Effect of Insulin on *SN*-1,2-Diacylglycerol Species and De Novo Synthesis in Rat Skeletal Muscle

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Insulin treatment increases the *SN*-1,2-diacylglycerol (DAG) concentration in skeletal muscle. Because DAG may participate in transmission or modulation of the insulin receptor signal, we examined the effect of insulin on total DAG and on different DAG species in isolated rat hemidiaphragms incubated with 5 mmol/L glucose. Five DAG species (16:0-18:1ω9, 16:0-18:1ω7, 18:0-18:1ω9, 18:0-18:2ω6, and 18:1-18:2) were identified and quantified. After a 5-minute incubation with 60 nmol/L insulin, neither total DAG nor a DAG species increased; exposure to insulin for 10 or 20 minutes increased the concentration of total DAG and of several DAG species. Insulin did not increase DAG in muscles incubated without glucose. Two sources for the insulin-mediated DAG increase were considered: phosphatidylcholine (PC) hydrolysis and de novo DAG synthesis from glucose. Concentrations of choline and phosphocholine in muscle were not increased after 10-minute incubations with insulin. However, insulin increased ^{14}C incorporation from [U-^{14}C]glucose into DAG, triacylglycerol (TAG), and total lipids approximately threefold. Okadaic acid (OKA), an inhibitor of phosphoprotein phosphatases 1 and 2A, increased muscle DAG content and synthesis from glucose, similar to the effect of insulin. Doses of OKA or insulin that increased DAG mass greatly exceeded those required for stimulation of glucose transport. The insulin-mediated, relatively slow increase in muscle DAG observed here likely reflects primarily de novo synthesis from glucose. This effect would be downstream of insulin stimulation of glucose transport. However, a possible insulin-mediated, rapid, transient increase in muscle DAG content and PC hydrolysis cannot be ruled out by our studies.

INSULIN HAS PLEIOTROPIC effects on skeletal muscle that include stimulation of glucose transport and glycogen synthesis. The initial events after insulin binds to the α-chain of its receptor include activation of insulin receptor β-chain tyrosine kinase and tyrosine phosphorylation of intracellular proteins such as insulin receptor substrate 1.[1] However, the signaling mechanisms that link the early events to specific metabolic pathways are poorly understood.[1,2] An insulin-stimulated *SN*-1,2-diacylglycerol (DAG) has been proposed as a signal for stimulation of glucose transport.[3,4] An increase in muscle DAG mass after insulin treatment has been reported in vivo and in vitro by some[3-6] but not other[7] laboratories. Furthermore, it was suggested that the increase in DAG mass may precede insulin-mediated stimulation of glucose transport in muscle.[3,4]

Because individual chemical species or subgroups of DAG may have distinct effects on signaling systems,[8] we investigated whether a particular species of DAG increases preferentially in muscle after insulin treatment. Consistent with the hypothesis of DAG species-specific activation of glucose transport, Stralfors reported that glucose transport in adipocytes is stimulated by only a few DAG species tested in vitro.[9]

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Several studies suggest that in muscle, at least part of the insulin-stimulated increase in DAG reflects de novo synthesis. Heydrick et al[10] and Ishizuka et al[3] reported that insulin increases [^{14}C]glucose and [^{3}H]glycerol incorporation, respectively, into DAG in muscle. An insulin-stimulated increase in glycerol-3-phosphate acyltransferase activity was observed in BC3H-1 myocytes, which may contribute to the increase in de novo synthesis.[11] However, other sources such as insulin-stimulated hydrolysis of membrane phospholipids, eg, phosphatidylcholine (PC), have also been suggested in BC3H-1 myocytes[12,13] and rat adipocytes[13] and hepatocytes.[14] PC can be hydrolyzed to DAG directly by phospholipase C (PLC) or by the sequential action of phospholipase D and phosphatidic acid phosphohydrolase (reviewed in Exton[15]). The products of these reactions are phosphocholine and choline, respectively. We therefore examined whether an increase in either of these compounds accompanied an insulin-mediated increase in muscle DAG.

Okadaic acid (OKA) is an inhibitor of phosphoprotein phosphatases 1 and 2A[16] that stimulates muscle glucose transport by an insulin receptor–independent mechanism.[17,18] Since several insulin receptor–independent activators of muscle glucose transport also increase DAG content, eg, PLC[5,19] and electrically induced contractions,[20] we measured the effect of OKA on DAG concentration in muscle.

MATERIALS AND METHODS

Animals

Male Wistar rats that weighed 50 to 80 g (Charles River Laboratories, Wilmington, MA) were studied after an overnight fast.

Muscle Incubations

Rats were decapitated, and the two hemidiaphragms along with adjacent ribs were rapidly dissected. Hemidiaphragms were incubated individually either in Dulbecco's modified Eagle's medium

([DMEM] 5.6 mmol/L glucose) supplemented with 0.1% bovine serum albumin with additions as indicated, or in the balanced salt solution of Gey and Gey (BSS) supplemented with 2 mmol/L pyruvate and 0.1% bovine serum albumin.[5,18] All incubations were performed at pH 7.4 and 37°C in media equilibrated and continuously gassed with 95% O_2–5% CO_2 in a rotating-shaker water bath. OKA stock solutions were prepared in dimethyl sulfoxide; media not containing these compounds were supplemented with equal concentrations of the solvent, which never exceeded 0.4% and did not affect the metabolic parameters measured (data not shown). In all experiments, one hemidiaphragm of each rat was incubated with the agent tested (insulin or OKA), and its pair served as a control incubated in the same medium supplemented with vehicle only.

Quantitation of DAG

The amount of DAG extracted from muscle as described below was measured by the DAG kinase assay[21] with modifications as previously described.[5,22]

Glucose Transport

Glucose-free media. Hemidiaphragms were preincubated for 30 minutes in BSS (supplemented with 2 mmol/L pyruvate) without or with insulin (60 nmol/L). Transport was measured by transferring muscles to identical media supplemented with 5 mmol/L 2-[1,2-^{3}H]deoxyglucose ([2-DG] 0.5 μCi/mL) and 0.1 mmol/L [U-^{14}C]sucrose (0.1 μCi/mL; included as an extracellular space marker) for 15 minutes as previously described.[5,23] Muscles were then dissolved in 0.5 mL 1N NaOH and neutralized with 2N HCl for quantitation by liquid scintillation spectrometry.

