Insulin Increases Distinct Species of 1,2-Diacylglycerol in Isolated Perfused Rat Heart

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Insulin and glucose increase the synthesis of 1,2-diacylglycerol (1,2-DAG), the physiological activator of protein kinase C (PKC) in a variety of tissues and cells. The effects of insulin and glucose on the abundance and fatty acid composition of 1,2-DAG were investigated in isolated perfused rat hearts with the use of capillary gas chromatography and 1,2-dipentadecanoin as an internal standard. A high concentration of insulin (25 mU/mL) significantly increased cardiac contractility and reduced coronary flow. In addition, perfusion with 25 mU/mL insulin induced significant increases of 18.2% and 26.4% in 1,2-DAG mass after 5 and 30 minutes, respectively, in the presence of 8.6 mmol/L glucose, whereas there was no increase in 1,2-DAG with 2.5 mU/mL insulin. Analysis of the fatty acid composition of 1,2-DAG showed that only species containing specific fatty acids (16:0, 18:1, and 18:2) were increased in response to insulin. In contrast, an increase in glucose concentration in the perfusion medium from 3 to 17 mmol/L had no effect on the total mass or fatty acid composition of 1,2-DAG, cardiac contractility, or coronary flow. Addition of a high insulin concentration to the high-glucose medium increased the abundance of 1,2-DAG containing 16:0, 18:1, and 18:2 fatty acids, as well as cardiac contractility. It is concluded that the effect of insulin on cardiac contractility may be related to the associated increase in 1,2-DAG abundance.

SOME HORMONES and neurotransmitters increase the content of 1,2-diacylglycerol (1,2-DAG), the physiological activator of protein kinase C (PKC), in their target cells. It is believed that increased activity of membrane-associated PKC in vivo is attributable to an increased abundance of 1,2-DAG in the affected cells.[1] PKC regulates vascular permeability,[2] contractility,[3] and proliferation,[4,5] as well as cardiac contractility[6] and hypertrophy,[7] and the abnormal cardiovascular properties associated with diabetes mellitus appear to be attributable in part to increased PKC activity.[8,9]

High glucose concentrations have been shown to increase 1,2-DAG in cultured cells.[9-11] The glucose-induced increase in 1,2-DAG abundance has been proposed to be mediated by hydrolysis of inositol phospholipids by phospholipase C,[10] de novo synthesis,[12-14] and hydrolysis of phosphatidylcholine.[10] Insulin also increases 1,2-DAG in cultured cells[15] and tissues,[16,17] although the exact source of 1,2-DAG has not been identified. There is no evidence that insulin increases the amount of 1,2-DAG in the heart.

Previously, we[18] and others[9,19] have shown that total 1,2-DAG mass is chronically increased in the myocardium of streptozotocin-induced diabetic rats. Such abnormalities in lipid metabolism may be related to the pathogenesis of diabetic cardiomyopathy. This animal model shows insulin deficiency and high concentrations of blood glucose. We have now investigated the effects of insulin and high glucose on 1,2-DAG generation in isolated perfused normal rat hearts. We analyzed 1,2-DAG and its fatty acids using capillary gas chromatography with 1,2-dipentadecanoin as an internal standard, instead of using the radioenzymatic method described by Preiss et al.[20]

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MATERIALS AND METHODS

Perfusion Techniques

Male Wistar rats with a body mass of 250 to 350 g were fed a standard pellet diet with water ad libitum. The animals were killed by decapitation 20 minutes after heparinization. After thoracotomy, the hearts were quickly excised, placed in ice-cold oxygenated Krebs-Henseleit solution, and perfused retrogradely by aortic cannulation according to the method of Langendorf. The perfusion medium was Krebs-Henseleit buffer (pH 7.4) containing 120 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.25 mmol/L $MgSO_4$, 1.25 mmol/L $CaCl_2$, and 8.6 mmol/L glucose, unless otherwise indicated. This solution was gassed continually with 95%/O_2 5% CO_2 and maintained at 37°C. The perfusate was delivered to the aortic inflow cannula at a pressure of 80 cm H_2O. After perfusion for a 25-minute period of stabilization, two groups were perfused with the same solution containing 3 or 8.6 mmol/L glucose (controls), and the other groups were subjected to insulin (Insulin Actrapid Human; Novo-Nordisk, Bagsvaerd, Denmark) or high-glucose conditions, as indicated. Immediately after perfusion, the hearts were frozen in liquid N_2 and stored at −70°C until lipid analysis.

To measure contractile force, the apex of the left ventricular free wall was connected by a hook with a silk thread. A resting tension of 2 g was applied to the heart at the start of perfusion. Isometric tension was recorded on a polygraph recorder (Recticorder; Nihon Kohden, Tokyo, Japan) by means of a force-displacement transducer (TB-611T; Nihon Kohden).

