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Biochemistry

Insulin stimulates the biosynthesis of *chiro*-inositol-containing phospholipids in a rat fibroblast line expressing the human insulin receptor

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ABSTRACT **HIRc-B cells (rat fibroblasts expressing the human insulin receptor) were incubated with *myo*-[^{3}H]inositol for 48 hr, and the biosynthesis of *chiro*-[^{3}H]inositol was investigated in the absence and presence of insulin following a time course up to 60 min. After phase separation, treatment with insulin for 15 min caused a 2.2-fold increase in the specific radioactivity of *chiro*-[^{3}H]inositol-containing phospholipids in contrast to a 1.2-fold increase in the specific radioactivity of *myo*-[^{3}H]inositol-containing phospholipids. No insulin-mediated change in the specific radioactivity was observed in the inositol phosphates or free inositols. Further detailed analysis of individual [^{3}H]inositol-containing phospholipids demonstrated marked increases in specific activity of the *chiro*-[^{3}H]inositol phospholipids after 15 min of incubation with insulin: phosphatidylinositol 4-phosphate and 4,5-bisphosphate, 4.2-fold; lysophosphatidylinositol, 1.5-fold; phosphatidylinositol, 3.2-fold. In contrast, *myo*-[^{3}H]inositol-containing phospholipids demonstrated relatively small increases (1.1- to 1.4-fold) after 5 min of incubation with insulin. These findings indicate that insulin stimulates *de novo* synthesis of *chiro*-inositol-containing phospholipids at the inositol phospholipid level.**

The combination of D-*chiro*-inositol and galactosamine was originally discovered as an unexpected species of rat liver inositol phosphoglycan insulin mediator (1). A second species of insulin mediator, first described by Saltiel and Cuatrecasas (2), contained *myo*-inositol and glucosamine, the usual inositol and hexosamine commonly present in the inositol phosphoglycans. The *chiro*-inositol-containing mediator has been shown to activate pyruvate dehydrogenase phosphatase and glycogen synthase phosphatase (3, 4). Glycogen synthase and pyruvate dehydrogenase are the rate-limiting enzymes which control nonoxidative and oxidative insulin-sensitive glucose metabolic pathways, respectively. These two phosphatases are activated by insulin and the two pathways are now recognized to be defective in postreceptor insulin resistance in primates with type II diabetes (5). A lack of *chiro*-inositol together with an excess of *myo*-inositol has been noted in type II diabetic urine and skeletal muscle (5), leading us to suggest that insulin resistance may result from a possible defect in synthesis or metabolism of *chiro*-inositol (6).

Accordingly, we investigated the biosynthesis of *chiro*-inositol. We reported that *myo*-[^{3}H]inositol was converted to *chiro*-[^{3}H]inositol in the intact rat. Although *chiro*-[^{3}H]inositol was present widely in all tissues examined and in inositol-containing phospholipids and glycosylphosphatidylinositols (GPIs), the highest conversions were found in insulin-sensitive tissues—namely, muscle, liver, and blood (6).

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Accordingly, to further study the biosynthesis of *chiro*-inositol from *myo*-inositol and its possible responsiveness to insulin, we incubated rat fibroblasts that overexpress the human insulin receptor (HIRc-B cells) with *myo*-[^{3}H]inositol and studied the conversion of *myo*-[^{3}H]inositol to *chiro*-[^{3}H]inositol in the absence and presence of insulin. We report that insulin induces a large increase in the *chiro*-[^{3}H]inositol phospholipid specific radioactivity (up to 4-fold) and a small increase (1.2-fold) in the *myo*-[^{3}H]inositol phospholipid specific radioactivity. In contrast, no marked increase was observed in the *myo*-[^{3}H]inositol or the *chiro*-[^{3}H]inositol specific radioactivity in either the cellular free inositol or the inositol phosphates.

EXPERIMENTAL PROCEDURES

Materials. Phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidic acid (PA) were obtained from Avanti Polar Lipids. PI 4,5-bisphosphate (PIP_2), PI 4-phosphate (PIP), and lyso-PI were from Sigma. Silica gel G plates for thin-layer chromatography (TLC) were from Merck. Analytical-grade anion-exchange (AG1-X8) and cation-exchange (AG50W-X8) resins were from Bio-Rad. All other reagents and chemicals were purchased from Sigma at analytical-grade purity.

