonal cells (X61) contain dopaminergic stimulatory substances which increase the dopamine content of MN9D cells [3]. Striatal cell lines (X61) provide a source for such substances and the mesencephalic-derived MN9D cell line provides a rapid assay method for detecting active molecules capable of modulating cellular dopamine. The crude cell lysate of X61 cells, as well as a partially purified ultrafiltrate preparation (UF4) of that lysate, also increases the dopamine content of primary dopaminergic neurons grown in reaggregate culture in the absence of target cells (i.e., mesencephalic cells co-cultured with tectum, a non-target region for dopaminergic neurons) as well as levels of homovanillic acid in the culture medium [15]. In such cultures, in which the majority of dopaminergic neurons are lost due to the absence of target cells, treatment with the crude lysate or UF4 ultrafiltrate results in a 2- (UF4) to 2.9- (X61 lysate) fold increase in the density of dopaminergic neurons in the treated cultures [15].

The UF4 ultrafiltrate contains active substances, probably peptides, of low molecular weight and high water solubility. It was, however, apparent that the bulk (two-thirds) of

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dopaminergic stimulatory activity present in the processed X61 cells resided in a fraction which did not pass through a YM-5 ultrafiltration membrane and was lipid soluble. The present study was conducted to determine the chemical nature and activity of this major fraction.

The UF4 ultrafiltrate was obtained from sonicated lysates of X61 cells which were allowed to "autodigest" at room temperature for 2 days and concentrated by pressure filtration through an Amicon YM-5 membrane (5000 Da molecular weight cut-off). Some form of digestion occurs in this process as evidenced by the fact that 2 days of incubation at room temperature results in the conversion of some (approximately 30%) of the dopaminergic stimulatory activity in the X61 cell lysate from a high molecular weight form to a size that can pass through a YM-5 membrane (see [15] for details).

The low molecular weight UF4 ultrafiltrate fraction contained significant dopaminergic stimulatory activity as assessed by effects on MN9D cells. However, the majority of the activity from the "autodigested" X61 cell lysate did not pass through the YM-5 ultrafiltration membrane. Approximately two-thirds of the activity resided in the material remaining on the high molecular weight side of the Amicon YM-5 membrane and is referred to as "X61 concentrate". This X61 concentrate was subsequently extracted with 2 M NaCl followed by a 1:1 mixture of isoamyl alcohol/chloroform. The isoamyl alcohol/chloroform extract was shown to contain materials capable of increasing MN9D dopamine levels. This activity is not extractable from fresh X61 cell lysate, but appears to require the autodigestion step with time for the activity to become liberated from some cell component and be available for organic extraction.

The isoamyl alcohol/chloroform soluble stimulatory activity was taken up by a C18 reverse phase column from a mixture of 70% acetonitrile/30% (0.05% trifluoroacetic acid in water) and then eluted by a linear gradient from the mixture to 100% acetonitrile. The active fractions from the column showed some absorbance at 215 nm, but the bulk of absorbance was seen in fractions devoid of activity, suggesting that the reverse phase separation resulted in considerable purification of the activity. The active fractions from the reverse phase column were then applied to a Phenomenex 5 μm, 50 Å Phenogel gel filtration sizing column. The dopaminergic stimulatory activity eluted from the Phenogel column in 100% acetonitrile within a single absorbance peak. The Phenogel fractions containing dopaminergic stimulatory activity were subjected to mass spectrographic analysis. Two peaks of high intensity were observed with molecular weights of 283 and 565.

NMR spectroscopy demonstrated that the single elution peak from the Phenogel column contained two chemical moieties, the greater of which constituted approximately 80–90% of the material (Fig. 1). The 600 MHz ^{1}H 1D NMR spectrum of the sample showed one major set of peaks and no significant minor peaks, suggesting a sample purity greater than 95%. The ^{13}C 1D NMR spectrum, however, showed two sets of peaks, indicating that the sample contained two species

