Nuclear production and metabolism of diacylglycerol

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The story of nuclear diacylglycerol is proving to be a complex one. Sub-pools of nuclear diglyceride that differ in their metabolism, nuclear localization and temporal regulation have been identified, suggesting potentially diverse signaling functions. One of the great remaining challenges is to assign functional roles to these diverse populations. In the last twenty years great strides have been made toward understanding the character and composition of nuclear DAG. Determining the functions of this nuclear lipid should make the next twenty years interesting, indeed.

Key words: nucleus, diacylglycerol, lipid metabolism, molecular species.

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The fact that cellular lipids serve critical roles in a vast array of signaling networks is becoming increasingly evident. The complexity of lipid signaling is proving to be surprisingly rich, with the number of potential signaling molecules increasing. One of the most celebrated lipid signaling molecules is diacylglycerol (DAG), most notable for its role in the activation of protein kinase C (PKC). This mini review focuses on the production and metabolism of DAG in the nucleus of the cell and its role in transducing cellular signals within the nucleus, with particular emphasis on the correlation between nuclear DAG and mitogenesis. The relationship of DAG fatty acid species to signal specificity and the significance of endonuclear compartmentalization of DAG are discussed.

Common precursors of nuclear diglycerides

Changes in the mass of nuclear diglyceride have been detected in a number of systems under a variety of conditions (Banfic et al., 1993; Cataldi et al., 1990; Divecha et al., 1991a, 1995; Jarpe et al., 1994; Martelli et al., 1995; Miguel et al., 2001; Trubiani et al., 1990) This observation led to a search for the source of the induced lipid; indeed, much of the effort spent analyzing nuclear diglyceride has focused on determining the parental source. Correlations were quickly revealed that linked the hydrolysis of $PI(4,5)P_2$ with the appearance of nuclear DAG in a number of cell types (Martelli et al., 1995; Miguel et al., 2001; Trubiani et al., 1990; Divecha et al., 1993; Neri et al., 1998). This, in turn, led to a directed search for the enzymes involved in the synthesis of $PI(4,5)P_2$, namely PI(4)-kinase and PI(4) 5K-kinase. The result has been the discovery of a differentially regulated nuclear PI cycle that is uncoupled temporally, as well as spatially, from the PI cycle previously thought to exist only at the plasma membrane. Divecha and coworkers showed convincingly that these two PI cycles are indeed uncoupled, by demon-

strating that bombesin treatment of Swiss 3T3 cells results in PI/DAG changes only at the plasma membrane, while IGF-1 treatment results in PI/DAG changes that are restricted to the nucleus (Divecha1991b).

While the identification of PIP_2 as a precursor for nuclear DAG has been an important discovery, the other glycerophospholipids also serve as direct precursors to DAG. Other likely sources of nuclear DAG are phosphatidic acid (PA), phosphatidylcoline (PE), phosphatidylethanolamine (PE), phosphatidylserine (PS), and *de novo* synthesis. Importantly, several potential sources of DAG constitute such large percentages of the total cellular lipid population that the small decreases that would result from DAG production are difficult to measure. Fortunately, early lipid research showed that each phospholipid is composed of a population of constituent molecules that vary in the fatty acid composition at the sn-1 and sn-2 positions of the glycerol backbone (Kuksis et al., 1969; Mahadevappa and Holub, 1982; Raben et al., 1990; Nishihira et al, 1995). Detailed analysis of each phospolipid revelaed that the fatty acid species (aslo called the molecular species) of the lipid often remained constant when the conditions of the cell culture reòained constant, but the profile could change in response to a stimulus, reflecting active metabolism of that lipid (Raben et al., 1990; Jones et al., 1999; Pettitt and Wakelam, 1993; Divecha et al., 1991a). By comparing changes in molecular profiles over time within a single lipid, and comparing profiles between lipids at a single time point, it was possible to identify the effects of agonists on lipid metabolism, and to identify parental precursors of molecules such as DAG and PA. Using this method, some success in tracking nuclear lipid metabolism has been achieved using gas chromatography and/or mass spectroscopy to catalog molecular profiles (Divecha et al., 1991a; Jarpe et al., 1994; Divecha 1991b; D'Santos et al., 1999; Jones et al., 2002; Pessin, 1991).

One weakness inherent in early profiling methods was that preparation of the samples could be cumbersome and time consuming, making complete analysis difficult. In the past, we have successfully used high performance liquid chromatography to separate the major glycerophospholipids prior to capillary gas chromatography with flame ionization detection (GC-FID) to identify molecular species profiles for each of the major glycerophospholipids, as well as diacylglycerol in the nuclei of IIC9 cells (Jarpe et al., 1994). This type of profiling clearly illustrates the variations in fatty acid composition between lipid populations, resulting in a *fingerprint* that can be used to follow the metabolic fate of a particular lipid class.

