methods

Cell-based multiwell assays for the detection of substrate accumulation and oxidation[1]

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Abstract **We describe multiwell assays for detecting the accumulation as well as the subsequent oxidation of ^{14}C-labeled substrates in cultured cells. Accumulation is monitored in real time by an established scintillation proximity assay in which the scintillator is embedded in the plate base primarily detecting cell-associated radiolabel. The substrate oxidation assay is a novel variant of previously described experimental approaches aimed at trapping $^{14}CO_2$ produced by isolated enzymes, organelles, or intact cells. This method uses a standard 96-well tissue culture plate and, on top, an inverted filter plate immersed with NaOH that are clamped into a sandwich sealed with a silicon gasket to obtain gas-tight compartments. $^{14}CO_2$ is captured in the filter and quantified by conventional scintillation. We demonstrate both the accumulation and subsequent oxidation of ^{14}C-labeled substrates in cultured human myotubes, adipocytes, and hepatocytes. Both methods are adaptable for compound screening; at the same time, these protocols provide easy-to-use and time- saving methods for in vitro studies of cellular fuel handling.**—Wensaas, A. J., A. C. Rustan, K. Lövstedt, B. Kull, S. Wikström, C. A. Drevon, and S. Hallén. **Cell-based multiwell assays for the detection of substrate accumulation and oxidation.** ***J. Lipid Res.*** **2007.** 48: **961–967.**

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Measurement of cellular processes such as the uptake and oxidation of energy-rich substrates is important in cell biology. Often, phenotypes of metabolic abnormalities may transmit into in vitro systems with whole cells or enzymes isolated from the affected individual. It has been shown that primary skeletal muscle cells from type 2 diabetics as well as severely obese patients have reduced capacity to oxidize fatty acids compared with cells isolated from healthy controls (1–3). Moreover, a recent investigation demonstrated correlations between different metabolic parameters, such as respiratory quotient dynamics, insulin sensitivity, O_2 consumption, body fat mass, plasma free fatty acids, and fatty acid oxidation measured in primary cells from the respective donor (4). These findings call for accessible multiwell assays for the measurement of substrate uptake and oxidation.

Scintillation proximity assays (SPAs) designed to detect radioligand accumulation in adherent cell layers have been commercially available for more than a decade (e.g., Cytostar SPA and Scintiplate) and used for numerous applications (5–7), but there are few published studies of nutrient metabolism. We present results on the accumulation of radiolabeled fatty acids and deoxyglucose (DOG) in human cells grown on 96-well SPA plates monitored noninvasively in real time. Numerous protocols designed for the measurement of cellular metabolism use ^{14}C-labeled substrates with simultaneous or subsequent capture of the $^{14}CO_2$ produced (8–11). The substrate oxidation assay presented here is a novel variant of previously described experimental approaches and uses standard 96-well tissue culture and filter plates. The accessibility and high throughput of the described in vitro techniques should make them attractive tools for studies of cellular energy metabolism.

MATERIALS AND METHODS

Materials

DMEM, MEM, DMEM/Nutrient Mix F12, fetal calf serum (FCS), L-glutamine, biotin, D-pantothenate, human apo-transfer-

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Abbreviations: CPT1, carnitine palmitoyl transferase-1; DOG, deoxyglucose; DPBS, Dulbecco's phosphate-buffered saline; EPA, eicosapentaenoic acid; FCS, fetal calf serum; IBMX, 3-isobutyl-1-methylxanthine; LA, linoleic acid; OA, oleic acid; PA, palmitic acid; P/S, penicillin/streptomycin; SGBS, Simpson-Golabi Behmel syndrome; SPA, scintillation proximity assay.