Cell Culture and *myo*-[^{3}H]Inositol Labeling. The HIRc-B cells (7, 8) were generously supplied by Donald McClain (University of Alabama, Birmingham). Cells were incubated at 37°C in air containing 5% CO_2 at 90% relative humidity. Before labeling, cells were grown to $\approx 2 \times 10^4$ per cm^2 in 10-cm-diameter dishes in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL) supplemented to contain 10% fetal bovine serum (GIBCO/BRL) and 100 nM methotrexate (9). The medium in each dish was replaced with 7 ml of inositol-free DMEM containing 10% dialyzed fetal bovine serum, 100 nM methotrexate, and *myo*-[1,2-^{3}H]inositol (22 μCi/ml; 46.0 Ci/mmol, DuPont/New England Nuclear; 1 Ci = 37 GBq). After incubation for 24 hr, the cells were serum-starved by replacing the medium with 7 ml of inositol-free DMEM supplemented with human transferrin (5 μg/ml), methotrexate (100 nM), and *myo*-[^{3}H]inositol (3 μCi/ml) and incubating for an additional 24 hr.

Insulin Stimulation of *myo*-[^{3}H]Inositol-Labeled HIRc-B Cells. The effect of insulin was tested by removing the medium from serum-starved cells, then adding 8 ml of inositol-free DMEM containing 0.1% bovine serum albumin (fraction V; Sigma) with or without 100 nM insulin (Humulin

Abbreviations: PI, phosphatidylinositol; GPI, glycosyl-PI; PIP, PI 4-phosphate; PIP_2, PI 4,5-bisphosphate; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid.

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R human insulin; Eli Lilly). Control and insulin-treated plates in triplicate were incubated at 37°C for 0, 5, 15, or 60 min. Control plates were treated with the same medium lacking insulin.

Extraction of Inositol-Containing Phospholipids. The reaction was terminated at each time point by removing the medium and adding 2 ml of cold CH_3OH. Individual plates of cells were scraped, transferred to glass tubes, and extracted with 4 ml of $CHCl_3/CH_3OH$/0.05 M HCl (2:1:1). After vigorous vortex mixing, tubes were centrifuged to separate the phases. The organic phase was pooled after further extraction of the aqueous phase with two additional aliquots of 2 ml $CHCl_3$. The aqueous phase was removed and saved for further analysis. The pooled organic phase was dried under N_2 and redissolved in 0.4 ml of $CHCl_3/CH_3OH$ (2:1) for TLC. Cells from control and insulin-treated plates were extracted in the same way immediately after each time point.

Isolation of [^{3}H]Inositol-Containing Phospholipids. Each total lipid extract from each individual plate was fractionated into individual phospholipid classes by TLC in a solvent system of $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (50:20:10:10:5) (solvent system A) as previously described (6, 10). Autoradiography was performed directly on the TLC plate with Amersham Hyperfilm-^{3}H (Amersham) for 24–36 hr at −70°C. Three major bands (1, 2, and 4) comigrating with authentic standards of PIP_2/PIP, lyso-PI, and PI, respectively, were located on both sides of the TLC plate with the autoradiogram for definitive identification of each [^{3}H]inositol-containing phospholipid. The area between bands 2 and 4 was designated as band 3 and analyzed along with bands 1, 2, and 4. Each band (nos. 1–4) was then scraped off the plate, eluted with $CHCl_3/CH_3OH$ (2:1) and $CHCl_3/CH_3OH/H_2O$ (10:10:3), and hydrolyzed in 6 M HCl at 110°C for 48 hr after evaporation of the solvents under N_2 (6, 10). Acid hydrolysates were chromatographed and quantified for *chiro*-inositol and *myo*-inositol mass by HPAE-Dionex high-performance liquid chromatography (HPLC) with an Aminex HPX-87C column (Bio-Rad) as described (6, 10). Each peak with the same retention time as standard *myo*-inositol or *chiro*-inositol was collected for radioactivity counting as described (6).

