Fatty acids modulate protein kinase C activation in porcine vascular smooth muscle cells independently of their effect on de novo diacylglycerol synthesis

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Abstract

Aims/hypothesis. Diabetes-induced activation of protein kinase C has been associated with the development of vascular complications. Elevated de novo diacylglycerol synthesis has been postulated to underlie this protein kinase C activation. Diabetes also increases the circulating concentrations of non-esterified fatty acids, which are immediate precursors of diacylglycerol through the de novo pathway. We hypothesized that increased fatty acids contribute to de novo diacylglycerol synthesis and activation of protein kinase C in vascular cells.

Methods. Primary cultures of porcine carotid smooth muscle cells were exposed to fatty acids, bound to albumin in physiologic ratios. Diacylglycerol and triacylglycerol were measured in extracts of these cells. Protein kinase C activation was measured as membrane translocation with isoform-specific antibodies.

Results. Saturated fatty acids caused considerable accumulation of diacylglycerol through de novo synthesis. Unsaturated fatty acids increased triacylglycerol,

but not diacylglycerol. Platelet-derived growth factor activated the α , ε and ζ protein kinase C isoforms. Activation of the α and ζ isoforms was amplified by oleate pretreatment but inhibited by palmitate. In the absence of growth factor stimulation, neither palmitate nor oleate had any effect on the membrane/cytosol distribution of any protein kinase C isoform.

Conclusion/interpretation. Saturated fatty acids elicited de novo diacylglycerol synthesis in vascular smooth muscle cells without activating protein kinase C. Effects of fatty acids on protein kinase C activation by platelet-derived growth factor did not correlate with the effects on de novo diacylglycerol synthesis. These results indicate that de novo diacylglycerol synthesis is, by itself, insufficient to activate protein kinase C. [Diabetologia (2000) 43: 1136–1143]

Keywords Diabetes mellitus, protein kinase C, diacylglycerol, non-esterified fatty acids, platelet-derived growth factor, vascular smooth muscle, cultured cells.

Diabetes-induced activation of protein kinase C (PKC) has been implicated in the development of chronic vascular complications [1–4]. Vascular cells

exposed to high glucose in vitro accumulate diacylglycerol (DAG) and have higher PKC activities [5]. Similar changes have been shown in animal models [6]. A specific PKC inhibitor attenuates vascular dysfunctions in diabetic animal models [7]. Such results strongly implicate DAG/PKC signalling in the pathogenesis of vascular diabetic complications.

Although many studies have shown diabetes increases in vascular DAG, the metabolic origin of this DAG and its relation to PKC activation are less apparent. Diabetes clearly increases de novo lipid synthesis by glycerophosphate acylation [5, 6]. High glucose concentrations also increase turnover of phos-

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Abbreviations: DAG, Diacylglycerol; ITS, insulin, transferrin and sodium selenite; MEM, (Eagle's) minimal essential medium; PDGF, platelet-derived growth factor; PKC, protein kinase C; VSMC, vascular smooth muscle cell.

phatidylcholine through a phospholipase D-initiated pathway [8, 9]. The fatty acyl composition of the DAG arising in diabetes would be consistent with either of these sources [6, 10]. Extrapolation of results from cell culture systems is further complicated by hyperglycaemia being only one of numerous metabolic abnormalities in diabetes. Of particular relevance to lipid signalling, there is an increase in concentrations of non-esterified fatty acids (NEFA) to a degree which tends to be proportional to the increase in blood glucose concentration [11]. Treatments that restore blood glucose concentrations to normal reverse these changes in NEFA [12]. Studies showing improved clinical outcomes with better glycaemic control thus do not exclude a contribution of NEFA to complications. Indeed, NEFA are increased in insulin-resistant people without overt diabetes and in non-diabetic offspring of parents with Type II (non-insulin-dependent) diabetes mellitus and could contribute to the increased vascular risk in these groups [13, 14].