Coronary flow was determined by collecting the coronary sinus effluent every 5 minutes throughout the experiment.

Determination of 1,2-DAG

Each heart was freeze-dried, and approximately 25-mg tissue samples from the left ventricle near the apex were placed in 5 mL ice-cold chloroform:methanol (2:1 vol/vol) containing 0.005% butylated hydroxytoluene as an antioxidant and 4 μg 1,2-dipentadecanoin (Nu-Chek-Prep, Elysian, MN) as an internal standard. The tissue was homogenized for 20 seconds in a motor-driven glass homogenizer kept on ice.[21] The homogenate was

maintained on ice for 30 minutes and then passed through a paper filter. The filtrate was evaporated to dryness under a stream of N_2 at 40°C. The dried lipids were resuspended in 30 μL chloroform: methanol (2:1 vol/vol) and applied to precoated silica gel plates (20 × 20 cm, Kieselgel 60 F_{254}; Merck, Darmstadt, Germany). The plates were developed twice in a solvent system of n-hexane: diethyl ether:acetic acid (80:35:1 vol/vol/vol). The marker 1,2-diolein was applied to the same plate. The area corresponding to 1,2-DAG was identified by exposure of only 1,2-diolein to iodine vapor and scraped into 2 mL chloroform:methanol (9:1 vol/vol).[21] The extract was evaporated to dryness with a stream of N_2 gas, and the fatty acyl moieties in this fraction were transmethylated with boron trifluoride-methanol as described by Morrison and Smith.[22] Methyl fatty acids were analyzed on a gas chromatograph (model GC 14-A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a HR-SS-10 fused silica capillary column (30 m × 0.25 mm internal diameter, Shinwakakoh, Kyoto, Japan). Peaks were identified by comparison to standards (Nu-Chek-Prep), and the peak areas were calculated with a Chromatopac C-R6A integrator (Shimadzu). The total amount of 1,2-DAG was calculated from the integrated signal of the gas chromatograph. This method provides the exact amount of 1,2-DAG in the myocardium expressed as each fatty acid component of equivalent amounts to its 1,2-DAG, although the fatty acid position on the glycerol moiety and the precise molecular species of 1,2-DAG were not identified.

Statistical Analysis

Results are expressed as the mean ± SEM. Analysis of the statistical significance of the mean change or percentage change from control values was performed using the Kruskal-Wallis *H* test to assess the presence of significant intergroup nonhomogeneity. The Mann-Whitney *U* test was used for further comparisons between control and treatment groups. *P* less than .05 was considered statistically significant.

RESULTS

Effects of Insulin and Glucose on Heart Performance

After a 25-minute period of stabilization, resting tension decreased from 2 g to approximately 1.7 g. In response to a high concentration (25 mU/mL) of insulin in the presence of 8.6 mmol/L glucose, a decrease in resting tension (Fig 1A) was accompanied by a significant increase in cardiac muscle contractility as indicated by developed force (Fig 1B) and its maximal first derivative with respect to time (dF/dt or −dF/dt), and a significant decrease in coronary flow (Fig 2A) with no significant change in heart rate (Fig 2B). In contrast, perfusion medium containing 2.5 mU/mL insulin induced only small effects on the performance of isolated hearts during the 30-minute perfusion period. After a 25-minute perfusion in the presence of 3 mmol/L glucose, increasing the concentration to 17 mmol/L had no effect on heart performance (Figs 1 and 2). Increasing the glucose concentration to 17 mmol/L and adding 25 mU/mL insulin resulted in increases in developed force and maximal dF/dt and −dF/dt and a decrease in resting tension, similar to the effect of insulin at this concentration in the presence of 8.6 mmol/L glucose.

The percent change in heart performance parameters 30 minutes after adding insulin or increasing the glucose concentration, relative to values after the stabilization