Isolation of [^{3}H]Inositol Phosphates. Pilot experiments demonstrated that inositol phosphate and inositol bisphosphate were eluted from AG1-X8 by 0.1 M HCl, indicating that the inositol mono- and bisphosphates would be eluted from the AG1-X8 column by 0.1 M HCl under these conditions. Each aqueous extract from each individual culture plate was dried, resuspended in 3 ml of H_2O, and adjusted to pH 6.5 with 30 μl of 0.5 M NaOH. Each sample was then applied to an AG1-X8 column (2-ml bed volume, formate form). The pass-through fraction, containing free inositols, was collected. The column was washed with 15 mM HCl and bound material was eluted with 0.1 M HCl. After lyophilization, the eluate was redissolved in 5 ml of H_2O, adjusted to pH 4.0–4.5, and applied to an AG50W-X8 (1-ml bed volume, H^+ form) column. The pass-through fraction, containing the inositol phosphates, was collected. All fractions purified from the aqueous extracts were then subjected to acid hydrolysis for analysis of *chiro*-[^{3}H]inositol and *myo*-[^{3}H]inositol by the HPLC method described above.

Statistical Analysis. Three independent experiments were performed, each with triplicate plates analyzed separately with similar results. Data shown were from one representative experiment performed and analyzed in triplicate. All results were expressed as means ± SEM and were evaluated by Student's *t* test. Significance was set at $P \leq 0.05$.

RESULTS

Insulin Action on *myo*-[^{3}H]Inositol-Labeled HIRc-B Cells. After phase separation, inositol phospholipids, inositol phosphates, and free inositols were first analyzed for inositol specific radioactivity (cpm/pmol). Fig. 1 shows the difference in the control versus insulin-stimulated changes in specific radioactivity of *myo*-[^{3}H]inositol (Fig. 1*A*) and *chiro*-[^{3}H]inositol (Fig. 1*B*) after a 15-min incubation. No change induced by insulin was seen (15 min) in the *myo*-[^{3}H]inositol specific radioactivity in inositol phosphate and the free inositol fractions (Fig. 1*A*). This is in keeping with a lack of an insulin effect on PIP_2 hydrolysis in insulin-sensitive tissues (11). *myo*-Inositol phospholipids showed a small (20%) increase in specific radioactivity (Fig. 1*A*), in keeping with insulin-stimulated *de novo* synthesis of inositol phospholipids through diacylglycerol generated from PC hydrolysis (12). However, when we further separated total phospholipids obtained at the 15-min time point into individual inositol-containing phospholipids (see Fig. 3*A*), an increase was detected only in band 1 (representing PIP/PIP_2), which showed a 30% increase. The *myo*-[^{3}H]inositol specific radioactivity in total phospholipids was 6.6 times that of free *myo*-[^{3}H]inositol. This probably reflects the fact that free *myo*-inositol in the cells, but not lipid-bound *myo*-inositol, equilibrated with inositol in the medium during the serum-starvation phase when the specific radioactivity in the medium was 3/22 of that present during the prior 24 hr.

In contrast, after 15 min of insulin treatment, the *chiro*-[^{3}H]inositol specific radioactivity (Fig. 1*B*) in the phospholipid fraction increased 2.2-fold. A slight increase (12%) was observed in *chiro*-[^{3}H]inositol specific radioactivity in the inositol phosphate fraction, with no change in the free *chiro*-[^{3}H]inositol specific radioactivity with insulin treatment (Fig. 1*B*). Interestingly, the *chiro*-[^{3}H]inositol specific