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rin, triiodo-L-thyronine, cortisol, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, sodium pyruvate, nonessential amino acid mix, BSA, Dulbecco's phosphate-buffered saline (DPBS; with Mg^{2+} and Ca^{2+}), and HEPES were from Sigma (St. Louis, MO). Palmitic acid (PA), oleic acid (OA), linoleic acid (LA), and eicosapentaenoic acid (EPA) were purchased from Nu-Chek Prep, Inc. (Elysian, MN). UltimaGold™, OptiPhase Supermix, MicroScint™ O, and [1-^{14}C]EPA (New England Nuclear, MA) were delivered by Perkin-Elmer (Shelton, CT). Ninety-six-well UniFilter® GF/B plates were from Whatman, Inc. (Clifton, NJ). Cytostar-T® was purchased from GE Healthcare (Chalfont St. Giles, UK), Ultroser G from Ciphergen (Cergy-Saint-Christophe, France), insulin (Actrapid®) from NovoNordisk (Bagsvaerd, Denmark), roziglitazone from Alexis Biochemicals Corp. (Lausen, Switzerland), HepG2 cells (human hepatoma cell line) from the American Type Culture Collection (Rockville, MD), and [1-^{14}C]PA, [1-^{14}C]OA, [1-^{14}C]LA, and [1-^{14}C]D-DOG from American Radiolabeled Chemicals (St. Louis, MO). [U-^{14}C]PA and $NaH^{14}CO_3$ were obtained from Amersham Bioscience. Coomassie reagent was delivered by Bio-Rad (Copenhagen, Denmark), BC assay reagent from Uptima (Montluçon, France), Corning® CellBIND® tissue culture plate from Costar, and non-heat-inactivated FCS from GIBCO (Paisley, UK). Human Simpson-Golabi Behmel syndrome (SGBS) cells were kindly provided by Dr. M. Wabitsch at the University of Ulm, Germany. All other chemicals used were of standard commercial high-purity quality.

Cell culture

Human myotubes. Satellite cells were isolated from vastus lateralis muscle by needle biopsy, split three to four times (12), and cultured on 96-well plates with DMEM (5.5 mM glucose), 2% FCS, 2% Ultroser G, and penicillin/streptomycin (P/S) until 70–80% confluent. Myoblast differentiation to myotubes was then induced by changing medium to DMEM (5.5 mM glucose) including 2% FCS, 25 pM insulin, and P/S and continued for 4 days before preincubations were started.

SGBS adipocytes. Preadipocytes were cultured on 96-well plates with growth medium consisting of DMEM/Nutrient Mix F12, 10% FCS (non-heat-inactivated), 8 mg/l biotin, 4 mg/l D-pantothenate, 2 mM L-glutamine, and P/S. Differentiation of confluent preadipocytes to adipocytes was initiated by washing the cells and adding serum-free growth medium supplemented with 10 mg/l human apo-transferrin, 0.2 nM triiodo-L-thyronine, 20 nM insulin, 100 nM cortisol, 500 μM IBMX, 25 nM dexamethasone, 1 μM rosiglitazone, 2 mM L-glutamine, and P/S for 4 days and then continued without IBMX, dexamethasone, and rosiglitazone for another 10 days.

Human hepatoma cell line. HepG2 cells were cultured on 96-well plates with MEM, 10% FCS, 1 mM sodium pyruvate, 100 μM nonessential amino acid mix, 2 mM L-glutamine, and P/S.

SPA

Radiolabeled substrates taken up and accumulated by adherent cells will be concentrated close to the scintillator embedded in the plastic bottom of each well; thus, they provide a stronger signal than the radiolabel dissolved in the medium alone (**Fig. 1**). Influx (increased signal intensity) as well as efflux/catabolism (decreased signal intensity) can be monitored by the SPA system. Optimization of the specific radioactivity must be done empirically to find the lowest value providing acceptable signal-to-background/noise values. SPA plates have counting efficiencies for cell-associated ^{14}C-labeled substrates of ~30%, compared with the ~90% efficiency of counting cell lysates by conventional liquid scintillation. Other β-emitting isotopes such as ^{3}H, ^{33}P, ^{35}S, or ^{125}I can be used, but counting efficiency will vary as a result of differences in the isotope energy spectra (e.g., ^{3}H has an SPA counting efficiency of only 0.5% to 1%). Measurements of [^{14}C]fatty acid and [^{14}C]DOG uptake by SPA were performed in either DMEM with 5.5 mM glucose (myotubes) or DMEM/Nutrient Mix F12 (SGBS cells). The cells were grown and differentiated on 96-well Cytostar-T® SPA plates as described above.

Fatty acid uptake. Differentiated myotubes or SGBS cells were washed with PBS and added to their respective media containing 1 μCi/ml [1-^{14}C]PA, [1-^{14}C]OA, [1-^{14}C]LA, or [1-^{14}C]EPA at concentrations of 50, 100, or 200 μM adjusted by the corresponding unlabeled fatty acid (PA, OA, LA, or EPA, respectively) bound to BSA at a ratio of 2.5:1.