Glucose-containing media. Hemidiaphragms were preincubated for 30 minutes in DMEM (5.6 mmol/L glucose) without or with insulin (60 nmol/L) and then transferred to identical media supplemented with tracer 2-DG (0.5 μCi/mL) and 0.1 mmol/L [U-^{14}C]sucrose (0.1 μCi/mL) for 15 minutes. Intracellular 2-DG content was calculated by subtraction of 2-DG in the extracellular space from that in the tissue[10,24] and normalization to tissue weight. Transport calculations (nanomoles per milligram per 15 minutes) are based on the concentration of 2-DG or glucose, respectively, in the media and assume that glucose and 2-DG have equal affinity for glucose transporters.[10]

Glucose Incorporation Into Lipids and Glycogen

Hemidiaphragms were preincubated for 20 minutes in DMEM (5.6 mmol/L glucose) and then transferred into fresh medium of identical composition but supplemented with [U-^{14}C]glucose (3 μCi/mL) without or with 60 nmol/L insulin. After a 10-minute incubation, muscles were dissected from the rib cage, frozen in liquid N_2, and cut into two segments. One was used for extraction of lipids with chloroform:methanol 2:1 (vol/vol) as previously described.[5] The other was dissolved in 30% KOH (wt/vol) for measurement of label incorporation into glycogen as described in method 2 by Burant et al.[23] DAG and triacylglycerol (TAG) fractions were separated from the total lipid extract by thin-layer chromatography on silica gel 60 thin-layer chromatography plates developed in petroleum ether:ethyl ether (1:1 vol/vol). Bands were identified by their co-migration with authentic standards and then scraped from the plate, and ^{14}C incorporation into each fraction was quantified by liquid scintillation spectrometry. Incorporation of medium glucose into different fractions was calculated based on specific activity of the medium and normalized to the wet weight of tissues.

Choline and Phosphocholine Assay

Diaphragms were incubated and extracted as already described, but the aqueous phase was taken for analysis. Choline and phosphocholine levels were measured using the method reported by Murray et al.[25] Briefly, the extract was supplemented with tracer [^{3}H]choline and [^{14}C]phosphocholine to correct for variability of extraction. Choline and phosphocholine were separated by differential extraction using the ion-pairing reagent sodium tetraphenylboron, and phosphocholine was converted to choline by incubation with alkaline phosphatase. The two choline fractions were phosphorylated by incubation with [γ-^{32}P]adenosinetriphosphate and choline kinase, and the unreacted [γ-^{32}P]adenosinetriphosphate was removed by ion-exchange chromatography. ^{32}P incorporation into phosphocholine was quantified by comparison to standards.

Gas Chromatography–Flame Ionization Detection

A known amount of DAG standard (5 μg 18:0-20:4ω6) previously shown to be absent from muscle (data not shown) was added to lipid extracts to correct for differences in extraction efficiency and for use as a retention time standard. DAG species were identified by their fatty acyl constituents esterified to the first and second glycerol carbons, respectively; fatty acids are listed as number of carbons in the chain: number of carbon-carbon double bonds:ω position of first double bond (when known). The DAG fraction of the lipid extract was separated by thin-layer chromatography. The DAG band (identified by co-migration with a standard) was derivatized to *tert*-butyldimethylsilyl-DAG and analyzed by gas chromatography (GC) with a flame ionization detector (FID) as previously described.[26] The signal from the FID was integrated. For measurements of the mass of each species, the area under each peak was normalized to that of the added standard and expressed per gram muscle.

GC–Mass Spectrometry

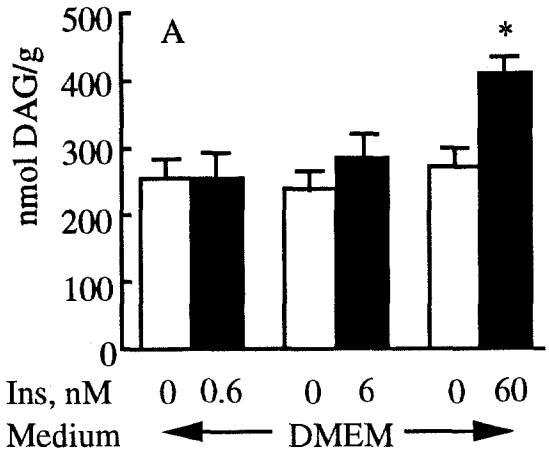
DAG species were derivatized and separated as described for GC-FID. Compounds that eluted from the column were detected by a Hewlett Packard 5780 mass selective detector (Wilmington, DE). To increase sensitivity in the high-mass range (M_r = 650 to 700), the detector was operated in the selected-ion monitoring mode. Data were collected and stored on a Hewlett Packard 2000 series Chemstation computer. The relative retention time (Tr) of compounds was calculated by comparison to the butylated hydroxytoluene peak. Species were identified by their M-57 ions. This fragment is formed upon the loss of the *tert*-butyl group from the *tert*-butyldimethylsilyl moiety.[27]