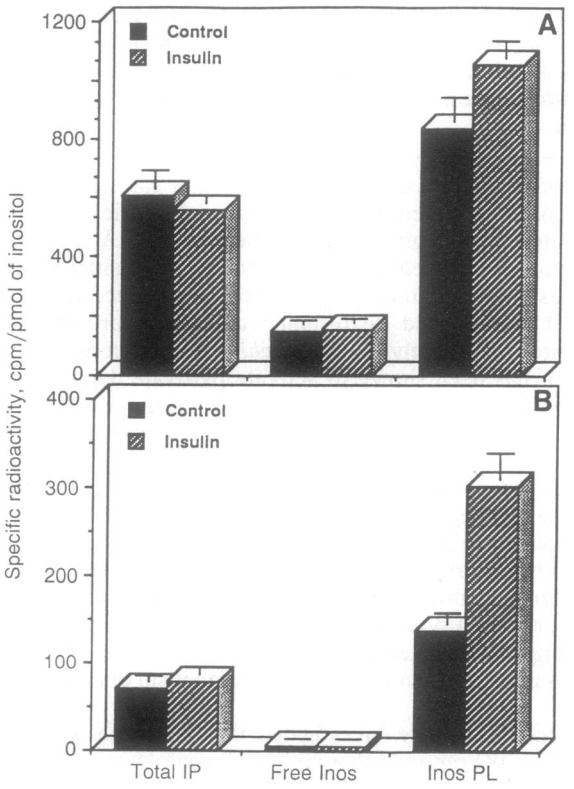

FIG. 1. Changes in the specific radioactivity of *myo*-[^{3}H]inositol (*A*) and *chiro*-[^{3}H]inositol (*B*) after a 15-min incubation with insulin. After phase separation, total inositol-containing phospholipids (Inos PL), from the organic phase, and total inositol phosphates (Total IP) and free inositols, (Free Inos) from the aqueous phase, were analyzed for inositol specific radioactivity. Results shown are mean ± SEM of one triplicate representative experiment from three triplicate independent experiments (all three experiments showed similar results).

radioactivity of the phospholipid fraction was 37 times that of the free *chiro*-[^{3}H]inositol. This indicates either that little or no hydrolysis of *chiro*-[^{3}H]inositol phospholipids had occurred (i.e., very low turnover of *chiro*-[^{3}H]inositol phospholipids) or that there had been a large dilution of free *chiro*-[^{3}H]inositol from nonlabeled precursor, possibly the medium glucose (50 mM in the DMEM cell growth medium).

Separation of [^{3}H]Inositol-Containing Phospholipids by TLC. To further examine which of the inositol phospholipid species were involved in the insulin-stimulated increases, the total lipid extract was fractionated into its individual inositol-containing phospholipids. As shown by TLC in solvent system A (Fig. 2*A*), the total lipid extract from each plate was typically separated into six phospholipid classes including PIP_2/PIP, PI, PS, PC, PE, and PA as visualized by exposure to I_2 vapor. Once the autoradiography was performed on the TLC plate (Fig. 2*B*), three definitive [^{3}H]inositol-labeled fractions—bands 1, 2, and 4, corresponding to authentic standards of PIP_2/PIP, lyso-PI, and PI, respectively—were revealed. Typically, by densitometric analysis, 7–10% of total radioactivity from each lipid extract was recovered in band 1, 12–16% in band 2, 4–6% in band 3 (which was diffuse over the area in between bands 2 and 4), and the remainder (70–80%) in band 4. Although minor differences in radioactivity distribution among the four bands were observed, we were unable to find a consistently different pattern of labeling in the bands comparing control and insulin-treated cells. Band 3, the area between band 2 (lyso-PI) and band 4 (PI), was included along with bands 1, 2, and 4, since GPI has been shown to be insulin-sensitive and to migrate between PIP_2/PIP and PI (13, 14). Each band was then located on the plate, scraped, eluted, acid hydrolyzed, and analyzed for *chiro*-[^{3}H]inositol and *myo*-[^{3}H]inositol by HPLC.

Insulin-Stimulated Biosynthesis of *chiro*-Inositol-Containing Phospholipids. The specific radioactivity of *myo*-[^{3}H]inositol in all four bands was not significantly increased with insulin treatment except that relatively small increases (band 1, 40%; band 2, 30%; bands 3 and 4, 10%) were observed in all four bands after 5 min of incubation with insulin (Fig. 3*A*). Interestingly, when the 20% increase previously discussed in total inositol phospholipids at 15 min was further analyzed in the separate bands, only band 1 showed an increase, 30%, with the other bands showing no increase. The data indicate that the insulin stimulation of *myo*-inositol phospholipid synthesis or turnover occurred primarily at 5 min and continued up to 15 min. Later in the time course, the *myo*-[^{3}H]inositol specific radioactivity of bands 2–4 decreased after the initial increase at 5 min either to control (i.e., zero-time incubation with no insulin treatment; Fig. 3) or below control at 15 min, continuing to decrease at 60 min, except for band 1, which increased by up to 40% even at 60 min of insulin treatment.