The aim of this study was to test the hypothesis that fatty acid exposure modulates DAG/PKC signalling in vascular cells. We exposed vascular smooth muscle cell (VSMC) monolayers to saturated and unsaturated fatty acids. The NEFA were bound to albumin before they were added to the culture media to avoid supraphysiologic concentrations of monomeric NEFA [15]. We measured the effects on DAG and triacylglycerol mass and on the membrane/cytosol distributions of individual PKC isoforms. We also examined the effects of NEFA on PKC activation by platelet-derived growth factor (PDGF), which elicits DAG accumulation in VSMCs by turnover of endogenous membrane phospholipids.

Materials and methods

Primary cultures of vascular smooth muscle cells (VSMCs). We prepared VSMCs from adult male porcine carotid arteries as described previously [16]. Cells were seeded into 75-cm² flasks in 30 ml of Eagle's minimum essential medium (MEM) supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B. Amphotericin was used only during the initial plating of cells and not for subsequent media changes or subculturing. The culture medium was replaced after 5 days and then every Monday, Wednesday and Friday. Cells reached confluence after 12 to 14 days. Subculturing was done every 7 days using 0.5 mg/ml trypsin and 0.53 mmol/l EDTA at a subculture ratio of 1:4. For experiments, 5×10^4 cells/ml from passages 3 to 7 were seeded into 100-mm dishes in 12 ml of MEM supplemented with 10% FCS, penicillin and streptomycin as above. The culture medium was replaced every 48 h. Cells reached near-confluence after 7 days. They were rendered quiescent by washing once with phosphate-buffered saline and replacing the medium with antibiotic-free MEM supplemented with 0.1% FCS for 24 to 48 h. To confirm that serum did not influence the results, some experiments were repeated with cells rendered quiescent in serum-free and antibiotic-free MEM supplemented with

5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite (ITS) and also with 1 mmol/l ascorbate [16].

Fatty acid supplementation of culture media. We added warmed aqueous solutions of fatty acid sodium salts dropwise, with stirring, to a 66 mg/ml solution of delipidated bovine albumin (Sigma, St. Louis, Mo., USA) in MEM minus the divalent metal ions [15]. This solution was dialysed overnight against 20 volumes of MEM. For unsaturated fatty acids, the solution was maintained under an N₂ atmosphere to prevent oxidation. The solution was sterilized by filtration and stored at -20 °C until use. We confirmed fatty acid concentrations by a colorimetric-coupled enzyme assay [17]. Albumin was measured by absorbance at 280 nm. An extinction coefficient of $41.6 \text{ cm}^{-1} \cdot (\text{mmol/l})^{-1}$ was used to calculate the final albumin concentration. We also prepared a stock albumin solution lacking fatty acids. These solutions were added to culture media to give the desired final concentrations of albumin and fatty acid. The albumin concentration in all experiments was 50 µmol/l (3.3 mg/ml).

Lipid extraction and assays. We harvested cells in 100-mm dishes by scraping and extracted the lipids into neutral CHCl₃/methanol as described previously [18]. Diacylglycerol in these extracts was measured using a standard radioenzymatic assay with *E. coli* diacylglycerol kinase [18]. Membranes from *E. coli* strain N4830/pJW10 were the source of this enzyme [19]. We measured Triacylglycerol using a glycerophosphate oxidase/Trinder assay [20]. A 300-µl sample of the CHCl₃/methanol extract was evaporated under N₂ and dissolved in 25 µl of isopropanol. We then added 1 ml of reconstituted triacylglycerol reagent (Roche Diagnostics, Indianapolis, Ind., USA). The samples were briefly sonicated in a water bath and incubated at 25 °C for 10 min. Absorbances were read at 500 nm and triacylglycerol calculated using a standard curve measured on a calibrated solution of triolein (Sigma). This assay will also measure DAG. Thus, the triacylglycerol concentrations reported were all corrected for DAG, which was measured as described above. Organic phosphate (phospholipid) in the extracts was measured as described previously [21]. All DAG and triacylglycerol concentrations are expressed as mmol acylglycerol/mol lipid phosphorus. Neutral lipids were isolated by passing the total lipid extracts over a small column of florisil [22]. The thin layer chromatography of the neutral lipids was done as described previously [23]. Positive ion spectra were obtained on a Sciex API III tandem mass spectrometer with electrospray ionization. Ammonium acetate (5 mmol/l) was added to the lipid solution to facilitate ionization.