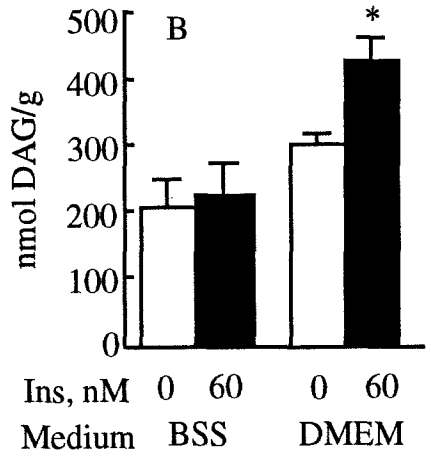
Materials

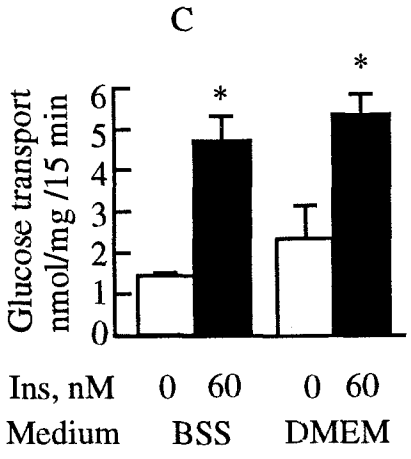
Monocomponent crystalline pork insulin was a gift from Dr Ronald Chance, Eli Lilly Research Laboratories (Indianapolis, IN). OKA was purchased from Kamiya Biomedical (Thousand Oaks, CA) and from LC Services (Woburn, MA). SP-2380 GC columns were from Supelco (Bellefonte, PA). GC-grade hexane, chloroform and methanol were from EM Science (Gibbstown, NJ). *Tert*-butyldimethylchlorosilane/imidazole reagent was from Alltech (Deerfield, IL). Sodium tetraphenylboron, heptan-4-one, and butylated hydroxytoluene were from Sigma (St Louis, MO). Lipid standards were from Serdary Research Laboratories (London, Ontario, Canada). Radiolabeled compounds were from American Radiolabeled Chemicals (St Louis, MO). All other reagents were purchased from previously identified sources.[5]

Data Presentation

Data are presented as the mean ± SE. Statistical analysis of differences between means was performed using Student's *t* test, paired Student's *t* test, or two-way ANOVA, as indicated.







RESULTS

Effect of Insulin on Total DAG

Insulin stimulates glucose transport in hemidiaphragms half-maximally at a dose of approximately 0.6 nmol/L and maximally at approximately 6 nmol/L.[18] We previously reported that muscle DAG increases after a 10-minute incubation with 60 nmol/L insulin.[5] In isolated skeletal muscle cells, glucose transport increases within 2.5 minutes after exposure to insulin.[28] To examine the correlation between insulin's effects on DAG content and glucose transport, we measured the time course and insulin dose-response of increases in DAG mass in hemidiaphragms. Insulin increased DAG content by 20% to 30% at the highest dose tested (60 nmol/L) during a 10-minute incubation (Figs 1A and 2b), but not at lower doses (Fig 1A) or after a 5-minute incubation (Fig 2a). DAG mass remained above basal levels after a 20-minute incubation with 60 nmol/L insulin (Fig 2c).

To determine whether insulin increases DAG mass in the absence of glucose, hemidiaphragms were incubated in either glucose-containing DMEM or glucose-free BSS supplemented with pyruvate. A 10-minute exposure to 60 nmol/L insulin did not affect DAG mass in hemidiaphragms incubated in BSS (Fig 1B). The response of muscles incubated in DMEM, which was measured in parallel (Fig 1B) as a positive control for the glucose-free experiments, was similar to that seen in Fig 1A. Insulin increased the rate of 2-DG transport similarly (~threefold) in muscles incubated with or without glucose (Fig 1C).

Effect of Insulin on DAG Species

To examine whether insulin selectively increases the concentration of an individual DAG species, we separated and identified five major DAG species in rat diaphragms. Although individual fatty acids found in DAG isolated from hindlimb muscle have been reported,[20] the intact DAG species from muscle have not been identified. The identity of these species was determined by GC-FID or GC-MS as described earlier. The DAG fraction separated into five peaks on GC-FID, designated A through E in Table 1 and on the sample GC-FID chromatogram shown in Fig 3. The Trs of the five species were consistent (standard deviation <0.5%).

Fig 1. Effect of insulin (Ins) on diaphragm DAG content. (A) Ins dose-response of DAG. Hemidiaphragms were preincubated in DMEM without additions for 30 minutes, and vehicle was added to one and Ins at the concentrations indicated to the other from the same rat. After 10 minutes, muscles were rinsed, rapidly excised from the rib cage, and frozen in liquid nitrogen. (B) Effect of Ins on DAG in muscles incubated with or without glucose. Hemidiaphragms were preincubated for 30 minutes in glucose-free BSS or in DMEM (5.6 mmol/L glucose), 60 nmol/L Ins or vehicle was added, and after 10 minutes muscles were frozen. In both studies A and B, DAG was extracted and measured. (C) Effect of Ins on 2-DG transport in diaphragms incubated without or with glucose. Hemidiaphragms were preincubated for 30 minutes in either BSS or DMEM without or with 60 nmol/L Ins; 2-DG uptake was measured in a subsequent 15-minute assay in identical media supplemented with 2-[1,2-^{3}H]DG and [U-^{14}C]sucrose. Data in studies A through C are the mean ± SE, n = 4 to 6. *Insulin > basal, $p < .05$ by Student's *t* test.