In contrast, the specific radioactivity of *chiro*-[^{3}H]inositol in band 1 (Fig. 3*C*), representing PIP_2/PIP, showed the most marked (4.2-fold) increase compared with control cells after 15 min of incubation with insulin. As seen in Fig. 3*B*, *chiro*-[^{3}H]inositol in band 4 (representing PI) had the highest specific radioactivity among all four bands and followed the same pattern as band 1 with a 3.2-fold increase in *chiro*-[^{3}H]inositol specific radioactivity at 15 min. Band 2 (Fig. 3*C*) also demonstrated a similar but smaller increase (1.5-fold). In general, the *chiro*-[^{3}H]inositol specific radioactivity of bands 1, 2, and 4, after increasing at 15 min, decreased to either control or below control level at 60 min. Band 3 also showed a 1.5-fold increase in specific radioactivity at 5 min and then a gradual decrease to the control level over 15–60 min (see Fig. 3*C*).

DISCUSSION

Recently we isolated and identified in bovine liver two GPI species which contained *chiro*-inositol together with galactosamine and suggested that they might represent lipid precursors for the *chiro*-inositol galactosamine-containing mediator (10). We previously reported a major deficiency in *chiro*-inositol in urine and muscle of type II diabetics including University of Virginia subjects, Pima Indian subjects, and *Macaca mulatta* monkeys. This deficiency has recently been

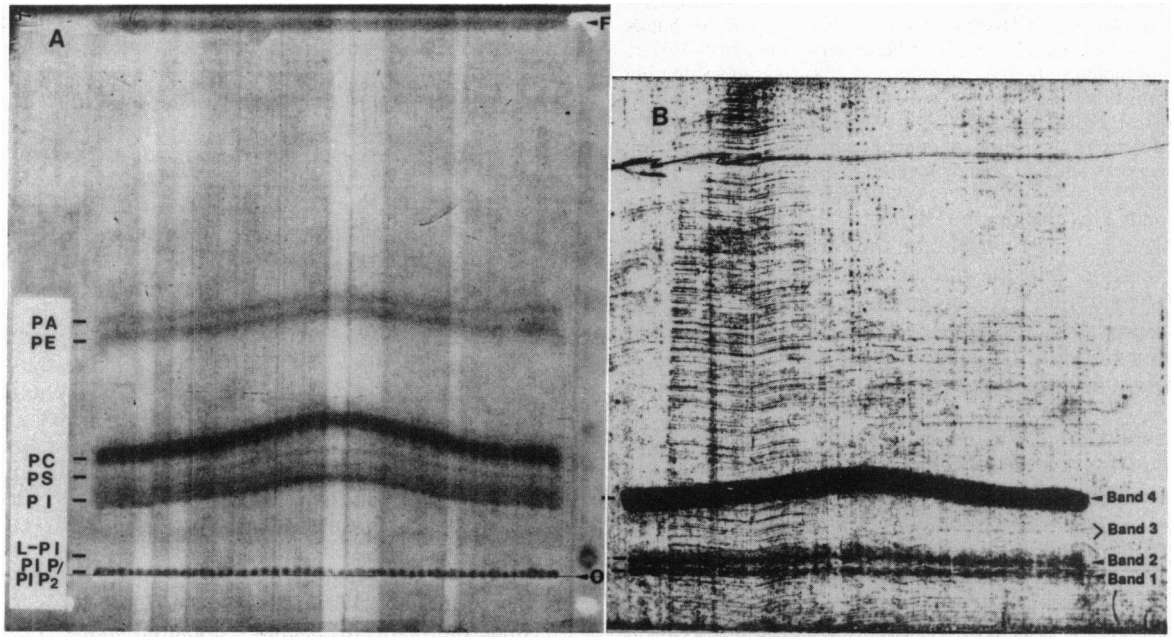

FIG. 2. TLC analysis of [^{3}H]inositol-containing phospholipids in solvent system A. (*A*) Total lipid extract from the organic phase was fractionated into individual phospholipids in parallel with authentic standards (indicated at left) and visualized by exposure to I_2 vapor. O and F denote origin and solvent front, respectively. (*B*) Autoradiography of the [^{3}H]inositol-containing phospholipids of the TLC plate (*A*). Band 1 (representing PIP/PIP_2), band 2 (representing lyso-PI), band 3 (area between bands 2 and 4), and band 4 (representing PI) were analyzed as described.