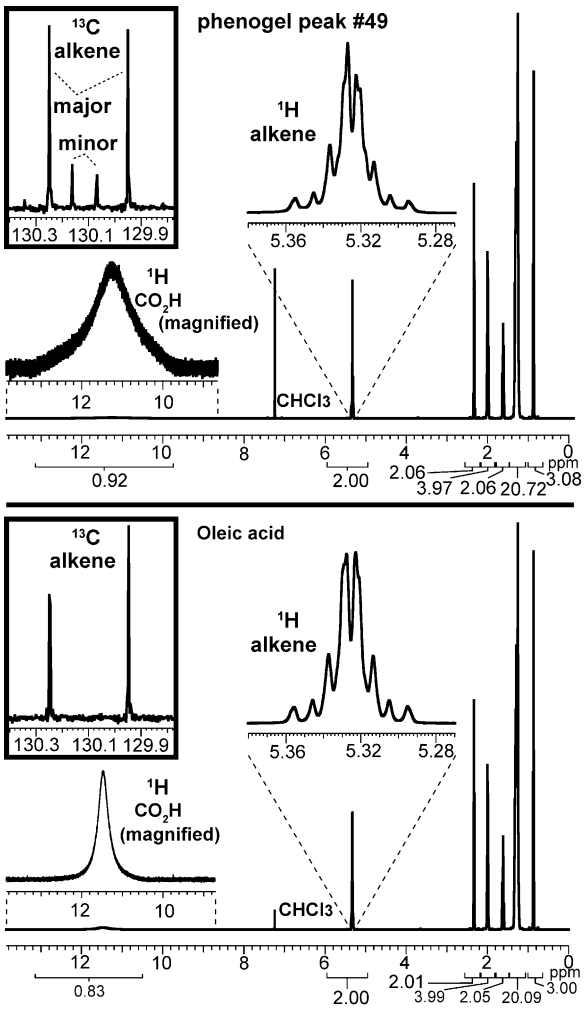

Fig. 1. The NMR spectra of peak #49 from the Phenogel column purified material (top) as compared to commercial oleic acid (bottom). The ^{1}H spectrum is shown at the bottom of each panel, and the peak integral values of the intensities are indicated below the axes. The carboxylic acid and alkene regions of the ^{1}H spectra are magnified and expanded. The alkene regions of the ^{13}C spectra are shown as insets. It is apparent that the Phenogel #49 sample contains one major and one minor species. The minor species was identified by NMR analysis to be *cis*-vaccenic acid.

so closely related that they gave rise to virtually identical ^{1}H NMR spectra. The minor component made up approximately 10–20% of the total sample, according to their relative intensities in the ^{13}C spectra. The positions of the ^{1}H and ^{13}C peaks in these spectra ruled out the possibility that the sample contains protein, peptide, DNA, RNA, carbohydrate, glycolipid, steroid, or other cholesterol-related molecules. The positions are instead consistent with those expected from a fatty acid. Comparison of the major species' spectra with ^{1}H and ^{13}C NMR spectra of various fatty acids showed that they were very similar to those of *cis*-9-octadecenoic acid (oleic acid). It remained uncertain whether the unsaturated bond was of *cis* or *trans* configuration. Pure *cis*-9-octadecenoic acid and *trans*-9-octadecenoic acid (elaidic acid) (Aldrich), and their ^{1}H and ^{13}C NMR spectra were compared to those of the sam-

ple. We found that the major species' peaks were essentially identical to those of the *cis* isomer (i.e., oleic acid) (Fig. 1).

A variety of compounds structurally related to oleic acid were obtained and examined by NMR spectroscopy to determine the identity of the minor compound in the sample. We found that the ^{1}H and ^{13}C NMR spectra of *cis*-vaccenic acid (*cis*-11-octadecenoic acid), which has the same empirical formula as oleic acid, were essentially identical to those of the minor species in the sample. Thus, our NMR data support the conclusion that the purified sample comprises a mixture of approximately 80–90% *cis*-oleic acid and 10–20% *cis*-vaccenic acid.

The NMR spectral analysis with known synthetic entities established the chemical composition of the majority of the purified material to be *cis*-9-octadecenoic acid (oleic acid). The mass spectroscopic analysis is in accord with the NMR data in that the molecular weight of 283 corresponds to that of oleic acid and/or *cis*-vaccenic acid and the larger sized 565 molecular weight species may well represent a dimerization of these long-chain fatty acids.