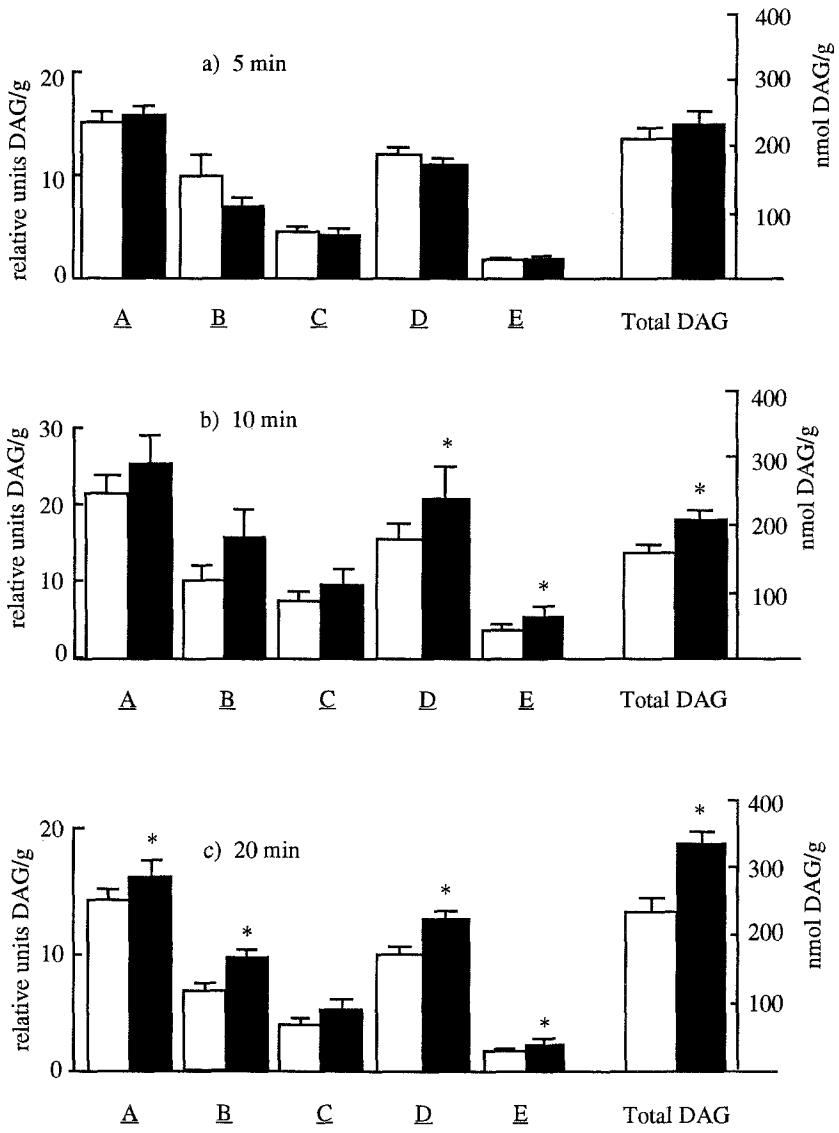


Fig 2. Time course of insulin's effect on total DAG mass and DAG species. Hemidiaphragms were preincubated for 20 minutes in DMEM and then transferred to DMEM without (□) or with (■) 60 nmol/L insulin for 5 (a), 10 (b), or 20 (c) minutes. Muscles were frozen, and DAG species (A through E) and total DAG contents were quantified (species by GC-FID, total DAG by the DAG kinase assay). Data are the mean ± SE in relative units (species) or nmol/g (total DAG) from at least 6 observations. Total DAG and species contents were measured in separate experiments and are displayed together to facilitate comparison of the insulin effect on each parameter. Relative units of DAG species were derived from the area under each chromatographic peak, normalized to that of the standard DAG and expressed per g muscle. *Insulin > basal, $P < .05$ by paired Student's t test.

Table 1. Identification of Muscle DAG Species by GC

Peak	Tr		Ions	Species
	GC-FID	GC-MS		
A	0.575*	3.8*	M-57	16:0-18:1ω9
B	0.588	NS		16:0-18:1ω7
C	0.732*	3.05*	M-57	18:0-18:1ω9
D	0.765*	4.26*	M-57	18:0-18:2ω6
E	0.800	4.35	M-57 (A, B, C, D)	18:1-18:2

NOTE. Total lipids were extracted from rat skeletal muscle, and DAG was separated by thin-layer chromatography and derivatized. DAG species were then separated by injection onto a GC column. DAG standards were treated similarly. Tr of each peak was derived by comparison to the internal standard.

Abbreviations: NS, not separated; A, R_1CO; B, R_2CO; C, $R_1CO + 74$; D, $R_2CO + 74$.

*Identified by co-elution with DAG standards.

As shown in Table 1, three of the five species (A, C, and D) were identified by comparing their Trs with those of standards. Species B was tentatively identified using the extensive list of standards reported by Pessin and Raben[26] and Myher and Kuksis.[27] They show that 16:0-18:1ω7 elutes at 1.02 to 1.03 times the Tr of 16:0-18:1ω9. This is the same proportion found between species A and B.

The identity of species A, C, and D was verified by GC-MS. They eluted with Trs similar or identical to those of known standards (Table 1). Species E was identified by the co-elution of the M-57 ion with fragments of molecular weights corresponding both to the carbonyl form of fatty acids 18:1 and 18:2 (R_1CO and R_2CO, respectively) and to the acid form of fatty acids bound to the glycerol moiety ($R_1CO + 74$ and $R_2CO + 74$). Although the position of the fatty acid on the glycerol is not determined by this method, a DAG species with the less-saturated fatty acid at the C1 position would be unusual.

As a positive control for studies of insulin's effects,

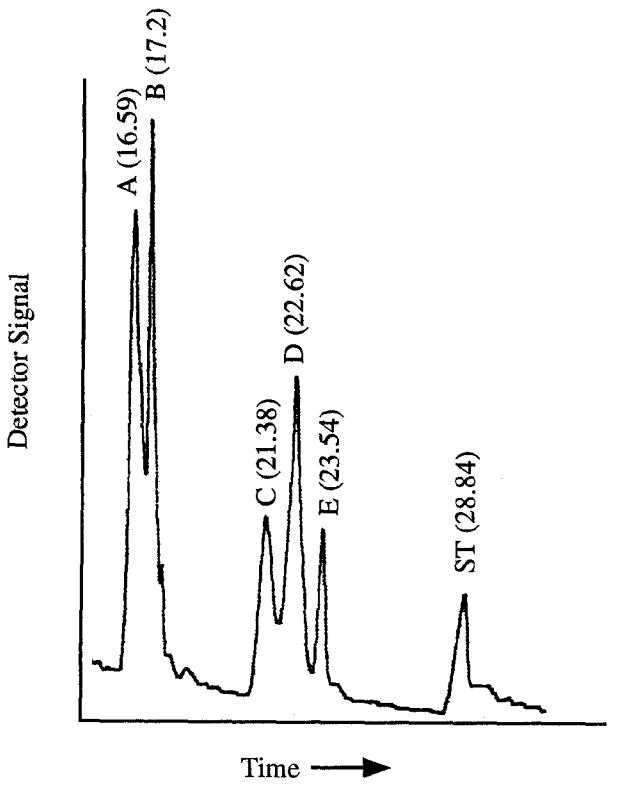


Fig 3. Separation of muscle DAG species by GC-FID. Lipids were extracted from diaphragm muscle, and DAG was isolated by thin-layer chromatography, derivatized, and separated by GC-FID. The plot is expressed as FID signal *v* time. Material that eluted before 16 minutes was also found in blank runs. The 5 peaks labeled A through E are as they appear in Table 1, with Tr in parentheses. Standard (ST) DAG (18:0-20:4ω6) was included in each assay.