Glucose uptake. Differentiated myotubes were preincubated for 24 h with 100 μM PA or 40 μM BSA (control) and then washed with PBS before incubation in medium containing [1-^{14}C] D-DOG (2 μCi/ml) with or without insulin (1 μM).

For measurement of glucose or fatty acid uptake with the SPA technique, cells were added to 50 μl/well of radiolabeled medium and incubated (5% CO_2 atmosphere at 37°C) for 240–300 min. The increase in cell-associated radioactivity was measured in a six channel MicroBeta® Trilux scintillation counter (Perkin-Elmer) at several time points during incubation. Each well was counted for 30 s, giving a total time of counting one plate of only 8–9 min. Some empty wells were also added to 50 μl of radiolabeled medium to correct for the background. Finally, the cells were washed three times with PBS, harvested with 0.05 M NaOH (200 μl/well), and homogenized by ultrasonography.

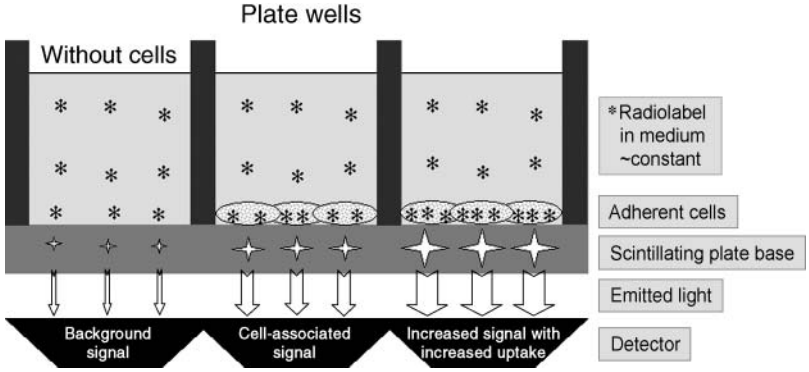

Fig. 1. Principles of the scintillation proximity assay (SPA).

Protein was determined by either Coomassie reagent for myotubes or BC assay reagent for SGBS cells. A total of 100–125 μl of cell homogenate per sample was added to scintillation fluid and counted by conventional liquid scintillation.

Substrate oxidation assay

HepG2 cells or human myotubes were grown on standard 96-well tissue culture plates or 96-well CellBIND® tissue culture plates as described above. Any preincubation substances were added directly to the medium. Substrate oxidation was monitored by incubating cells with ^{14}C-labeled fatty acids, with subsequent capture of liberated $^{14}CO_2$. Substrate medium was made of DPBS supplemented with 10–20 mM HEPES and 1 μM L-carnitine, adjusted to pH 7.3, and added to 20 or 100 μM (1 μCi/ml) fatty acid (either OA or PA), and the cells were added at 50 μl/well. A 96-well filter plate (UniFilter® GF/B) was activated for capture of CO_2 by the addition of freshly made NaOH (1 M, 25 μl/well). The plate harboring the cells was clamped together with the filter plate, forming a "sandwich," with the following bottom-to-top order: cell plate/silicon gasket/inversed filter plate (**Fig. 2**). The sandwich was placed in an incubator at 37°C, and substrate oxidation was allowed for 2–4 h. CO_2 produced from cellular respiration during incubation was continuously trapped in the filters. After incubation, the amounts of $^{14}CO_2$ captured in the filters were monitored by the addition of scintillation fluid (e.g., OptiPhase Supermix or MicroScint™ O; 50 μl/well). Radioactivity was counted in a scintillation counter for multiwell plates (e.g., MicroBeta® Trilux or TopCount from Perkin-Elmer). Capacity and linear range for CO_2 trapping were evaluated by adding acid (5 M H_2SO_4) to medium containing increasing amounts of $NaH^{14}CO_3$ dissolved in PBS on the 96-well plate (up to 1 μmol/well) with subsequent CO_2 capture for 5 h at room temperature.