In order to determine whether the Phenogel purified material and synthetic oleic acid produce similar effects on MN9D cellular dopamine, MN9D cells were plated into six-well culture plates and cultured in Dulbecco's Modified Eagle's medium containing 5% (v/v) Fetal Clone III and 1% (v/v) penicillin–streptomycin (5000 units penicillin/5000 μg streptomycin). The cells were exposed to increasing concentrations of oleic acid or the Phenogel purified material, diluted in dimethylsulfoxide (DMSO), for 48 h and then collected for analysis of cellular dopamine content using high performance liquid chromatography. Protein content of the cultures was determined spectrophotometrically [11].

In agreement with the NMR data, the Phenogel purified material and synthetic oleic acid showed identical concentration–response in terms of increasing MN9D dopamine as shown in Fig. 2. The concentration–response of MN9D cells to oleic acid has been repeated in two other experiments and the results obtained were identical. Exposure of MN9D cells to concentrations of oleic acid or the Phenogel purified material greater than 124 μM produced either less of a dopaminergic stimulatory effect or was actually toxic to the cells. Thus, the response at 124 μM was considered to be maximal. The maximal effect of oleic acid and the Phenogel purified material in this experiment represents an approximate five-fold increase in dopamine level over controls. The EC_{50} for oleic acid is approximately 5.5×10^{-5} M. The effects of oleic acid are not secondary to either an increase in MN9D cell proliferation or differentiation. This issue was tested directly in a separate experiment in which dopamine levels, cell number and the state of cell differentiation were examined. MN9D cells were exposed to DMSO or 124 μM oleic acid for 48 h. An increase in dopamine of 8.3-fold was observed in this experiment. The protein content of MN9D cells exposed to oleic acid was essentially identical to that of the DMSO group and the number of cells in the oleic acid-treated group (0.89 ± 0.13 million cells, mean ± S.E.M., $n = 6$) was not significantly different from DMSO vehicle (1.05 ± 0.10 million cells, mean ± S.E.M., $n = 6$). The oleic acid-exposed cells showed none of the characteristics of differentiated MN9D cells, i.e. a reduction in cell number or increased process outgrowth [1].

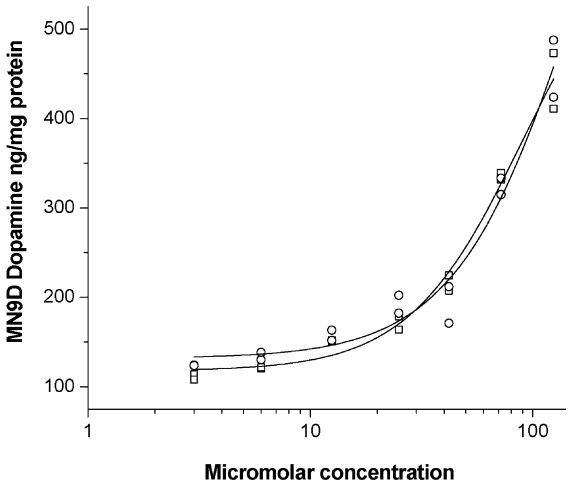

Fig. 2. Effect of 48 h exposure to increasing (log scale) concentrations (3–124 μM) of *cis*-9-octadecenoic acid (oleic acid, squares) or to the Phenogel column purified material from X61 cells (circles) on MN9D dopamine levels. MN9D dopamine level of DMSO vehicle control = 109.9 ng/mg protein.

Given that the assays were carried out in serum-containing medium and the known capacity of oleic acid to bind to serum proteins [8,12], the free EC_{50} for oleic may be considerably lower than this estimate. The level of oleic acid required to increase dopamine levels under serum-free conditions cannot be tested with MN9D cells since they do not grow well under conditions of low serum or serum-free medium. The MN9D cells, in addition, only permit the examination of effects on the catecholaminergic phenotype. The issue of whether oleic acid affects other transmitter phenotypes will require primary neuronal cultures.

While *cis*-vaccenic acid, the minor constituent of the Phenogel purified fraction, is active (see below), a comparison of the concentration–response curve of *cis*-vaccenic with oleic acid on MN9D dopamine, suggests that *cis*-vaccenic acid has a slightly lower potency.