hemidiaphragms were incubated for 30 minutes with PLC (from *Clostridium perfringens,* 0.5 U/mL) and DAG species were quantified by GC-FID. Each species increased significantly (mean ± SE, % increase over basal: species A, 40 ± 16; B, 96 ± 26; C, 128 ± 30; D, 63 ± 16; E, 74 ± 28; n = 7, $P < .05$). To study the effects of insulin on relative abundance of each species, muscles were incubated in DMEM without or with 60 nmol/L insulin for 5, 10, or 20 minutes and species were quantified by GC-FID. No species was increased significantly after a 5-minute exposure to insulin (Fig 2a). After 10 minutes (Fig 2b) all species showed some increases, but only species D and E had increased significantly. After 20 minutes (Fig 2c) species A, B, D, and E were significantly increased. Insulin-mediated increases in tissue concentrations of individual DAG species were relatively small (10% to 40% of basal) and similar to the increase in total DAG mass. Although it is possible that insulin induces changes below the sensitivity of the assay (coefficient of variation, ~30%), these data indicate that no species or subgroup of DAG accounts for most of the insulin-stimulated increase in total DAG content. Furthermore, they do not suggest a DAG species-specific activation of glucose transport, since no species was increased after only a 5-minute exposure to insulin.

Effect of Insulin on Choline and Phosphocholine Content of Muscle

To assess adequacy of the analytical methods in skeletal muscle, concentrations of choline and phosphocholine were measured in rat hemidiaphragms after a 30-minute incubation without or with PLC (0.5 U/mL). The concentration of phosphocholine increased 87% in PLC-treated muscles, and no change in choline content was observed (Fig 4A). The PLC-induced increase in phosphocholine was similar in magnitude to that previously reported for total DAG in muscle.[5] The effect of insulin on choline and phosphocholine content was then measured. Under conditions where insulin increased DAG mass (Fig 1), there was no increase in either of these compounds (Fig 4B). Therefore, hydrolysis of the predominant phospholipid in muscle apparently did not account for the insulin-stimulated increase in DAG after a 10-minute incubation with 60 nmol/L insulin.

Insulin Regulation of Glucose Flux in Muscle

Diaphragm muscles were incubated with [U-^{14}C]glucose without or with 60 nmol/L insulin for 10 minutes as described earlier, and lipid and glycogen labeling were measured (Fig 5). Insulin stimulated ^{14}C incorporation into DAG, TAG, and total lipids approximately threefold (Fig 5A, B, and C) and glycogen synthesis approximately 10-fold (Fig 5D).

Effect of OKA on DAG Mass in Diaphragm

OKA stimulates glucose transport in muscle, although a maximal response requires 45 to 50 minutes.[17,18] Muscles were incubated for 50 minutes without or with varying OKA concentrations, and total DAG content was measured. OKA at 0.5 and 1 μmol/L increased DAG mass in diaphragms to a degree similar to that of insulin (Table 2). However, also like insulin, a higher concentration of OKA (500 nmol/L) was required for the DAG response than for half-maximal stimulation of glucose transport (~100 nmol/L[18]).

To determine whether OKA increases de novo synthesis of DAG, we performed labeling studies similar to those for insulin. OKA (1 μmol/L) increased [^{14}C]glucose incorporation into DAG, TAG, and total lipids (Fig 6), but as previously reported,[18] no significant effect on glucose incorporation into glycogen was detected.

DISCUSSION

Insulin receptor–activated signal-transduction mechanisms are difficult to study because the hormone regulates such diverse aspects of metabolism. This renders it difficult to discriminate between downstream products of insulin-activated pathways and authentic second messengers. In the current report, we have sought to clarify the role of DAG in insulin signaling.

We identified five DAG species in muscle. Although Pessin and Raben identified 25 species in fibroblasts,[26] Cleland et al[20] identified only three fatty acids (16:0, 18:0, and 20:4ω6) in DAG from rat hindlimb muscle. The first two species were identified in peaks A and B (16:0) and C

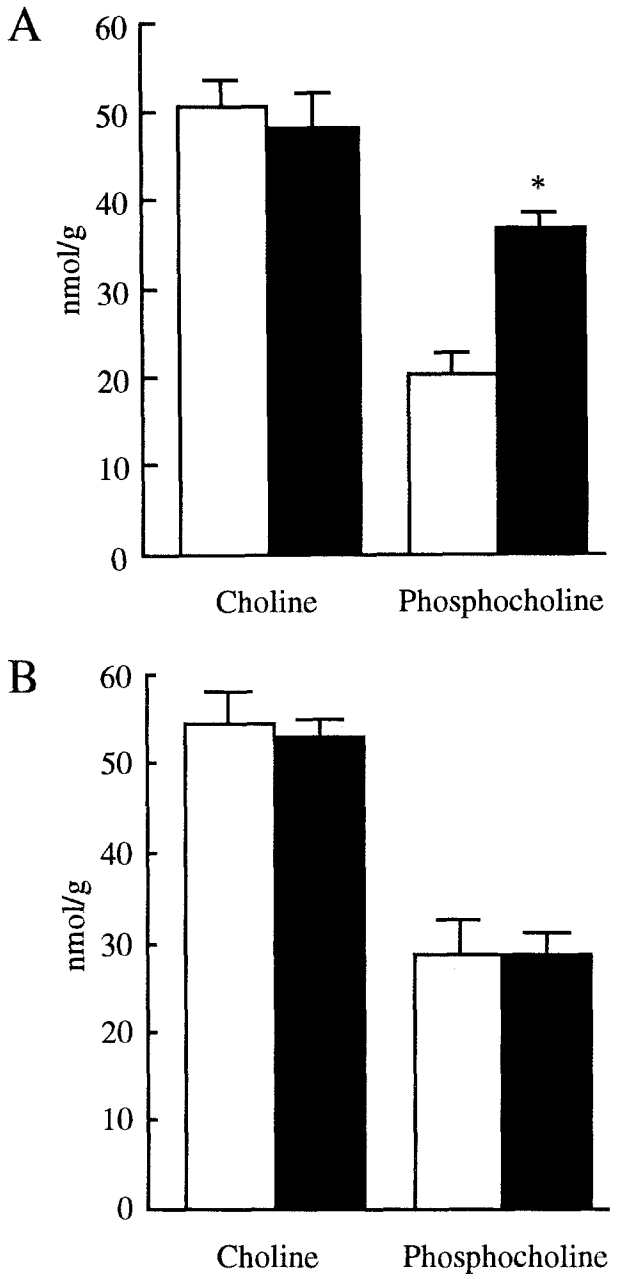


Fig 4. Effect of PLC or insulin on muscle choline and phosphocholine content. (A) Hemidiaphragms (n = 3) were incubated for 30 minutes in DMEM without (□) or with (■) PLC (0.5 U/mL, from *Clostridium perfringes*). (B) Hemidiaphragms (n = 11) were preincubated for 30 minutes in DMEM without additions. Muscles were then transferred to fresh DMEM without (□) or with (■) 60 nmol/L insulin for 10 minutes. After incubation, tissues were frozen and powdered, and choline and phosphocholine were extracted and measured. Results are the mean ± SE.