Comparison with conventional substrate oxidation assay

Trapping of metabolized CO_2 was also monitored with a conventional method using HepG2 cells seeded in 12.5 cm^2 flasks and cultured to confluence. During measurement of OA oxidation, cells were incubated with DMEM supplemented with 20 μM [1-^{14}C]OA (1.1 μCi/ml), 1 mM L-carnitine, and 10 mM HEPES. The flasks were made airtight with rubber caps, and after 2 h, each flask was injected with 300 μl of phenylethylamine-methanol (1:1, v/v) through the rubber cap into a center well containing a folded filter paper. Subsequently, 300 μl of perchloric acid (1 M) was added to the cells, and the flasks were allowed to stand at room temperature to trap labeled CO_2. The center wells with filters were transferred to separate counting tubes, scintillation fluid added and the samples counted in a β-counter.

Presentation of data and results

All values are reported as means ± SEM. All experiments were performed with three to six parallel assays.

RESULTS

Long-chain fatty acid accumulation in human myotubes and SGBS adipocytes

Figure 3 shows the time- and concentration-dependent increases in cell-associated [^{14}C]PA, [^{14}C]OA, [^{14}C]LA, and [^{14}C]EPA measured noninvasively using SPA plates. Whereas for myotubes (Fig. 3A–D) there were small or no differences between the accumulation of PA, OA, and LA,

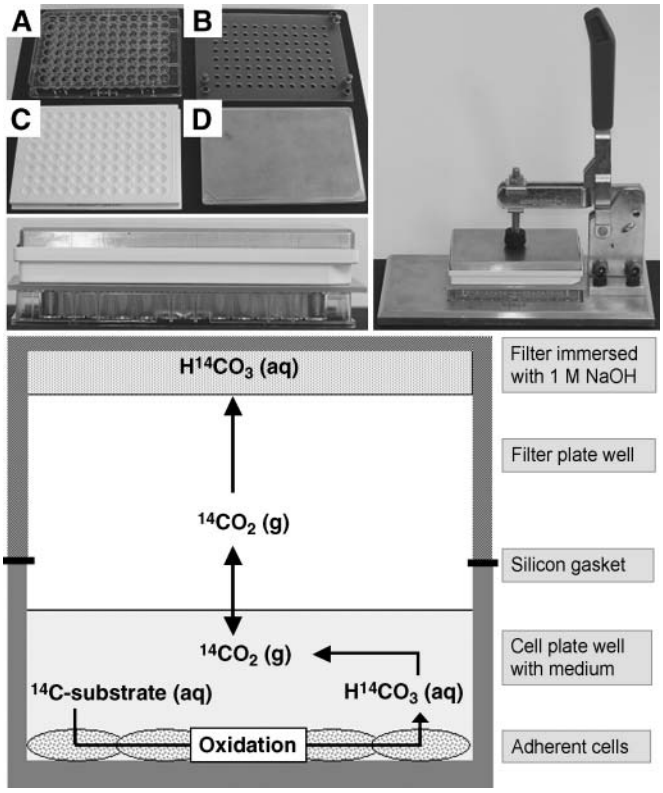

Fig. 2. The substrate oxidation appliance. The top left panel shows all of the different parts before assembly of the apparatus. A: 96-well tissue culture plate; B: silicon gasket with stabilizing knobs for each corner well; C: 96-well filter plate; D: metal plate for applying even pressure. The parts are assembled into a sandwich (left panel below) and put under pressure by the apparatus (top right panel). The lower panel illustrates the principles of the CO_2 trapping system. When a ^{14}C-labeled substrate is oxidized, the released CO_2 will be trapped in the alkaline suspension in the top filter.

they clearly accumulated less EPA at all concentrations tested. The processes governing fatty acid accumulation in myotubes also seemed saturated at the highest concentrations. The SGBS cells, however (Fig. 3E–H), did not show signs of saturation, and there were no apparent differences between the uptake rates of the different fatty acids. SGBS adipocytes also displayed two to four times greater accumulation of all fatty acids compared with myotubes.

Moreover, we compared the amounts of cell-associated fatty acids after 4 h of incubation using the SPA method with the conventional technique of washing, harvesting, and scintillation counting of the dissolved cells. The correlations between the two methods were 0.93, 0.96, 0.97, and 0.95 for PA, OA, LA, and EPA, respectively.