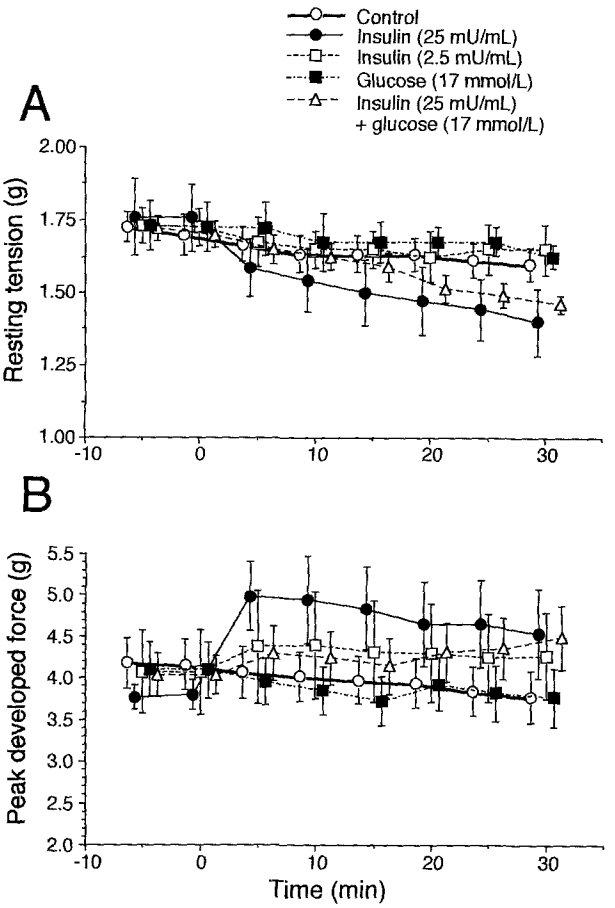

Fig 1. Time course of the effects of insulin and glucose on resting tension (A) and peak developed force (B) of isolated perfused rat hearts after a 25-minute stabilization period. Control and insulin groups were perfused in the presence of 8.6 mmol/L glucose. Glucose and insulin + glucose groups were perfused in the presence of 17 mmol/L glucose, after perfusion with 3 mmol/L glucose during the stabilization period. Test perfusions began at time 0. Each point represents the mean ± SEM for 6 or 7 hearts.

period, are shown in Figs 3 and 4. The high concentration of insulin (25 mU/L) induced significant increases in peak developed force of 32.3% and 17.8% without and with an increase in glucose concentration, respectively. Maximal dF/dt and −dF/dt were also increased by the addition of insulin, regardless of glucose concentration. The increase in maximal −dF/dt was greater than the increase in dF/dt, indicating that insulin improves diastolic function more effectively than systolic function. The low concentration of insulin (2.5 mU/mL) also increased maximal dF/dt (16.5%) and −dF/dt (25.7%). Heart performance at 30 minutes was affected by increasing the glucose concentration of the perfusate.

Effects of Insulin and Glucose on 1,2-DAG Mass and Fatty Acid Composition

Addition of 25 mU/mL insulin to the perfusion medium increased total 1,2-DAG mass by 18.2% (499 ± 22 *v* 422 ± 15 ng/mg dry weight, $P < .05$) after 5 minutes; after

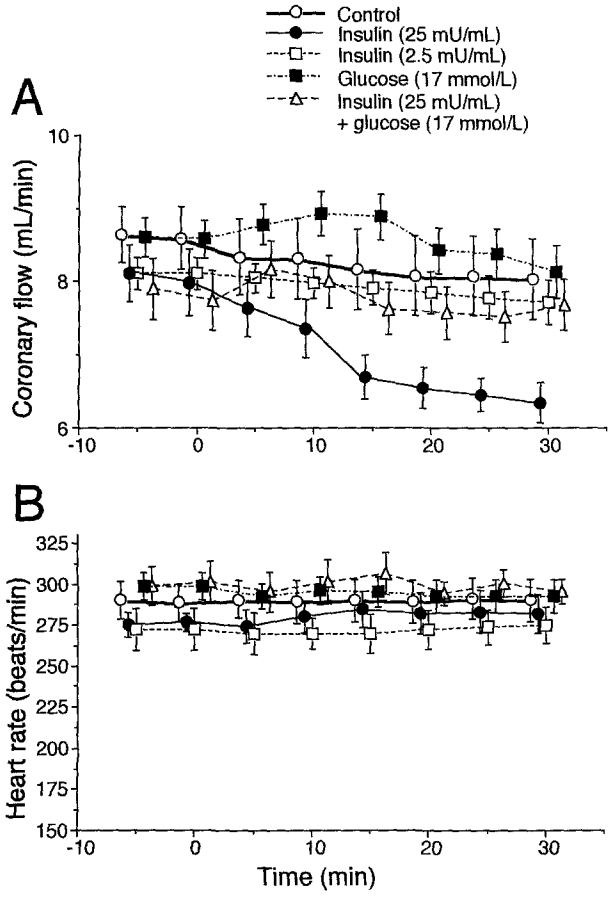

Fig 2. Time course of the effects of insulin and glucose on coronary flow (A) and heart rate (B) of isolated perfused rat hearts after a 25-minute stabilization period. Control and insulin groups were perfused in the presence of 8.6 mmol/L glucose. Glucose and insulin + glucose groups were perfused in the presence of 17 mmol/L glucose, after perfusion with 3 mmol/L glucose during the stabilization period. Test perfusions began at time 0. Each point represents the mean ± SEM for 6 or 7 hearts.