More recently, the application of electrospray ionization mass spectroscopy (ESI MS/MS) has provided a high throughput technique for rapid profiling of molecular species. A major benefit to this technique is that monitoring changes in relative lipid mass and species profiles of the various constituent lipids from a single sample is rapid, and sample preparation is minimal. ESI MS/MS promises to be an extremely sensitive method of analysis for the changes in lipid metabolism that take place following a stimulus.

One interesting outcome of the GC-FID molecular profiling of DAG described above is that increases in nuclear (and cellular) DAG appear to be derived from only a few parent lipids, primarily PC and PIP_2 (D'Santos et al., 1999; Pessin et al., 1990; Pessin and Raben, 1989). This is surprising considering the number of reactions that potentially result in the production of DAG. This suggests that production of DAG is tightly regulated, and that there are only a few major pathways (and therefore, only a few major sources) of this signaling molecule.

There is evidence that at least some of the saturated nuclear DAG may result from the transfer of the phosphorylcholine headgroup of PC to ceramide during the formation of sphingomyelin (SM). Characterization of the lipids associated with chromatin in rat liver nuclei revealed a significant population of both SM and PC, along with sphingomyelinase and sphingomyelin synthase activities (Albi and Magni, 1999; Manzoli et al., 1977). Based on a correlation between DNA synthesis and SMase activity, Albi and coworkers suggest that SM may be important in maintaining the structural rigidity of chromatin, and that hydrolysis of this lipid (producing ceramide and phosphorylcholine) is important for relaxation of the chromatin during DNA synthesis (Albi and Magni, 2003; Albi et al., 2003a). Conversely, formation of SM may be an important gene regulator, acting at the level of chromatin structure analogous to histone acetylation.

The synthesis of SM following proliferation would necessarily result in a population of chromatin associated DAG. However, DAG has not been detected on chromatin to date, though this may be due to the

presence of detergent used in the preparation of the chromatin fraction.

In a separate study, this group also determined that treatment of purified nuclei with SMase results in loss of 48% of total nuclear RNA, while DNase digestion does not affect the RNA content of isolated nuclei (Albi et al., 1996). Micheli et al isolated an RNase resistant population of RNA from the nuclei of rat hepatocytes, and discovered that treatment with SMase renders this population of RNA susceptible to RNase. This led them to conclude that SM is involved in RNA stability, with a possible role in transcriptional regulation (Micheli et al., 1998). If SM protection of RNA is a common event, then it is likely that DAG is formed when the SM is metabolized.

Common metabolic fates of nuclear diglycerides

Currently, there are two major recycling pathways for nuclear DAG: conversion to phosphatidic acid (PA) by diacylglycerol kinase (DGK) with subsequent recycling into PI, PG or PS, or direct recycling of DAG into PC or PE via the CDP-alcohol pathway. There is an extensive literature available on regulation and potential physiologic roles of the various mammalian DGKs (for reviews see van Blitterswijk and Houssa, 1999; Martelli et al., 2002; Kanoh et al., 2002) but details of these enzymes are not included in this discussion. However, what has become apparent from studies on various DGK isoforms, and on the metabolic fate of nuclear DAG, is that the in vivo substrate for this enzyme appears to be primarily those molecules containing a polyunsaturated fatty acid at the sn-2 position (i.e., DAG derived from hydrolysis of PIP_2). This conclusion is supported by argentation analysis of DGK-produced PA (which reveals the amount of unsaturation in the fatty acids: Kennerly, 1987) and, most convincingly, by matching the fatty acid profile of newly synthesized PA to the profile of endogenous PIP_2 (Jones et al., 2002; Hodgkin et al., 1998). There is evidence that this in vivo preference of DGK for polyunsaturated DAG is due to topological restriction since in vitro studies of DGK activity show that only DGKe, a somewhat uncommon DGK expressed primarily in the cells of the retina, exhibits a preference for polyunsaturated DAG (Tang et al., 1996). A more convincing demonstration that the in vivo preference exhibited by many DGK isoforms for polyunsaturated DAG was given by van der Bend and coworkers, who showed that pretreatment of cells fibroblasts and Jurkat cells with exogenous PI-PLC (to generate DAG) did not result in an increase in PA production unless the cells were solubilized with detergent to disrupt compartmentalization (van der Bend et al., 1992).