A comparison of the effect of oleic versus the *trans* isomer (elaidic acid), using 11 concentrations ranging from 0.3 to 353 μM, on MN9D dopamine revealed that elaidic acid showed only minimal activity (less than 10% elevation in dopamine even at the highest concentration of 353 μM) (data not shown).

A number of more detailed studies have been initiated on the effect of oleic acid in increasing MN9D dopamine. The first of these studies, a time course on the effect of the Phenogel purified material and oleic acid, demonstrates a linear increase in MN9D dopamine over 48 h (Fig. 3). The effect of both the known compound and the Phenogel purified material in this experiment is impressive, resulting, after 48 h, in at

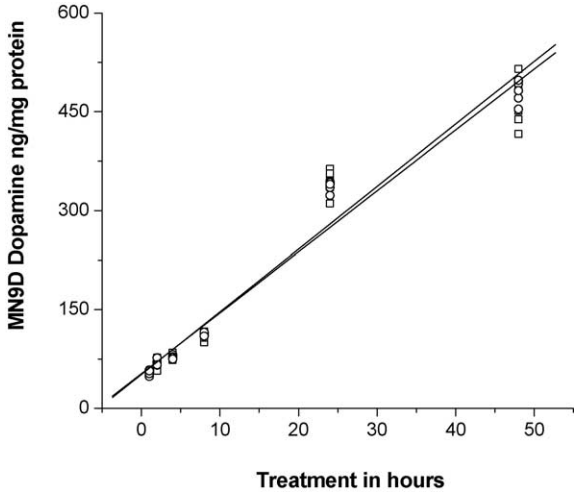

Fig. 3. Time course of the effect of exposing MN9D cells to 124 μM of *cis*-9-octadecenoic acid (oleic acid, squares) or 124 μM of the Phenogel column purified material from X61 cells (circles) on cellular dopamine levels. $N = 6$ cultures per time point. MN9D dopamine level (ng/mg protein) of DMSO vehicle control for the various time points: 1 h, 54.1; 2 h, 63.3; 4 h, 63.6; 8 h, 65.3; 24 h, 77.3; 48 h, 67.5.

least an 8.5-fold increase in MN9D dopamine as was seen in the experiment on cell proliferation described above. Given that the MN9D cells, which are fusion products of mesencephalic cells and the N18TG2 neuroblastoma, are doubling every 24 h, this result would suggest that the effect of the active chemical is to induce an increase in the dopaminergic phenotype of MN9D cells. If that is the case, then the effect should be present in the daughter cells as they appear.

As part of this study, a limited structure–activity analysis was conducted using a variety of common long-chain unsaturated fatty acids. It is clear from this study, that the ability of oleic acid to increase MN9D dopamine content over a 48 h period is shared by a number of other long-chain fatty acids containing one to four double bonds, as shown in Table 1. Concentration–response curves were determined for each of the indicated compounds and compared to oleic acid using seven concentrations over a range of 3–124 μM. The majority of the compounds tested were active, but in most cases, a maximal response was not obtained even at 124 μM. For this reason, Table 1 provides the cellular MN9D dopamine level (ng/mg protein) following 48 h of treatment with 124 μM of each respective compound, and the amount of increase this represents over the DMSO vehicle control.

Table 1
Effect of unsaturated long-chain fatty acids on MN9D cellular dopamine

Compound	Dopamine (ng/mg protein)	Fold-increase over DMSO control
Oleic acid	332	4.8
Arachidonic acid	365	5.3
Linoleic acid	300	4.3
Linolenic acid	297	4.3
Palmitoleic acid	280	4.0
cis-Vaccenic acid	264	3.8
cis-13-Octadecenoic acid	134	1.9
Petroselenic acid	92	1.3
Oleic anhydride	81	1.2

MN9D cells were treated for 48 h with 124 μM of each of the compounds indicated above and then cellular dopamine content determined. All compounds were dissolved in dimethylsulfoxide (DMSO). Dopamine level of DMSO vehicle control = 69 ng/mg protein.