and D (18:0), respectively. In addition, 18:1ω9 was identified in peaks A and C, 18:1ω7 in peak B, and 18:2ω6 in peak D. However, arachidonic acid was not identified in any of the five major DAG species isolated. Differences in DAG composition may reflect, in part, differences in fat composition of the diets. Arachidonic acid is likely present in minor DAG species that were not identified by our methods. Furthermore, hindlimb and diaphragm DAG species may differ in composition. We did not detect any of the species reported to stimulate glucose transport in adipocytes in vitro.[9]

In this study, insulin increased DAG mass in rat hemidiaphragms after 10 and 20 minutes, but not before. In a previous report,[3] total DAG content increased within 2 minutes after exposure of rat solei or hemidiaphragms to insulin in vitro. Although the reason for this discrepancy is not clear, it may reflect methodologic differences in tissue processing. In the present study, muscles were frozen immediately after incubation and the frozen muscle powder was weighed, homogenized for 30 seconds, and extracted at 4°C.[5] In isolated skeletal muscle cells, glucose transport is activated by insulin within 2.5 minutes.[28] In the current report, no DAG species had increased after a 5-minute exposure to insulin. The time course of insulin's effect on DAG species reflects the overall response of the whole tissue. However, we cannot rule out the possibility that some DAG species increased transiently as insulin diffused to each muscle-cell layer or that an early increase in a DAG species or in a subcellular compartment escaped detection.

Insulin increased muscle DAG content at a supraphysiologic concentration (60 nmol/L), but no significant increase in DAG was observed in response to 0.6 or 6.0 nmol/L insulin, doses that cause half-maximal and maximal stimulation of glucose transport, respectively.[8] Skeletal muscle cells, like adipocytes, have "spare" insulin receptors, ie, maximal stimulation of glucose transport requires activation of only a fraction of the cell-surface receptors.[29] Detection of insulin-induced increases in muscle DAG may require more complete receptor occupancy.[6] Insulin at 60 nmol/L activates most of the cell surface receptors in muscle in 5 minutes in vivo.[29]

Consistent with the observations of Heydrick et al[10] in rat soleus, we observed an insulin-stimulated approximately threefold increase in glucose incorporation into DAG, TAG, and total lipids in rat hemidiaphragms. However, others found little insulin-stimulated incorporation of glucose carbon into DAG in soleus muscle under conditions where insulin increased both glucose transport and incorporation of glycerol into DAG.[30] In the latter study, soleus muscles from rats that weighed 100 to 150 g were incubated; the thickness of such muscles may limit O_2 diffusion, which results in an anoxic core and concomitant metabolic alterations.[31,32] In rodent muscles, insulin stimulates glycolytic flux less than glucose transport or glycogen synthesis; maximal stimulation of glycolysis by insulin is twofold or less.[10,17] Thus, the ≥ threefold increases in glucose incorporation into DAG and other lipids (Fig 5 and Heydrick et al[10]) suggest that insulin activates lipid synthetic enzymes distal to glycolysis in muscle. The insulin dose-response of muscle DAG content supports this hypothesis, because the dose required to increase DAG was greater than that required for maximal stimulation of glucose transport. Insulin-mediated activation of glycerol-phosphate acyltransferase, a rate-limiting enzyme in lipid synthesis, has been suggested.[11]