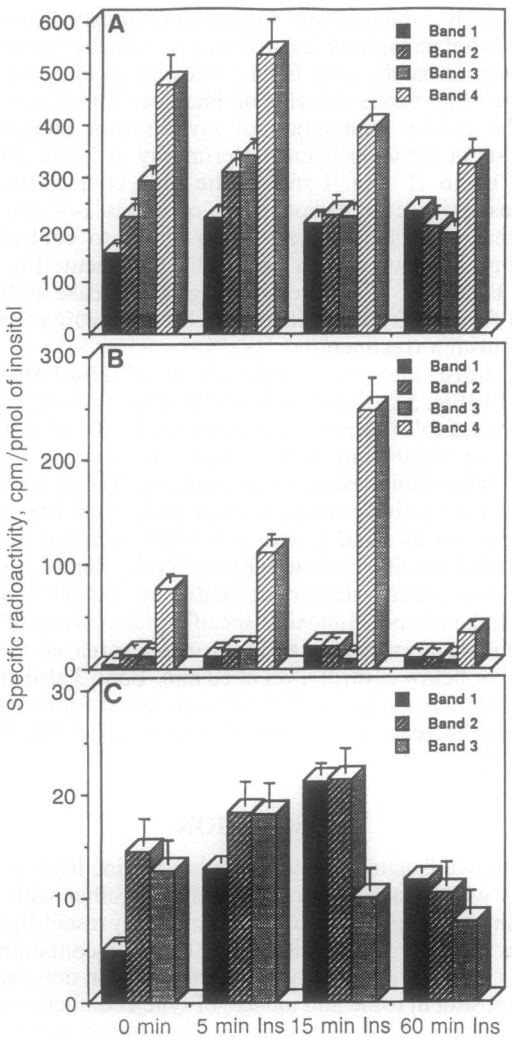

FIG. 3. Time course of insulin action on individual [^{3}H]inositol-containing phospholipids. (*A*) *myo*-[^{3}H]Inositol specific radioactivity of bands 1–4. (*B*) *chiro*-[^{3}H]inositol specific radioactivity of bands 1–4. (*C*) *chiro*-[^{3}H]inositol specific radioactivity of bands 1–3. Bars labeled 0 min indicate the control values at zero time of incubation with no insulin treatment. Bands 1–4 are designated as in Fig. 2*B*. Results (mean ± SEM) are from one triplicate representative experiment out of three triplicate independent experiments performed (all three experiments showed similar results).

directly related to the degree of postreceptor insulin resistance in *M. mulatta* by correlating the rate of urinary *chiro*-inositol excretion with the rate of insulin-mediated glucose disposal (M value), *in vivo* activation of skeletal muscle and adipose tissue glycogen synthase, and inactivation of skeletal muscle phosphorylase (15). From these studies a defect in *chiro*-inositol biosynthesis or metabolism was postulated (6, 16).

Our previous study (6) demonstrated that administered *myo*-[^{3}H]inositol was converted to *chiro*-[^{3}H]inositol widely in tissues of the intact rat. Since glucose was not labeled, this suggested that it was not a significant intermediate in *myo*-inositol breakdown and that the conversion was most likely a conversion of *myo*-inositol to *chiro*-inositol. Glucuronic acid, the established product of *myo*-inositol oxidation was labeled up to 1.5% of the *myo*-[^{3}H]inositol. In the same study, we demonstrated that among the tissues examined the ratio of *chiro*-[^{3}H]inositol to *myo*-[^{3}H]inositol was highest (≈8%) in liver, muscle, and blood. Inositol phospholipids and GPI from those three tissues revealed significant amounts of *chiro*-[^{3}H]inositol (up to 60% in blood). This suggested the possibility of an insulin-regulated synthesis of *chiro*-inositol or *chiro*-inositol phospholipid in those insulin-sensitive tissues.