30 minutes, the increase was 26.4% (522 ± 29 *v* 413 ± 19 ng/mg dry weight, $P < .05$; Fig 5). After 5 minutes of insulin perfusion, palmitic (16:0), oleic (18:1, n-9), and linoleic (18:2, n-6) acids in 1,2-DAG showed significant increases of 17.2%, 39.8%, and 46.3%, respectively, when compared with controls (Fig 6A). A similar pattern was apparent at 15 minutes (Fig 6B) and 30 minutes (Fig 6C); at the latter time point, 16:0, 18:1(n-9), and 18:2(n-6) showed significant increases of 33.2%, 39.0%, and 41.5%, respectively. Other fatty acid species of 1,2-DAG remained largely unaffected. These results indicated that the distribution of different species of 1,2-DAG changed in response to insulin perfusion, and that the increased 1,2-DAG mass contained predominantly 16:0, 18:1(n-9), and 18:2(n-6) fatty acids. Perfusion of hearts for 30 minutes with 2.5 mU/mL insulin had no significant effect on either total 1,2-DAG mass (Fig 7) or the fatty acid composition of 1,2-DAG (Fig 8A). Increasing the glucose concentration of the perfusion medium to 17 mmol/L failed to increase the total mass of

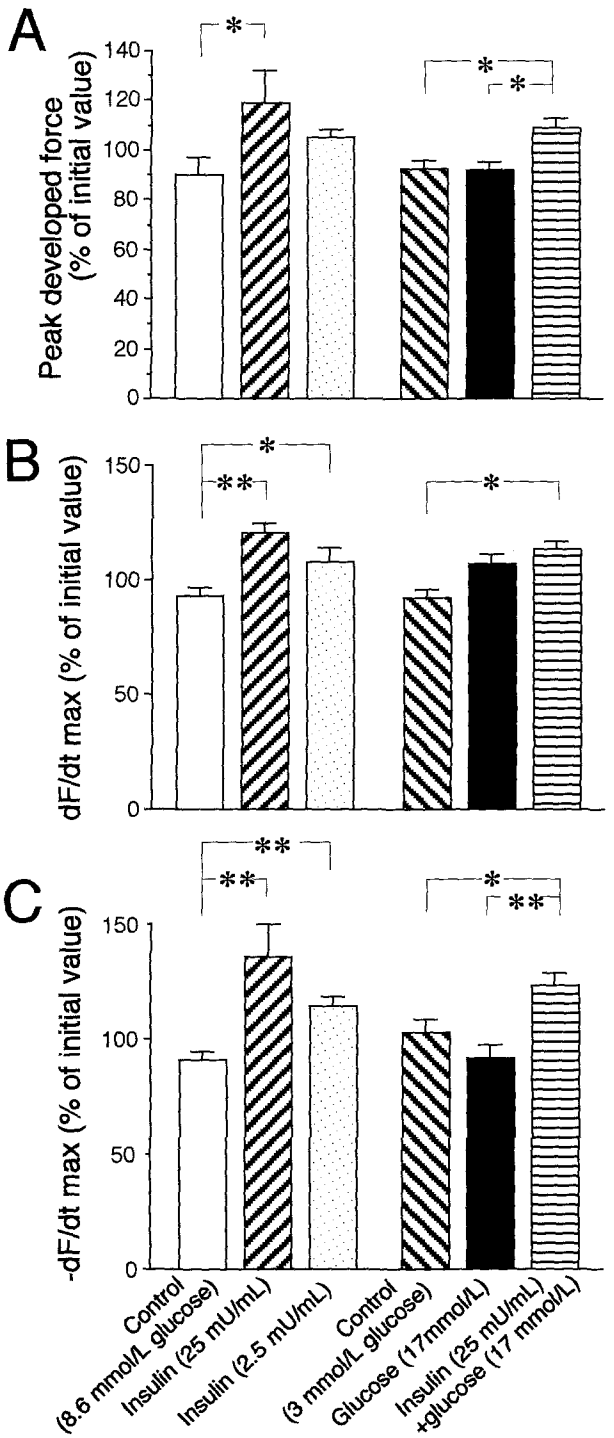

Fig 3. Effects of perfusion with insulin and glucose for 30 minutes on peak developed force (A), maximal dF/dt (B), and maximal −dF/dt (C) of isolated perfused rat hearts. Control and insulin groups were perfused with 8.6 mmol/L glucose. Glucose and insulin + glucose groups were perfused with 17 mmol/L glucose, after perfusion with 3 mmol/L glucose during the 25-minute stabilization period. Values are expressed as a percentage of values obtained after the stabilization period and represent the mean ± SEM for 6 or 7 hearts. *$P < .05$; **$P < .01$.