Two of the compounds tested showed only minimal effects: oleic anhydride, formed by the fusion of two molecules of oleic acid with the splitting out of water, and petroselenic acid, an 18 carbon monoenoic acid in which the double bond is at position 6, in contrast to oleic acid with a double bond at position 9. The remaining seven long-chain fatty acids were all active and included both monoenoic acids (oleic, palmitoleic, *cis*-vaccenic, and *cis*-13-octadecenoic acid) and polyenoic acids (linoleic with two double bonds, linolenic with three double bonds and arachidonic with four double bonds).

Thus, it is clear that a variety of long-chain fatty acids are capable of increasing the dopamine content of a mesencephalic-derived immortalized monoclonal cell line expressing a dopaminergic phenotype. While the number and variety of fatty acids tested is too limited to reach systematic conclusions regarding the optimal structure necessary to produce an increase in MN9D dopamine, the ability to significantly elevate MN9D dopamine content appears to depend on the presence of a carboxylic acid group and the relative position of an unsaturated double bond. Both petroselenic and oleic acid are C18 fatty acids. Petroselenic acid, which shows minimal effects (see Table 1), has a *cis* double bond that is located three carbons further from the terminal carboxylic acid than that of oleic acid, thus suggesting that the length of the side chain may be a critical determinant of effect.

The MN9D cell line has served as a useful test object for monitoring the presence and purification of dopaminergic stimulatory activities from lysate of immortalized monoclonal cell lines (X61) derived from the striatum. In addition, at least with respect to the small, water-soluble activity (UF4) which appears to be peptide in nature, effects on the MN9D line were predictive of the ability of this substance to increase the dopamine content of primary dopaminergic neurons and prevent their loss in the absence of striatal targets [15]. Whether the increases in MN9D dopamine seen following treatment with known long-chain fatty acids described here will be replicated on primary dopaminergic neurons is obviously a critical question and is currently being examined using three-dimensional reaggregate culture in a similar manner to the previous studies [3,15].

The striatal lines were developed specifically for the purposes of providing a substantial source of monoclonal cells which could be probed for substances that might influence dopaminergic function, either with respect to dopamine levels, cell survival, or the maintenance of the phenotype. It is worth noting that while an isoamyl alcohol/chloroform extract of a lysate concentrate of the X61 cell yielded a fraction capable of markedly increasing MN9D dopamine, that fraction on purification turned out to contain

long-chain fatty acids (oleic and *cis*-vaccenic acid) which could have been extracted from any number of sources. The presence of these active moieties in the X61 cell line, however, directed our attention to their isolation and purification.

Although this is the first description of an ability of long-chain fatty acids to increase cellular dopamine, there are many reports of effects of oleic and other unsaturated fatty acids on neuronal function. Oleic acid is an activator of protein kinase C activity [4,7] and, as has been reported, its synthesis has been linked to neuronal differentiation during development [6,13] and the promotion of axonal growth and induction of MAP-2 expression (microtubule associated protein-2), a marker of dendritic differentiation [9]. Arachidonic acid markedly stimulates, in a dose-dependent fashion, the spontaneous release of dopamine in purified synaptosomes from rat striatum and inhibits dopamine uptake [5]. In addition, reduced dietary intake of omega-6 (arachidonic acid) and omega-3 (docosahexanoic acid) fatty acids in piglets during the first few weeks of postnatal life has been shown to result in lower brain monoamine concentrations which can be reversed upon supplementation with adequate levels of these fatty acids [2]. We are not aware of any reports of beneficial effects of dietary unsaturated fatty acids on the pathogenesis or clinical course of Parkinson's disease.

While the treatment of the motor sequelae of Parkinson's disease has received intense study for over four decades with some very notable successes [10], it remains clear that additional treatment modalities would be helpful and are being sought. It is intriguing in this regard that a long-chain fatty acid such as oleic acid, which is essentially a benign dietary material, can markedly increase the dopamine content of a cell expressing a dopaminergic phenotype. If it should prove to be the case, that oleic acid or other active fatty acids increase the dopamine content of primary dopaminergic neurons, as we previously reported for the crude X61 lysate and partially purified ultrafiltrates (UF4), the long-chain fatty acids may provide an interesting addition to pharmacological approaches to the investigation of the disease and its treatment.

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