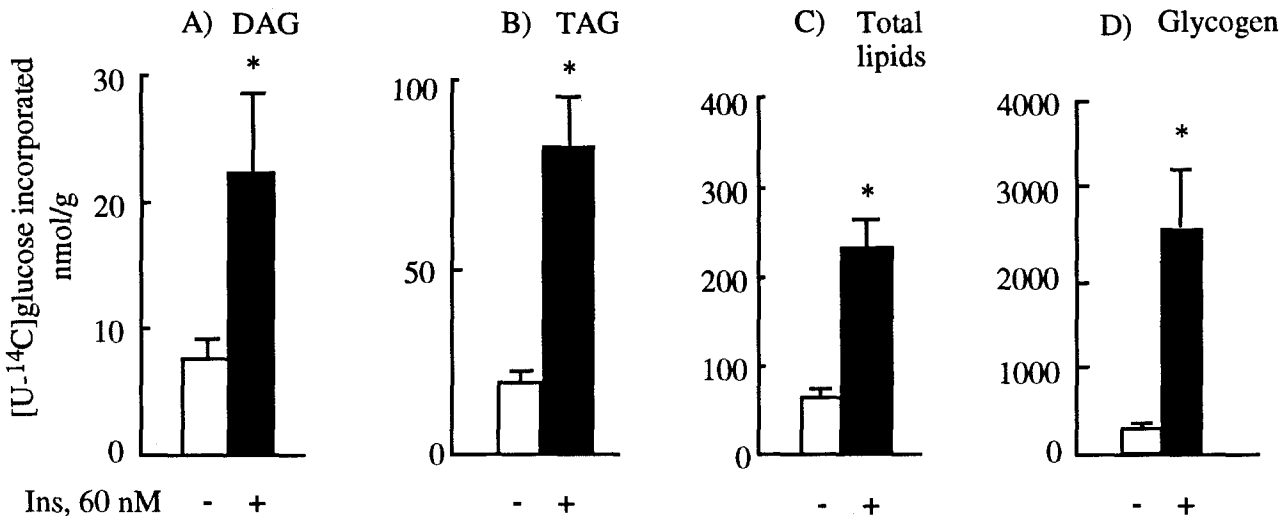


Fig 5. Effect of insulin on [U-^{14}C]glucose incorporation into lipids and glycogen. Hemidiaphragms were preincubated for 30 minutes in DMEM (5.6 mmol/L glucose) and then transferred to identical media supplemented with [U-^{14}C]glucose without (□) or with (■) 60 nmol/L insulin for 10 minutes. After incubation, muscles were rinsed with ice-cold DMEM, dissected from the ribs, frozen in liquid N_2, and cut into two approximately equal portions, one for lipid studies and the other for glycogen measurement. (A) DAG and (B) TAG were extracted, separated by thin-layer chromatography, scraped from the plates, and counted. An aliquot of the lipid extract was saved for measurement of glucose incorporation into (C) total lipids. Tissues saved for (D) glycogen measurements were solubilized, and glycogen was precipitated and washed, and [U-^{14}C]glucose incorporated into glycogen was quantified by liquid scintillation spectrometry. Data are expressed as nmol medium glucose incorporated per g muscle (mean ± SE, n > 6). *Insulin > basal, $P < .05$ by ANOVA.

The insulin-mediated increment in muscle DAG content shown in Figs 1 and 2 was, on average, approximately 100 nmol/g muscle/10 min, whereas [^{14}C]glucose incorporation into DAG increased by approximately 15 nmol/g muscle/10 min (Fig 5). Since each DAG molecule contains only half the carbons of [U-^{14}C]glucose, glucose incorporation into DAG would account for approximately 30% of the insulin-mediated increase in DAG if the specific radioactivities of glucose in the medium and of intracellular glycerol-3-phosphate were equal. However, since labeled glucose was added to the medium at the same time as insulin, the intracellular precursor pool was being labeled during the experiment and would likely not have reached isotopic equilibrium even at the end of the 10-minute incubation. De novo synthesis from glucose could account for the observed insulin-mediated increase in DAG content only if the median specific radioactivity of the intracellular precursor pool was ≤ approximately 30% of the specific radioactivity of glucose in the medium.

Table 2. Effect of OKA on DAG Content in Muscle

OKA (nmol/L)	DAG (nmol/g)	Increase (% of basal)	No. of Observations
0	347 ± 22.8	4.3 ± 10.0	4
100	362 ± 26.7		
0	319 ± 24.4	23.6 ± 8.1*	8
500	388 ± 26.4		
0	361 ± 69.4	43.5 ± 11.8*	4
1,000	525 ± 128.6		

NOTE. Hemidiaphragms were incubated in DMEM for 50 minutes. One hemidiaphragm from each rat was treated without and the other with OKA at concentrations indicated. After incubation, muscles were frozen and DAG content was measured by the kinase assay. Data are the mean ± SE.

*OKA > basal, $P < .05$ (paired Student's t test).

In previous studies, insulin increased DAG in solei incubated in glucose-free BSS[5]; however, in diaphragm, the insulin-stimulated increase in DAG was only apparent after incubation in glucose-containing medium (Fig 1B). Glycogen stores in solei of young rats are approximately twofold greater than in diaphragm.[23,33] Therefore, endogenous glucose may have served as a substrate for DAG synthesis in solei incubated without glucose. The fact that in diaphragms the insulin-mediated DAG increase required medium glucose would mitigate against DAG's role as a primary mediator of insulin-stimulated glucose transport, because the response of 2-DG transport was similar in muscles incubated with or without glucose. Glucose-free media are routinely used for measurements of insulin-stimulated transport of nonmetabolized glucose analogs[5,18,19,23,33] in various muscles, including diaphragms.[18,33]

The role of DAG/protein kinase C (PKC) signaling in insulin action remains controversial. Insulin stimulates PKC translocation to the plasma membrane[4,10] and phosphorylation of endogenous PKC marker substrates in skeletal muscle.[4] PKC activation may serve as a positive modulator of insulin action, eg, by increasing glucose transport, and/or as a negative downstream modulator involved in the termination of insulin's signal. Indeed, both effects may be promoted through phosphorylation of separate PKC substrates with disparate temporal responses. The role of DAG/PKC in stimulation of muscle glucose transport is supported, for example, by reports showing that PLC, an enzyme that increases endogenous DAG levels, stimulates glucose transport in muscle.[5,19] However, the effect of PLC on glucose transport has been suggested to