Protein kinase C assay. We chilled cells in a 100-mm dish on ice, washed them once with PBS, and harvested them by scraping them into 0.5 ml of 20 mmol/l TRIS-HCl, pH 7.5, 0.1 mmol/l EDTA, 0.2 g/ml glycerol, 0.25 mmol/l Triton X-100, 1 mmol/l dithiothreitol, 1 mmol/l benzamidine, 1 mmol/l phenylmethylsulphonyl fluoride, 1 µg/ml aprotinin and 1 µg/ml leupeptin. They were lysed by 20 strokes with a dounce homogenizer and centrifuged for 1 min at $15,000 \times g$. Each PKC assay contained, in a volume of 60 µl, 25 mmol/l triethanolamine HCl (pH 7.5), 5 mmol/l Mg acetate, 0.425 mmol/l CaCl₂, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 40 µmol/l *sn*-1-palmitoyl-2-oleoyl phosphatidylserine (Avanti, Birmingham, Ala., USA), 10 µmol/l *sn*-1-palmitoyl-2-oleoyl diacylglycerol (DAG), 10 µmol/l [γ -³²P]ATP (New England Nuclear, Wilmington, Del., USA) and 25 µmol/l NKRPNSRSKY, a protein kinase C-specific substrate [24]. The free Ca²⁺ concentration was 50 µmol/l, based on published stability constants [19]. Diacylglycerol was prepared as described previously [23]. Chloro-

Fig. 1A–D. Induction of acylglycerol synthesis by non-esterified fatty acids. Quiescent VSMCs were treated with NEFA as indicated. Cells were harvested and assayed for DAG and triacylglycerol. **A** and **C** show the concentrations of DAG and triacylglycerol in cells exposed to 150 μmol/l palmitate or 150 μmol/l oleate for the indicated times. **B** and **D** show the DAG and triacylglycerol concentrations of cells exposed to varying concentrations of NEFA for 24 h. The albumin concentration was 50 μmol/l in all cases. All data are shown as means ± SEM of three or four independent determinations. Symbols used include: elaidate, (▼); linoleate, (◆); myristate, (○); oleate, (■); palmitate, (●); palmitoleate, (□); and stearate, (▲).

form solutions of phosphatidylserine and DAG were evaporated under a stream of N_2 in a test tube and dispersed into ice-cold 25 mmol/l triethanolamine buffer by sonicating twice with a microprobe tip at 10 mW for 5 s. The phosphatidylserine/DAG vesicles were combined with the Ca^{2+} , Mg^{2+} , EDTA, water and cell lysate (5 μl) to give a volume of 40 μl. The reaction was started by adding 20 μl of a solution containing [γ - ^{32}P]ATP and NKRPNSRSKYL and allowed to proceed at 25 °C for 10 min. It was terminated by adding 30 μl of 0.275 mol/l H_3PO_4 and 70 μl of the mixture was spotted onto a 2.3 cm P11 phosphocellulose disc (Whatman, Clifton, N.J., USA). The P11 discs were washed once in 75 mmol/l H_3PO_4 , once in 75 mmol/l Na_2HPO_4 and then twice in 75 mmol/l H_3PO_4 . The discs were dried briefly on paper towels and counted for ^{32}P in 4 ml of scintillation fluid. Background activities were measured by excluding phosphatidylserine and DAG from the assay and subtracted from the total activity, which was measured in the presence of phosphatidylserine and DAG as described above. We measured protein concentrations using a bichinchoninic acid assay after trichloroacetic acid precipitation [25].