In the present experiments, we adapted a protocol designed for obtaining maximum insulin action in intact cells. We utilized HIRc-B cells (expressing 1.25×10^6 insulin receptors per cell) and labeling conditions originally developed for demonstrating insulin activated PI 3-kinase (9) to achieve significant [^{3}H]inositol incorporation into all of the inositol-containing phospholipids and to maximize detection of minor inositol phospholipids in intact cells. The final 24-hr serum-starvation period allowed the cells to reach quiescence at high cell density in the presence of additional *myo*-[^{3}H]inositol of lower radioactivity (3/22 of previous amount) and further facilitated possible insulin (100 nM) action on the biosynthesis of *chiro*-inositol and *chiro*-inositol phospholipids.

After the phase separation, the effect of insulin was investigated first by measuring the specific radioactivity of total *myo*-[^{3}H]inositol and *chiro*-[^{3}H]inositol of inositol-containing phospholipids and the specific radioactivity of inositol phosphates and free inositols, comparing control and insulin-treated cells. Interestingly, at 15 min (Fig. 1*B*) we found a significant (2.2-fold) insulin stimulation of the specific radioactivity of the total *chiro*-[^{3}H]inositol-containing phospholipids, with a small (1.2-fold) increase in the *myo*-[^{3}H]inositol-containing phospholipids (Fig. 1*A*). Examination of the inositol phosphates and free inositols (Fig. 1 *A* and *B*) revealed no insulin-mediated change in the specific radioactivity, except that in the *chiro*-[^{3}H]inositol of the inositol phosphates a possible small, 12% increase with insulin was detected at 15 min. Moreover, significant *chiro*-[^{3}H]inositol was present in the acid hydrolysate of the inositol phosphate fraction (up to 65% of total inositol; data not shown). The structures of these *chiro*-inositol phosphates are not known.

Analysis of the time course of insulin action on individual [^{3}H]inositol-containing phospholipids revealed a dramatic insulin stimulation at 15 min in the *chiro*-[^{3}H]inositol-containing phospholipids (band 1, 4.2-fold; bands 2 and 3, 1.5-fold; band 4, 3.2-fold increase) compared with control (Fig. 3 *B* and *C*), in contrast to the *myo*-[^{3}H]inositol-containing phospholipids where a small increase (1.1- to 1.4-fold) was observed at 5 min (Fig. 3*A*), in keeping with data of others (12). In all three experiments, with each triplicate sample, increased specific radioactivity of *chiro*-[^{3}H]inositol occurred with increased radioactivity. In two of three experiments an increased mass of *chiro*-inositol was also detected, chiefly in bands 1 and 4 (data not shown).

Overall, the evidence clearly indicates that an insulin-stimulated conversion of *myo*-[^{3}H]inositol to *chiro*-[^{3}H]inositol occurs most effectively in the *myo*-[^{3}H]inositol-containing phospholipids, either at the PI or at the PIP/PIP$_2$ level. Although we anticipated that the specific radioactivity of both *myo*-inositol and *chiro*-inositol of the total inositol phosphates, free inositols, and inositol phospholipid fractions would be similar after 48 hr of labeling, we found that the specific radioactivity of *chiro*-inositol was lower than *myo*-inositol in all fractions. The reasons for this difference can be several. One possibility is that *chiro*-[^{3}H]inositol is synthesized from a nonradioactive precursor as well as from *myo*-[^{3}H]inositol, or that *chiro*-[^{3}H]inositol is synthesized from a *myo*-[^{3}H]inositol pool with a lower specific radioactivity.

Since there was no insulin-stimulated change observed in the specific radioactivity of the *chiro*-[^{3}H]inositol in the free inositol or inositol phosphate fractions, and the specific radioactivity in inositol phospholipids was higher than that in either the inositol or inositol phosphate fraction, these data indicate that *chiro*-inositol is probably not synthesized from

free *myo*-inositol as occurs in plants and insects through the oxidoreductive epimerase action (17).

In connection with the present studies, it is of considerable interest that a genetic rat nonobese type II diabetes model, the GK rat, has been shown to manifest insulin resistance associated with markedly decreased *chiro*-inositol in urine (18) and tissue (Susumu Suzuki, personal communication).

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