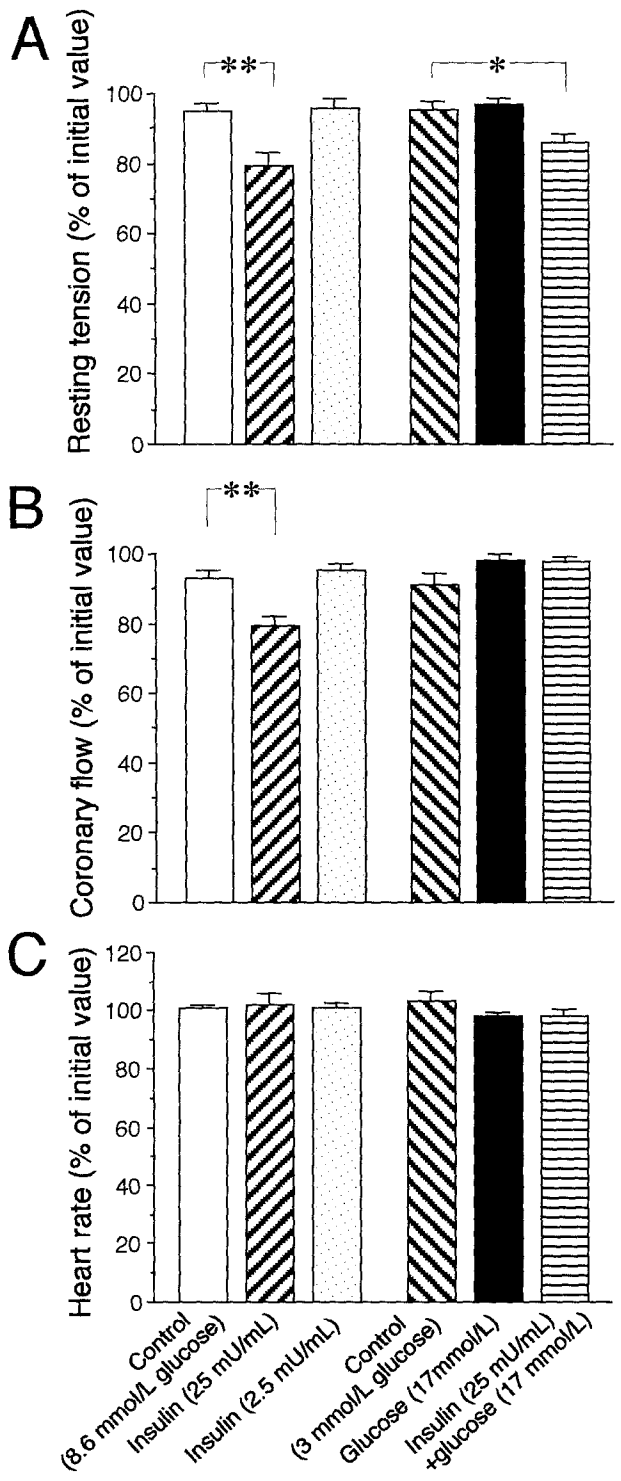

Fig 4. Effects of perfusion with insulin and glucose for 30 minutes on resting tension (A), coronary flow (B), and heart rate (C). Control and insulin groups were perfused with 8.6 mmol/L glucose. Glucose and insulin + glucose groups were perfused with 17 mmol/L glucose, after perfusion with 3 mmol/L glucose during the 25-minute stabilization period. Values are expressed as a percentage of values obtained after the stabilization period and represent the mean ± SEM for 6 or 7 hearts. *$P < .05$; **$P < 0.01$.

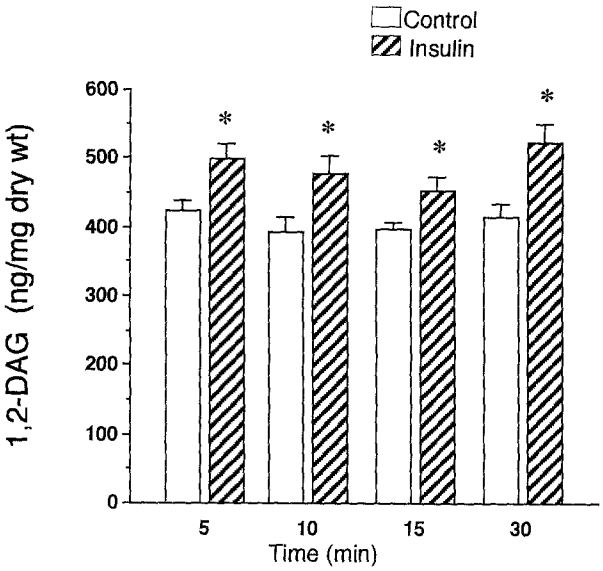

Fig 5. Total 1,2-DAG mass in isolated perfused rat hearts at various times of perfusion with 8.6 mmol/L glucose in the absence (control) or presence of insulin (25 mU/mL). Values are expressed as the mean ± SEM for 7 or 8 hearts. *$P < .05$ v corresponding control group.

1,2-DAG or the abundance of specific fatty acids, although the combination of 25 mU/mL insulin and 17 mmol/L glucose significantly increased total 1,2-DAG mass, as well as 16:0, 18:1(n-9), and 18:2(n-6), by 29.4%, 24.4%, and 29.1%, respectively (Figs 7 and 8B). There was no significant difference in total 1,2-DAG mass or fatty acid composition between hearts perfused with 8.6 or 3 mmol/L glucose in the absence of insulin.