Insulin-stimulated glucose uptake in human primary skeletal muscle cells

Figure 4 shows the accumulation of [^{14}C]DOG/5.5 mM glucose in human myotubes with or without 1 μM insulin after preincubation with control medium (Fig. 4A) or 100 μM palmitate (Fig. 4B). Preincubation with palmitate decreased the insulin-dependent portion of radiolabeled glucose uptake (Fig. 4C).

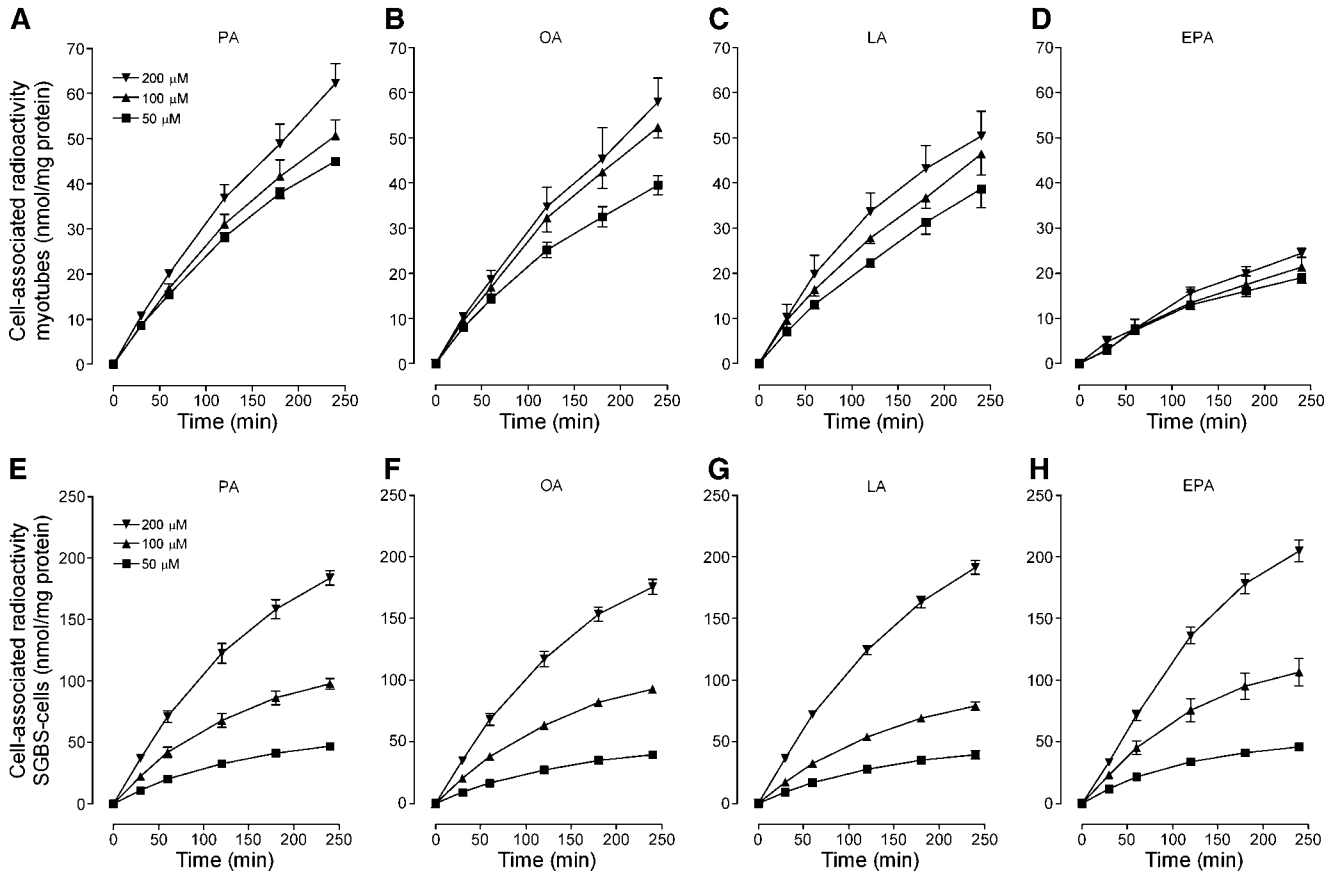

Fig. 3. Cell-associated fatty acid accumulation in differentiated human myotubes and Simpson-Golabi Behmel syndrome (SGBS) adipocytes. The cells were incubated with [^{14}C]palmitic acid (PA; A, E), [^{14}C]oleic acid (OA; B, F), [^{14}C]linoleic acid (LA; C, G), or [^{14}C]eicosapentaenoic acid (EPA; D, H). Measurements of cell-associated radioactivity were monitored by SPA at 0, 30, 60, 120, 180, and 240 min during the incubation. Results represent means ± SEM of three separate experiments.