Immunoblotting of membrane and cytosol fractions. We investigated membrane translocation of individual PKC isoforms by immunoblotting with isoform-specific antibodies. Monoclonal antibodies against PKC α , β and ϵ were from Transduction Laboratories, San Diego, Calif., USA. Polyclonal antibodies against PKC δ and ζ were from Santa Cruz Biotechnology, Santa Cruz, Calif., USA. Quiescent cultures (0.1 % FCS, 48 h) were treated with NEFA/albumin for 4 to 24 h. To investigate effects of PDGF, cells were washed once with MEM and incubated in the same medium for 30 min. We prepared PDGF-BB (R&D Systems, Minneapolis, Minn., USA) as a 1 μg/ml stock solution in MEM. It was added at a final concentration of 10 ng/ml and the cells were incubated for 0 to 60 min. Cytosolic and membrane-bound PKC fractions were prepared as described previously [26]. We measured protein concentrations in these fractions by a modified Lowry assay (Bio-Rad DC assay, Bio-Rad, Hercules, Calif., USA). The fractions were electrophoresed on 7.5 % SDS-PAGE gels and transferred to nitrocellulose. We detected PKC isoforms by immunoblotting using an enzyme-derived chemiluminescence kit (Amersham, Arlington Heights, Ill., USA) and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Phosphotyrosine-containing proteins in membrane and cytosol fractions of PDGF-stimulated cells were detected using a phosphotyrosine-specific monoclonal antibody (Upstate Biotechnology, Lake Placid, N. Y., USA).

Results

Saturated fatty acids induce DAG accumulation in cultured VSMCs. Primary cultures of vascular smooth muscle cells were rendered quiescent and exposed to non-esterified fatty acids at concentrations up to

Fig. 2. Mass spectrometry of neutral lipids from vascular smooth muscle cells exposed to heptadecanoate. The positive ion spectrum of neutral lipids from cells exposed to 150 $\mu\text{mol/l}$ heptadecanoate for 24 h is shown. Acylglycerol molecular species seen include: diC17 $\cdot \text{NH}_4^+$, 614 and triC17 $\cdot \text{NH}_4^+$, 866. The peak at $m/z = 579$ is a carbonium ion which is identical to the main breakdown product of both the 614 and 866 ions in ms/ms [64]. Several peaks clustered around the molecular ion at 866 correspond to triacylglycerol species with two C17:0 groups and one other fatty acyl moiety, derived either from modification of the exogenous C17:0 (odd chains) or from the endogenous NEFA pool (even chains). These include: C15:0, 838; C16:1, 850; C16:0, 852; C17:1, 864; C18:1, 878; C18:0, 880; C19:1, 892; C19:0, 894

150 $\mu\text{mol/l}$. The NEFA were bound to albumin before adding them to the culture medium. The ratios of NEFA:albumin used are representative of those seen in vivo [11]. Palmitate caused DAG accumulation dependent on time and concentration (Fig. 1). Diacylglycerol concentrations reached maximum at 8 to 24 h and decreased slightly thereafter. Diacylglycerol accumulation was seen only with saturated fatty acids including myristate, palmitate and stearate. The unsaturated fatty acids palmitoleate, oleate and linoleate caused a slight decrease in total DAG. Elaidate, the *trans*-unsaturated analogue of oleate, also failed to induce DAG accumulation. We also examined NEFA effects on triacylglycerol, the final product of the *de novo* pathway. Both saturated and unsaturated fatty acids caused triacylglycerol accumulation in these cells (Fig. 1). Much more triacylglycerol was, however, seen with the unsaturated NEFA species. Palmitate and oleate had nearly identical effects on DAG and triacylglycerol if cells were rendered quiescent in serum-free ITS medium instead of 0.1 % FCS (data not shown).