DISCUSSION

The effects of insulin on cardiac muscle contractility are controversial.[23,24] Most studies have shown that insulin slightly increases myocardial contractility but has no effect on heart rate in the isolated perfused heart,[23,25] as also demonstrated in the present study. The conflicting observations on the effect of insulin may be attributable to differences in species or experimental conditions. Isolated Langendorff perfused hearts are considered to be under slightly anaerobic conditions; therefore, insulin may increase cardiac contractility, because insulin influences cardiac performance more favorably in anoxic hearts.[26] In general, insulin improves the utilization of carbohydrate, with a resulting decrease in the accumulation of fatty acids and their metabolites.

The action of insulin at the cellular level is initiated by binding to a cell-surface receptor, which results in the generation of second messengers. Several studies, including the present, have shown that insulin increases total DAG mass in tissues[17,18,27,28] and cells.[15,16] The results presented herein suggest that the insulin-induced increase in contractility in isolated perfused rat hearts may be related to the increased amount of 1,2-DAG in the myocardium. Al-

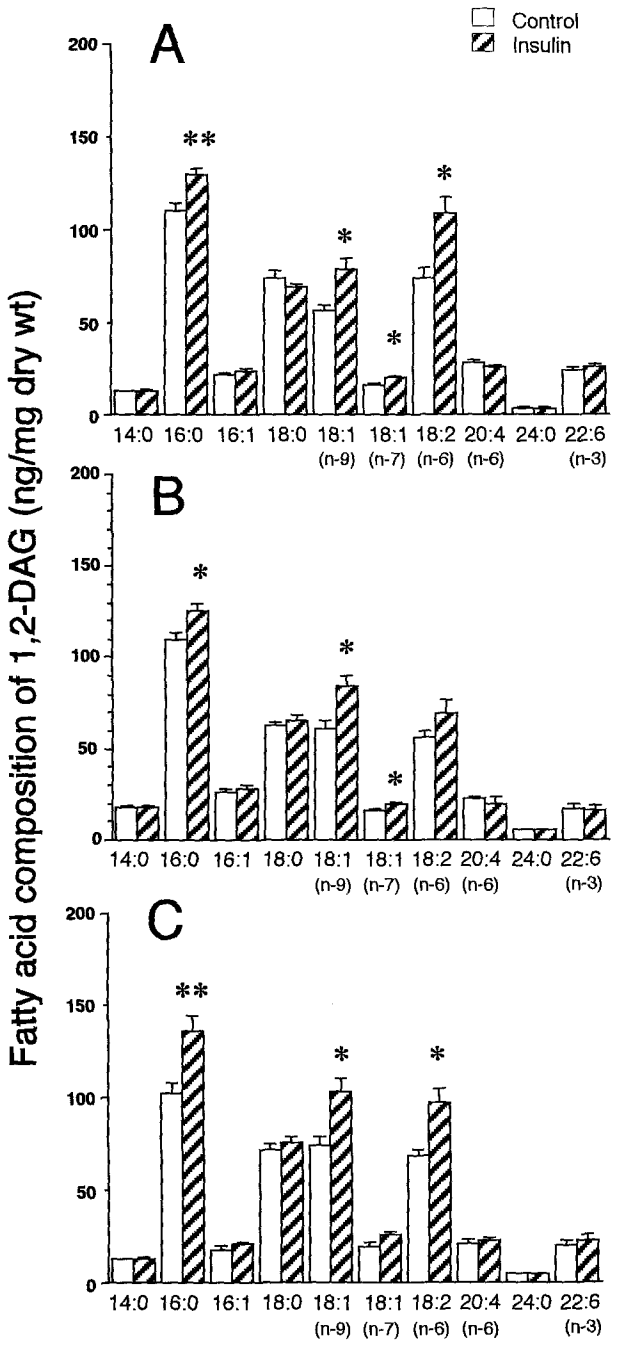

Fig 6. Fatty acid composition of myocardial 1,2-DAG at 5 minutes (A), 15 minutes (B), and 30 minutes (C) of perfusion with 8.6 mmol/L glucose in the absence (control) or presence of insulin (25 mU/mL). Values are expressed as the mean ± SEM for 7 or 8 hearts. *$P < .05$, **$P < .01$: v corresponding control group.

though the insulin-induced increase in 1,2-DAG abundance was only moderate, it may have a marked effect on the activation of PKC.[29] Insulin induces a biphasic increase in 1,2-DAG in cultured rat hepatocytes: the early phase is attributable to the stimulation of phosphatidylcholine-dependent phospholipase D, resulting in an increase in phosphatidic acid, and the delayed phase is attributable to the activation of phosphatidylcholine-dependent phospholipase C.[30] In contrast, Turinsky et al[31] detected no significant changes in DAG abundance after injection of insulin into rat skeletal muscle.