The conversion of DAG to PA by DGK is consistent with the notion that the ultimate fate of this pool of DAG is resynthesis into PI. This is further supported by the fact that all of the enzymes involved in the PI-cycle have been identified in the nuclei of cells (Saito et al., 1997; Payrastre et al., 1992; Baker and Chang, 1990). Details of the regulation of this cycle remain to be clarified, since the various enzymes of the nuclear PI cycle are in several subnuclear compartments, indicating that shuttling of the intermediates is necessary. Recent work by Hunt and coworkers on rapid remodeling of endonuclear PC hints that this polyunsaturated class of DAG could, theoretically, also be converted into PC. In these experiments, IMR-32 cells were fed polyunsaturated fatty acids (PUFA) for several days, and the molecular species of steady-state and newly synthesized PC were compared. Newly synthesized PC reflected the incorporation of PUFA-enriched DAG into the PC pool, but the saturated profile was restored in the main population, suggesting remodeling by desaturases or acyl transferases are employed to control the molecular profile of endonuclear PC (Hunt et al., 2002).

The fate of PC-derived DAG may be quite straightforward. Work in whole cells by Rosemary Cornell revealed a cycling between DAG and PC for several hours following stimulation of quiescent IIC9 cells with thrombin (Northwood et al., 1999). Indeed, work from the Hunt laboratory has revealed the presence of a complete endonculear PC cycle, which would support the plausibility of this view (Hunt and Burdge, 1998). However, the method by which the nucleus orchestrates this recycling is unclear.

Recently, a reverse sphingomyelinase activity was identified in a chromatin fraction from rat liver cells (Albi et al., 2003b). This reaction would result in the transfer of the phosphorylcholine headgroup from sphingomyelin back to DAG, producing PC and ceramide. Indeed, the specific activity of the sphingomyelinase responsible for this reaction (37.01 +/- 2.05 pmol/mg/min) is sufficient to account for a considerable conversion of DAG into PC. What is becoming evident is that nuclear DAG cannot simply be partitioned into groups according to its

molecular profile (saturated vs. unsaturated), but must be further subdivided into endonuclear populations based on the location of the lipid. Only in this manner will potential functions of nuclear DAG be revealed.

Subnuclear locations of diglycerides

Determining the subnuclear locations of nuclear DAG is a pressing issue, since this information will provide insights into possible functions, and help direct future research. An obvious location is the nuclear envelope, and indeed, much of the diglyceride produced in nuclei can be extracted using 1% TRITON-X 100, indicating that it resides in a membranous structure. Since no membranes have been observed inside nuclei by electron microscopy, one conclusion is that this lipid is located in the nuclear envelope. While this is certain to be the case for a significant portion of the detergent-soluble lipid, the concentration of detergent commonly used to lyse cells or to remove the nuclear envelope is 20-fold greater than the minimal amount required (Fricker et al., 1997; Irvine, 2002). This precludes a precise determination of how much of the Triton-extractable lipid was actually endonuclear. In any case, some detergent-resistant lipid remains even after this treatment indicating that it is complexed to protein, perhaps proteins of the nuclear matrix.

A more indirect way to determine locations of nuclear DAG is to determine the locations of its parental precursors and of the enzymes involved in the synthesis and metabolism of this lipid. PIP_2 can be identified using an anti-PIP_2 monoclonal antibody (Fukami et al., 1988; Fukami and Takenawa, 1989; Matuoka et al., 1988). Indeed, this technique has been used in a study by Boronenkov et al in rat liver nuclei, and more recently, by Tabellini and coworkers, in HeLa, PC12, and MDA-MB-453 cell lines (Boronenkov et al., 1998; Tabellini et al., 2003). In rat liver nuclei PIP2 was detected by EM in interchromatin regions (a region often associated with active gene transcription), as well as in the nucleolus. Therefore, there is the potential (though not the guarantee) for DAG production in these regions. Tabellini identified PIP_2 in the nuclear speckles of each cell line; regions in the nuclear interior rich in RNA splicing factors. DGKq was also shown to localize to this region, supporting the notion that regions of nuclear DAG can be identified by the location of its metabolizing enzymes. Nuclear PC and sphingomyelin, and the enzymes involved in their synthesis, have been identified in a triton X-100-resistant chromatin fraction in rat liver nuclei, as well as in the nuclear envelope. Taken together, these data indicate that reasonable locations for nuclear DAG are: the nuclear envelope, the nuclear matrix (site of PI-PLCb1), nuclear speckles (site of DGKq and PIP_2), nucleolus (site of PIP_2), and within the intrachromatin/euchromatin (sites for PC, SM and associated SM lipases).