To confirm that the acylglycerols derived from *de novo* acylation, cells were exposed to heptadecanoate, an odd chain fatty acid which is normally only a trace component of animal lipids. Thin layer chroma-

tography of the neutral lipids from heptadecanoate-treated cells showed DAG and triacylglycerol to be the predominant lipids present (data not shown). The neutral lipids were also analysed by tandem mass spectrometry (Fig. 2). Odd chain fatty acids are not synthesized by animal cells to any great extent and their presence reflects incorporation of the exogenous NEFA. The DAG and TG each consisted largely of a single molecular species derived from heptadecanoate (Fig. 2).

Fatty acids modulate activation of protein kinase C. Human and rat aortas express PKC α , δ , ϵ , ζ , ι/λ and very low concentrations of η [27]. Vascular smooth muscle cells in culture have been reported to express PKC α , β , δ , ϵ and ζ [28]. There is some variability among isolates. All VSMC lines express PKC α and PKC ζ together with one or both of the novel isoforms, PKC δ and PKC ϵ [29–34]. Most reports of PKC β expression are in cell lines derived from rodents [29–31]. Porcine carotid VSMCs expressed the α , ϵ and ζ PKC isoforms (Fig. 3).

We examined the cells for NEFA-induced translocation of individual PKC isoforms. Treatment with NEFA for 4 h did not alter the concentration or distribution of any PKC expressed by these cells (Fig. 4). Identical results were obtained after 24 h of NEFA treatment (data not shown). Some reports have suggested that high concentrations of fatty acids directly stimulate cytosolic PKCs without translocation to the particulate fraction [26, 35]. To exclude this, we assayed the total PKC activity of lysates prepared from cells treated with palmitate or oleate. There was no evidence for fatty acid effects on cytosolic PKC activity (Fig. 5).

We also examined the effects of NEFA on PKC activation induced by platelet-derived growth factor. Phorbol ester induced rapid translocation of PKC α and PKC ϵ to cell membranes (Fig. 4). Phorbol also induced the appearance of a higher M_r autophosphorylated, form of PKC ϵ in the membrane fraction [36].

Fig. 3. Protein kinase C isoforms expressed in VSMCs. Proteins from cultured porcine VSMCs were examined for presence of PKC α , β , δ , ϵ and ζ by immunoblotting with isoform-specific antibodies. An extract of porcine thymus (THYM) was included as a positive control. Note that the PKC ζ antibody cross-reacts with PKC α , which migrates at a slightly higher M_r .

Treatment with PDGF elicited these same changes and also induced translocation of PKC ζ , an isoform independent of DAG and phorbol (Fig. 6). In the absence of fatty acid, PDGF had only modest effects on PKC translocation. Pretreatment with oleate increased and prolonged the effects of PDGF on translocation of PKC α and PKC ζ (Fig. 6). Membrane binding of both isoforms was greatly increased at 30 min, when untreated cells were returning to baseline. In contrast to oleate, palmitate abolished PDGF-induced activation of PKC α and PKC ζ . These effects were selective among PKC isoforms, as neither fatty acid had any effect on PDGF-induced autophosphorylation of PKC ϵ (data not shown). Identical results were seen with all PKC isoforms if cells were treated with NEFA for 24 instead of 4 h (data not shown). Fatty acids had no effect on PDGF receptor autophosphorylation or on the appearance of other tyrosine phosphorylated proteins in response to PDGF (Fig. 7).

Discussion

Diabetes-induced activation of protein kinase C has been linked to vascular changes leading to atherosclerosis, glomerulopathy, retinopathy and neuropathy [1–4]. Prolonged exposure of cultured cells to in-

creased glucose concentrations results in DAG accumulation and translocation of PKC to particulate fractions. The metabolic origin of diabetes-induced DAG is less certain. Diacylglycerol arises through several pathways at multiple intracellular locations (Fig. 8) [37, 38]. It is unlikely that all these DAG