Capacity and linear range of 96-well CO_2 trapping

The appliance for capturing metabolic CO_2 (Fig. 2) was tested for capacity and linearity using $NaH^{14}CO_3$. **Figure 5A** shows the amount of $^{14}CO_2$ trapped in the filters plotted against the total amount of $NaH^{14}CO_3$ present on the lower 96-well plate. The amount of $^{14}CO_2$ captured in the filters showed a linear relation with the total amount of CO_2 released (between 0 and 1 μmol), which covers the range of CO_2 expected to be produced by any cultured cell type.

Comparison of the multiwell assay with the conventional "flask" method

We compared our new method for trapping and quantification of $^{14}CO_2$ with the conventional use of tissue culture flasks equipped with small cups. Similar amounts of $^{14}CO_2$ were trapped with both methods after incubation of HepG2 cells with [^{14}C]OA (Fig. 5B). The cells also grew equally well in both formats, as shown by measurement of cell protein (Fig. 5C).

Acute dose response of a carnitine palmitoyl transferase-1 inhibitor

To further investigate the usefulness of the substrate oxidation method, we examined different conditions related to fatty acid metabolism. The mitochondrial import of long-chain fatty acids for β-oxidation is controlled by carnitine palmitoyl transferase-1 (CPT1). HepG2 cells or human myotubes were incubated with increasing concentrations of the irreversible CPT1 inhibitor, etomoxir, and the amount of $^{14}CO_2$ from [U-^{14}C]PA during a 2 h incubation was trapped. The calculated IC_{50} values are 0.4 and 0.25 μM for HepG2 cells and myotubes, respectively (**Fig. 6**). This demonstrates that the multiwell substrate oxidation method can be used for compound screening and dose-response testing.

Long-term incubation of myotubes with a peroxisome proliferator-activated receptor δ agonist

The peroxisome proliferator-activated receptor δ agonist GW501516 may induce genes involved in the transport and oxidation of fatty acids in cells (13, 14). We exposed myotubes to increasing concentrations of GW501516 for 3 days and then monitored the oxidation of [^{14}C]OA. The oxidation was increased in a concentration-dependent manner up to 10 nM of the agonist ($EC_{50} \sim 0.6$ nM), with a maximum increase of ~2-fold compared with the control (**Fig. 7A**). Preincubation with GW501516 increased cell-associated [^{14}C]OA similar to the oxidation (Fig. 7B), although the relative effect was markedly smaller.

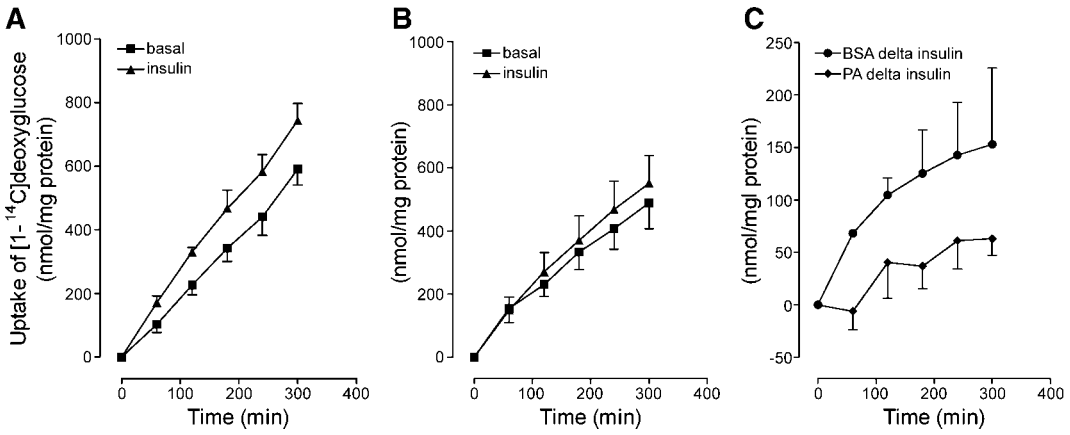

Fig. 4. Cell-associated glucose accumulation in differentiated human myotubes. Glucose uptake was measured after 24 h of preincubation with either BSA (40 μM; A) or PA (100 μM; B) with and without insulin (1 μM) from time zero. Uptake of [^{14}C]deoxyglucose was monitored by SPA at 0, 60, 120, 180, 240, and 300 min during the incubation. The insulin responses after 24 h of preincubation with BSA or PA are shown in C. Results represent means ± SEM of three separate experiments.