Potential functions of nuclear diglycerides

We have saved the most difficult, yet most interesting, question for last. What, indeed, are the functions of nuclear DAG? Correlations have been made between levels of nuclear DAG and a wide array of cellular fates such as mitogenesis, apoptosis, differentiation, gene regulation, and DNA synthesis. However, the mechanism whereby DAG exerts its effects in these processes is unclear. Certainly there is overwhelming evidence that DAG recruits PKC (for a review on nuclear PKC, see (Buchner, 1995). Indeed, many studies have focused on the effect of nuclear DAG on PKC, and there are a number of reports showing that PKCbII is recruited to the nucleus by the DAG increase at the G_2/M transition (Murray et al., 1994; Deacon et al., 2002; Sun et al., 1997; Kosaka, et al., 1996). Once at the nucleus, a major substrate for this PKC is lamin B; phosphorylation of lamin B on two serine residues promotes dissolution of the nuclear lamina necessary for entry into mitosis (Murray et al., 1994; Deacon et al., 2002). The translocation of PKCbII appears to be in response to PIP2 hydrolysis, and this has led to the conclusion that in vivo, PKC has some specificity for DAG containing polyunsaturated fatty acids, particularly for DAG containing arachadonic acid at the sn-2 position. This is supported by in vitro studies that show PKCa, -b1 and -d translocations are maximally stimulated by DAG containing highly unsaturated fatty acids at the sn-2 position (O'Flaherty et al., 2001). Interestingly, PKCa has also been observed to translocate to the nuclear envelope and nuclear interior in response to increases to both PI-derived and PC-derived DAG (Divecha1991b; Buchner, 1995; Martelli et al., 1999). While PKCa can also phosphorylate lamin B, its affinity for this substrate is several-fold lower than PKCbII; however, there is *in vivo* evidence that the PKCa isoform can also phosphorylate lamin B (Shimizu et al., 1998).

Numerous substrates have been identified for

PKC in the nucleus in addition to lamins (for a review see (Martelli et al., 2003). Interestingly, PKC has been shown to regulate the nuclear localization of diacylglycerol kinase-z (DGKz) in Cos 7 cells. The nuclear localization sequence present in DGKz has been shown to contain a MARCKS domain; a motif recognized and phosphorylated by PKCa. Topham and coworkers showed that PKCa controls the nuclear presence of DGKz, which exits the nucleus following phosphorylation of the MARCKS domain. By mutating the MARCKS domain, this group was able to force the nuclear retention of DGKz, resulting in a decrease in nuclear DAG and an increase in the percentage of cells in the Go/G1 phase of the cell cycle. This interplay between PKCa and DGKz in the nucleus represents a point of regulation for the cell: expulsion of DGKz from the nucleus produces a temporally extended DAG signal (and presumably PKC activity) in the nucleus, while phosphorylation of DAG by DGKz decreases the nuclear DAG signal and downregulates nuclear PKC (Topham et al., 1998).

PKC and DGK are not the only DAG-binding proteins in cells, however. To date, there are three other DAG-binding proteins that have been identified: b-chimaerin (a GTPase activating protein), Munc13 (a protein involved in exocytosis) and RasGRP (for a review on DG-binding proteins see (Kazanietz, 2000), and it seems likely that other DAG-binding proteins remain to be identified. To date, only PKC and DGK have been identified in the nucleus. Identifying and characterizing additional nuclear DAG-binding proteins is an important issue, central to understanding the roles of nuclear DAG.

One important consideration in analyzing nuclear DAG is determining which pools of diglyceride serve as signaling molecules, and which pools are used for lipid synthesis. For example, as previously mentioned, treatment of isolated nuclei or purified nuclear matrix with PI-PLC, the enzyme that hydrolyzes PIP2 to DAG and IP3, results in the loss of approximately 45% of newly synthesized nuclear RNA (Albi et al., 1996). This suggests that PIP_2 is somehow necessary for maintaining a connection between RNA synthesizing or modifying proteins and the nuclear matrix. Ultimately, one can imagine that hydrolysis of PIP_2 could be one step required for the release of mRNA from the nucleus. This could result in the appearance of matrix-associated DAG, which may, or may not, have an independent signaling role.

A similar situation may occur in chromatin, with the synthesis of sphingomyelin from PC and ceramide. A significant amount of sphingomyelin and PC have been detected in isolated chromatin fractions, and a correlation has been noted between levels of sphingomyelin, DNA synthesis and active gene transcription. Functionally, SM may act to stabilize chromatin, or negatively regulate DNA transcription or synthesis (Albi and Magni, 2003; Albi et al., 2003a). A product of sphingomyelin formation is DAG; therefore, DAG levels may increase in chromatin when SM is being synthesized and the DAG may or may not serve as signaling molecules.

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