Fig. 4. Effects of NEFA on membrane/cytosol distribution of VSMC PKC isoforms. Quiescent VSMCs were exposed to 150 $\mu\text{mol/l}$ palmitate (Pal), oleate (Ole), or elaidate (Elai) for 4 h. Membrane (Memb) and cytosol (Cyto) fractions were prepared and examined for PKC isoforms by immunoblotting. Fractions from control cells (Cont) exposed to albumin only are shown on the left and fractions from cells exposed to phorbol myristate acetate (PMA) for 10 min as a positive control are shown on the right. Note that the PKC ζ antibody cross-reacted with PKC α , which is located slightly above the PKC ζ band. This experiment was done three or more times with similar results for each fatty acid/PKC isoform combination.

Fig. 5. Effects of non-esterified fatty acids on protein kinase C activity in vascular smooth muscle cells. Quiescent VSMCs were exposed to 150 $\mu\text{mol/l}$ palmitate (●) or 150 $\mu\text{mol/l}$ oleate (▲) for the indicated times. Protein kinase C activity was measured in cell lysates. The data are shown as means $\pm$ SEM of four independent measurements.

pools contribute to PKC activation in diabetes. Several studies have used [^{14}C]glucose labelling to show increased de novo DAG synthesis during hyperglycaemia [1, 39]. Glucose labelling cannot directly measure DAG arising from other sources. There is also evidence that hyperglycaemia increases the turnover of phosphatidylcholine to DAG [8, 9]. Phosphoinositide turnover is decreased during hyperglycaemia [40].

In our study, induction of de novo DAG accumulation by saturated fatty acid (palmitate) failed to activate protein kinase $\text{C}\alpha$, ϵ or ζ . Other studies have also shown that DAG arising from phospholipid turnover outside of the phosphoinositide pathway does not invariably activate PKC [41]. Dipalmitoylglycerol activates PKC in vitro similarly to other DAGs [42–44]. Thus it is unlikely that these results are simply due to the molecular species of DAG accumulating when cells are exposed to palmitate. A more likely explanation for the failure of de novo DAG to activate PKC is that other factors that are required, including phosphatidylserine and specific anchoring proteins, are not present at the site of de novo synthesis [45]. De novo DAG synthesis arises in the endoplasmic reticulum, which has a low concentration of phosphatidylserine [37, 38]. Our results suggest that DAG arising from the de novo pathway is, by itself, unable to activate PKC in these cells. Thus, although increased de novo DAG synthesis does occur in diabetes, it does not necessarily contribute to PKC activation.

In the absence of PDGF stimulation, NEFA had no effect on PKC activation. Because albumin was included in these experiments, the NEFA monomer concentration was in the order of 1 nmol/l [15]. Previ-

Fig. 6. Oleate potentiation of PDGF-induced PKC α and PKC ζ translocation. Cells treated for 4 h with 150 $\mu\text{mol/l}$ of oleate, palmitate or no NEFA were stimulated with PDGF for 10, 20 or 30 min, as indicated. Membrane fractions were examined for PKC isoforms by immunoblotting. In separate experiments, both with and without oleate addition, membrane-bound PKC α and PKC ζ had returned to baseline 60 min after PDGF stimulation. Membrane PKC comprised only a small fraction of the total in all cases and no differences were observed in the concentrations of PKC isoforms in cytosol fractions of these cells (data not shown). These blots are representative of three independent experiments with different VSMC preparations.

ous studies showing PKC activation by oleate did not use albumin and would have exposed cells NEFA monomer concentrations of 10 to 100 $\mu\text{mol/l}$. In this study fatty acids did modulate activation of two PKC isoforms in response to PDGF. Enhanced PKC α activation was observed only with NEFA that did not increase DAG. Fatty acids that did increase DAG actually inhibited PKC α activation by PDGF. Protein kinase $\text{C}\zeta$, the other isoform modulated by NEFA, is not even DAG sensitive. Neither palmitate nor oleate altered the pattern of phosphotyrosine-containing proteins elicited by PDGF, indicating that their effects involve postreceptor mechanisms. Our findings suggest that NEFA, by modulating PKC activation in response to other signals, contribute to PKC activation in diabetes independently of their effects on de novo DAG synthesis. It is attractive to speculate that fatty acids also modulate glucose-induced activation of PKC. Platelet-derived growth factor and other cytokines are thought to have roles in both atherogenesis and glomerular mesangial proliferation. Fatty acid modulation of growth factor signalling might thus contribute to the development of vascular lesions in diabetes. Notably, activation of PKC ζ , a