The mass of 1,2-DAG in cells and tissues is usually determined with DAG kinase using the method of Preiss et al.[20] However, Paterson et al[32] highlighted several problems with this method: in particular, it determines not only 1,2-DAG but also 1-alkyl-2-acylglycerol and 1-alkenyl-2-acylglycerol as diacylglycerols and cannot distinguish between these diradylglycerol species. We analyzed 1,2-DAG and its fatty acids by capillary gas chromatography, with 1,2-dipentadecanoin as an internal standard, after separation from alkylacylglycerols, alkenylacylglycerols, and 1,3-diacylglycerol by thin-layer chromatography.[21] Although this method does not identify the fatty acid position on the glycerol moiety or the precise molecular species of 1,2-DAG, the exact amounts of 1,2-DAG and its component fatty acids in the myocardium were determined.

Phosphorylation of intracellular proteins by PKC is activated physiologically by 1,2-DAG.[1] In general, 1,2-DAG derived from the stimulus-triggered hydrolysis of phosphatidylinositol 4,5-bisphosphate is thought to be the natural effector of PKC. However, the 1,2-DAG derived from other pathways, such as the hydrolysis of phosphatidylcholine, may also activate PKC.

In addition to the total amount of 1,2-DAG in the tissue, the molecular species of 1,2-DAG is also important in terms of its physiological role, because different analogs show different abilities to activate PKC.[33-35] Few studies have

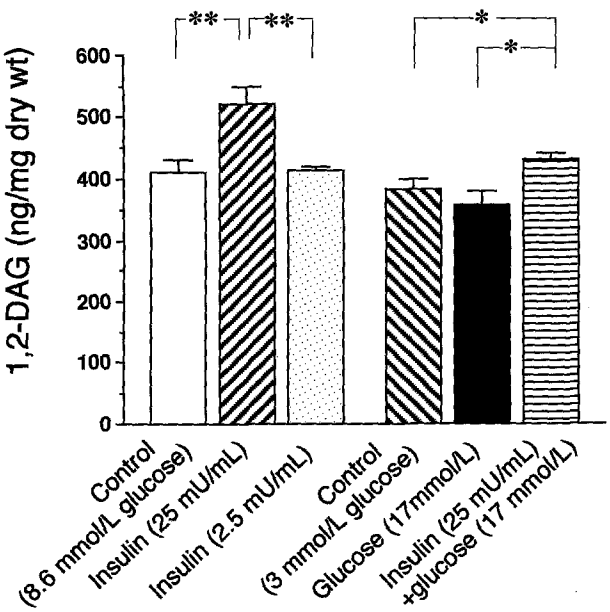

Fig 7. Total 1,2-DAG mass in isolated rat hearts after perfusion for 30 minutes with insulin and glucose. Control and insulin groups were perfused with 8.6 mmol/L glucose. Glucose and insulin + glucose groups were perfused with 17 mmol/L glucose, after perfusion with 3 mmol/L glucose during the 25-minute stabilization period. Values are expressed as the mean ± SEM for 7 or 8 hearts. *$P < .05$; **$P < .01$.