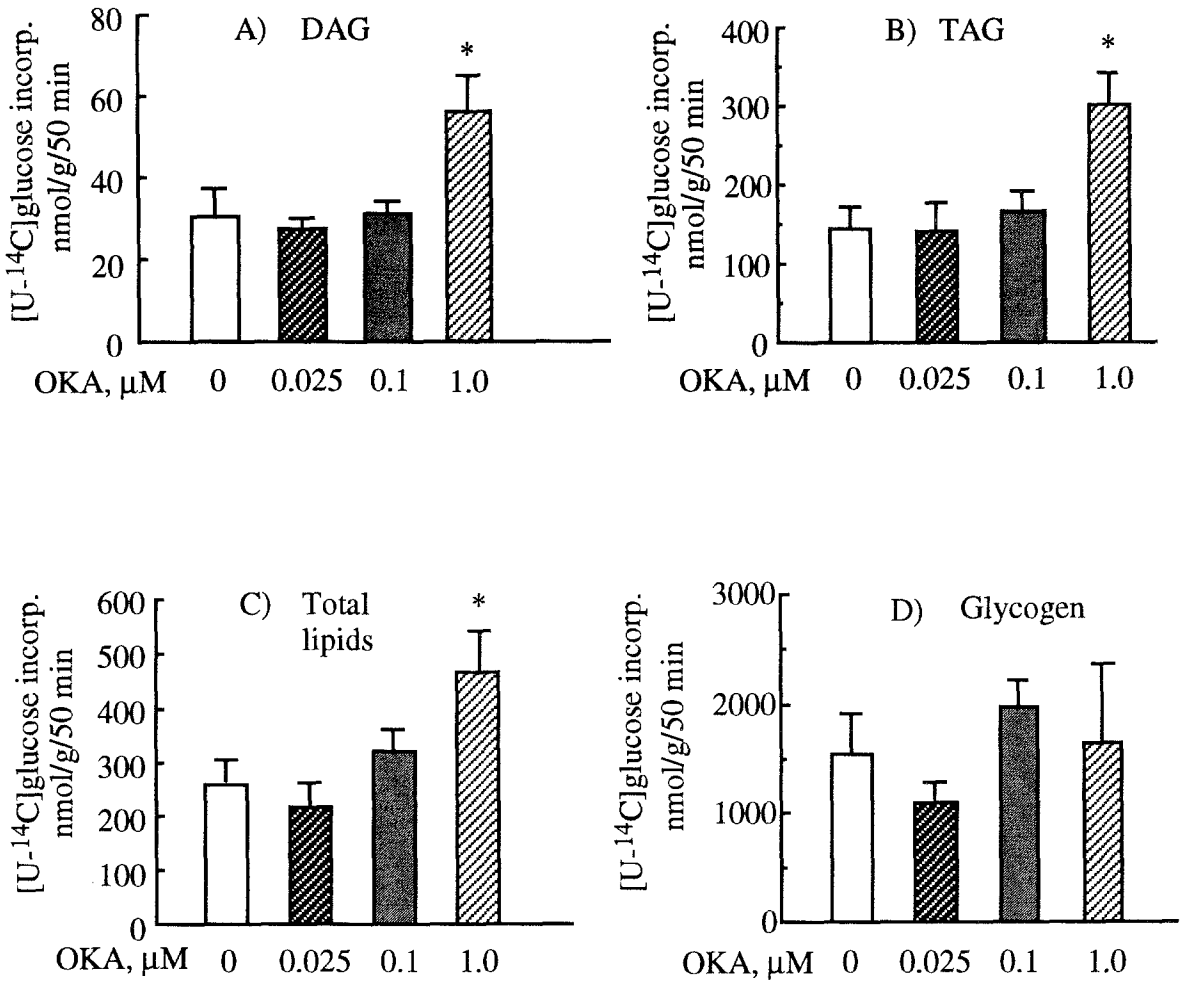


Fig 6. Effect of OKA on [U-^{14}C]glucose incorporation into lipids and glycogen. Hemidiaphragms attached to ribs were incubated in DMEM supplemented with [U-^{14}C]glucose without or with OKA at the indicated doses for 50 minutes. Muscles were then rinsed, cut from the ribs, and frozen. Glucose incorporation into each fraction DAG (A), TAG (B), total lipids (C), and glycogen (D) was estimated as described in Fig 5. Data are expressed as nmol medium glucose incorporated per g muscle (mean ± SE, n = 6). *OKA > basal, $P < .05$ by ANOVA.

mimic an exercise/anoxia-stimulated pathway rather than that regulated by insulin.[19] On the other hand, DAG/PKC may act to limit the physiologic response to insulin stimulation through effects on the insulin receptor itself. Ser/Thr phosphorylation of the insulin receptor inhibits its tyrosine kinase activity.[34] Consistent with this, several PKC isozymes inhibit insulin-activated tyrosine phosphorylation of an endogenous protein substrate of the insulin receptor, phosphatidylinositol 3-kinase.[35] Pre-exposure of muscles to PLC diminishes insulin-mediated in vivo activation of insulin receptor tyrosine kinase, which may reflect PLC-mediated PKC activation.[5] In fibroblasts that overexpress the human insulin receptor, glucose-mediated inhibition of insulin receptor tyrosine kinase activity is paralleled by translocation of several PKC isoforms and prevented by treatment with PKC inhibitors.[36] PKC activation inhibits glycogen synthase[19] and glucose incorporation into glycogen[24] in muscles.

PC is the most abundant phospholipid in muscle membranes[20]; its hydrolysis is a source of DAG upon stimulation of several G-protein–linked receptors.[37] In several models, agonist-induced PC hydrolysis is a delayed and sustained response, which follows the more immediate and transient hydrolysis of phosphatidylinositol phosphates (reviewed in Exton[37]). Although some evidence indicates that insulin may transiently stimulate hydrolysis of the latter in adipocytes,[38-40] this is still controversial in adipocyte, and has not been observed in skeletal muscle (reviewed in Exton[15]). We found no evidence for insulin-stimulated PC hydrolysis in muscle, ie, no increases in phosphocholine or choline were observed in response to insulin at a time when DAG was increased. Possibly, the former metabolites were used along with DAG to resynthesize PC in the de novo synthesis (Kennedy) pathway. However, following a single burst of electrically induced muscle contractions, DAG mass in muscle remains elevated for 30 minutes or longer.[20] Insulin-stimulated PC hydrolysis has been reported in frog oocytes,[41] C-6 glioma cells,[42] and cultured myocytes, rat adipocytes, and hepatocytes.[12-14] In the latter,[12-14] the insulin effect was rapid and transient; it may be mediated by

activation of phospholipase D.[14] Such small, transient effects would be difficult to demonstrate in intact incubated muscles, where insulin permeates by diffusion and interacts with different cell layers asynchronously.

Initially, we speculated that stimulation of glucose transport by OKA is mediated by increased DAG. Indeed, OKA increased the DAG content of hemidiaphragms. However, the dose required (0.5 μmol/L) was fivefold greater than that required for half-maximal stimulation of glucose transport in rat hemidiaphragms and 20-fold greater than the minimal dose that significantly stimulates glucose transport.[18] This dose-response suggests that OKA-stimulated glucose transport is not mediated through increased DAG content. In adipocytes, OKA stimulates glucose transport, lipolysis (via activation of hormone-sensitive lipase), and glucose incorporation into total lipids[16]; the latter may reflect, in part, re-esterification of fatty acids with glucose-derived glycerol. OKA mimics or potentiates the action of numerous protein kinases, including PKC, which in turn may activate PC phospholipases and increase DAG.[37] Furthermore, reversible phosphorylation modulates activity of regulatory enzymes in lipid synthesis,[43] which may contribute to the effects of OKA on muscle DAG content and to the increased flux of glucose into DAG, TAG, and total lipids.

In conclusion, the relatively slow insulin- and OKA-induced increases in muscle DAG content observed here likely reflect primarily downstream effects of changes in glucose transport and metabolism, which result in increased glucose flux into DAG. However, we cannot rule out the possibility of a biphasic response to insulin.[12-14] An early, transient increase in DAG that arises from PC hydrolysis may escape detection in this model.

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