Fig. 7. Tyrosine phosphorylation of proteins in response to PDGF. Cells treated for 4 h with 150 $\mu\text{mol/l}$ of oleate, palmitate, or no NEFA were stimulated with 10 ng/ml PDGF for 5 min. Membrane and cytosol fractions were prepared and examined for tyrosine phosphorylated proteins by immunoblotting. 1. No NEFA, control; 2. No NEFA, PDGF; 3. oleate, control; 4. palmitate, control; 5. oleate, PDGF; 6. palmitate, PDGF. The arrow indicates the position of the PDGF receptor

DAG-independent isoform, was particularly enhanced by oleate. Protein kinase C ζ is involved in stimulation of VSMC proliferation by PDGF and unsaturated NEFA (oleate) stimulate VSMC proliferation [26, 29]. Saturated NEFA (palmitate) do not have this effect. Modulation of PKC ζ signalling might thus be a specific mechanism through which NEFA contribute to vascular complications.

The observation that saturated fatty acids elicit DAG accumulation is itself of note. The induction of

triacylglycerol synthesis by unsaturated fatty acids indicates that all NEFA induce the de novo lipid pathway but that the final acylation of saturated DAG species to triacylglycerol is somehow impaired. Consistent with this interpretation, fully saturated DAG species have been shown to be poor in vitro substrates for DAG acyltransferase [46]. Other studies have shown that lymphocytes [47], neutrophils [48] and cultured fibroblasts [49] exposed to oleate or linoleate synthesize substantial amounts of triacylglycerol. Saturated NEFA (palmitate) did not induce triacylglycerol synthesis in these systems. These earlier studies did not examine DAG. Our results suggest that saturated NEFA impaired DAG acylation in these cells as well. Saturated NEFA-induced DAG accumulation could thus occur in many cell types. Elaidate, a C18 *trans*-unsaturated fatty acid, induced triacylglycerol synthesis similar to other unsaturated NEFA and did not induce DAG accumulation. As the physical properties of *trans*-unsaturated fatty acids are similar to those of their fully saturated homologues, our results are unlikely to simply be due to differences in the physical properties of saturated and unsaturated fatty acids. Rather, they must reflect some difference in the metabolic handling of NEFA species by these cells.

How fatty acids modulate PKC activation is not known. In addition to serving as precursors for DAG synthesis, fatty acids exert diverse effects on signalling pathways. Non-esterified fatty acids directly stimulate a variety of protein kinases and phosphatases and directly modulate the activities of several ion channels and GTP-binding proteins [26, 35, 50]. They also regulate transcription through specific binding to PPAR α receptors [51]. Metabolites derived from NEFA, including acyl coenzyme A thioesters, lysophosphatidic acid and phosphatidic acid, also have signalling functions [52–56]. Diacylglycerols elicit signalling events that are independent of their effects on PKC activation [57–59]. Indeed,

Fig. 8. Major reactions of animal glycerolipid metabolism. Metabolic relations of the major glycerolipids are shown. Diacylglycerol is a central intermediate in the synthesis and turnover of all major glycerolipids [38]. As such, DAG derives from multiple sources

NEFAs contribute to the insulin resistance and increased hepatic gluconeogenesis associated with Type II diabetes [60–62] and there is evidence that they alter glucose oxidation by VSMCs [63]. Any of these signalling effects might modulate PKC activation and contribute to the development of chronic diabetic complications.

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