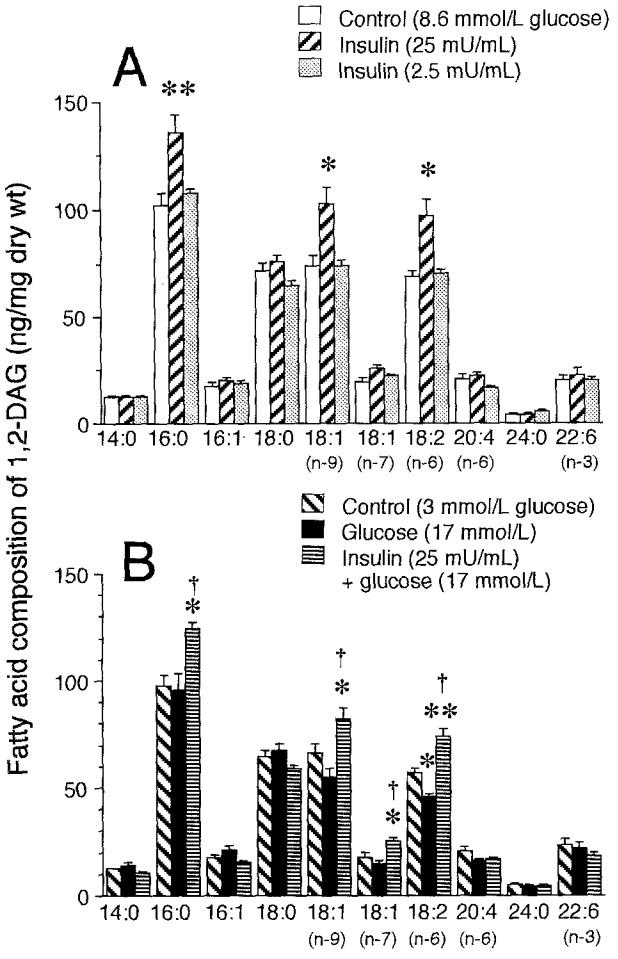

Fig 8. Fatty acid composition of myocardial 1,2-DAG after perfusion for 30 minutes with insulin and glucose. Control and insulin groups were perfused with 8.6 mmol/L glucose. Glucose and insulin + glucose groups were perfused with 17 mmol/L glucose, after perfusion with 3 mmol/L glucose during the 25-minute stabilization period. Values are expressed as the mean ± SEM for 7 or 8 hearts. *$P < .05$, **$P < .01$: *v* corresponding control group; †$P < .01$ *v* glucose 17 mmol/L group.

analyzed the fatty acid composition of 1,2-DAG produced in response to cellular stimulation; most studies have labeled lipids with specific fatty acids,[30] or have measured only total DAG with DAG kinase as described by Preiss et al.[20] The molecular nature of 1,2-DAG generated in response to an agonist may depend on the specific agonist and on the lipid hydrolyzed. Thus, the precise molecular species of increased 1,2-DAG may be indicative of the source lipid and its subcellular localization.[36]

Our results with isolated perfused rat hearts have demonstrated that total 1,2-DAG mass was already increased after 5 minutes and remained increased after 30 minutes of exposure to a high concentration (25 mU/mL) of insulin. A low concentration of insulin (2.5 mU/mL) had no effect on 1,2-DAG mass and only a small effect on cardiac performance. The increase in 1,2-DAG in response to high-dose insulin was attributable to increases in distinct 1,2-DAG species containing 16:0, 18:1, and 18:2 fatty acids. In cultured fibroblasts, 1,2-DAG generated in response to α-thrombin,[36] epidermal growth factor, or platelet-derived growth factor[37] consisted mostly of 16:0, 18:1, and 18:2 fatty acids. Furthermore, we showed that triiodothyronine decreased 1,2-DAG mass in rat hearts in vivo; in this instance, 16:0, 18:1, and 18:2 fatty acid components were specifically decreased, whereas other components remained virtually unchanged.[21] These observations indicate that 1,2-DAG species containing these fatty acyl moieties are most susceptible to regulation by cellular stimuli. Boggs and Buse[28] recently showed that 1,2-DAG species containing 18:0 also increased in rat skeletal muscle after insulin treatment. In contrast, Chen et al[38] detected no effect of insulin on the molecular species profile of DAG in skeletal muscle; these researchers showed that a 20% to 25% increase in total 1,2-DAG content induced by 10 mU/mL insulin was mainly attributable to synthesis other than the incorporation of glucose carbon. It should be noted that the amount of arachidonyl 1,2-DAG was relatively low and was not affected by insulin. Although it is of interest to determine the source of 1,2-DAG, fatty acid analysis of this compound was not helpful in this respect.

A high glucose concentration has also been shown to induce a rapid increase in the formation of 1,2-DAG from glycolytic intermediates such as dihydroxyacetone phosphate and glyceraldehyde-3-phosphate[39] via de novo synthesis of phospholipids, such as phosphatidic acid, in a variety of tissues.[11,14,40] Such increases in tissue 1,2-DAG were apparent without the addition of insulin. Furthermore, glucose-induced activation of PKC has been demonstrated in retinal capillary endothelial cell[41] and mesangial cell[11] cultures. In contrast, an increase in glucose from 5 to 20 mmol/L failed to produce DAG or phosphatidic acid in cultured retinal-pigment epithelial cells[42]; reciprocal osmoregulatory depletion of *myo*-inositol occurred rather than phosphatidylinositol synthesis. This discrepancy may depend on differences in species or in the duration of exposure to high glucose. We have shown herein that increasing glucose from 3 to 17 mmol/L had no effect on total 1,2-DAG mass, 1,2-DAG fatty acid composition, or contractility in isolated perfused rat hearts. However, an increase in 1,2-DAG and cardiac performance was observed in response to a high insulin concentration in the presence of high glucose. Phorbol esters that mimic the effect of 1,2-DAG on PKC activation also induce a positive inotropic response in isolated hearts[6] and myocytes.[43] Therefore, changes in total 1,2-DAG mass and fatty acid composition appear to be associated with changes in cardiac muscle contractility.

In conclusion, insulin, but not high glucose, increased both the amount of 1,2-DAG and cardiac muscle contractility in isolated perfused rat hearts. The insulin-induced increase in 1,2-DAG abundance may be attributable to increased amounts of 1,2-DAG containing specific fatty acids.

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