DISCUSSION

We describe two multiwell in vitro assays for the detection of cellular uptake and oxidation of fatty acids and glucose. We have demonstrated the potential of the SPA technique for measurements of both fatty acid and glucose accumulation in muscle cells and SGBS adipocytes. The myotubes exhibit saturation, whereas the SGBS cells demonstrate a linear relationship between fatty acid concentration and cell-associated accumulation of radioactivity up to 200 μM concentration of fatty acids. Furthermore, the results obtained using the Cytostar-T® plates correlated very well with the total radioactivity monitored with conventional scintillation after solubilizing the cells.

The noninvasiveness of the SPA procedure allows continuous monitoring of the cells and thereby makes it feasible to study the accumulation as well as the efflux of cell-associated radiolabeled substrates. In addition to high throughput, the SPA approach will also be valuable for studies of primary cells, of which there might be a limited amount available, and for other cell systems that are time-consuming to grow and differentiate (e.g., SGBS cells and myotubes). The only limitations with the detection of substrate accumulation by Cytostar-T® plates are the need for the cells to be adherent to the plate bottom and the fact that the substrate has to be labeled with a suitable β-emitter (preferably ^{14}C or ^{3}H). An alternative approach for the measurement of substrate flux ex vivo/in vitro is the use of positron autoradiography, in which the accumulation and, if applicable, secretion of β^{+}-emitting tracers (e.g., ^{11}C or ^{18}F) can be followed noninvasively in tissue slices (15, 16). This method elegantly

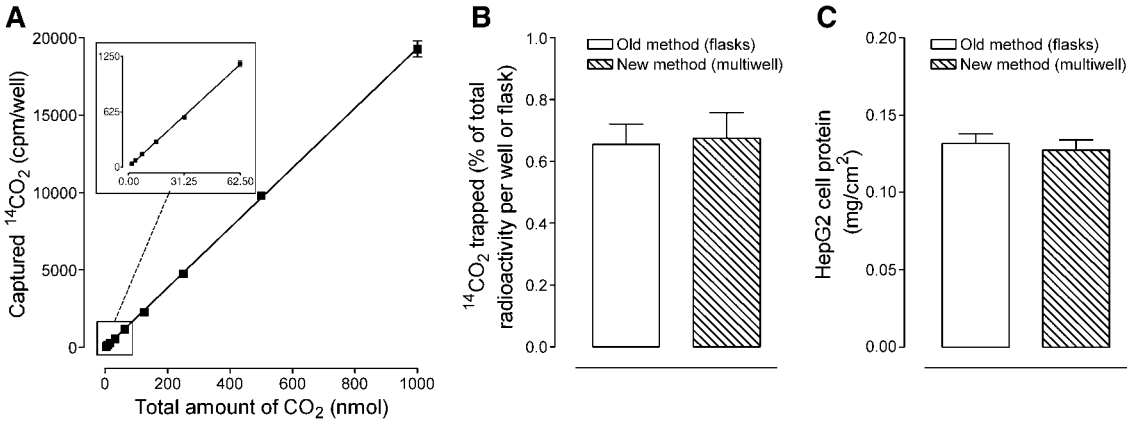

Fig. 5. A: Relationship between released $^{14}CO_2$ from $NaH^{14}CO_3$ at the bottom of the plate and trapping of $^{14}CO_2$ in the top filter alkaline suspension. Increasing amounts of $NaH^{14}CO_3$ in 100 μl of Dulbecco's phosphate-buffered saline (DPBS; pH 7.3) were acidified by adding 50 μl of 5 M H_2SO_4 per well, and the released CO_2 was captured during a subsequent 5 h incubation. The data presented represent one experiment (n = 6). B, C: Comparison between the new and conventional substrate oxidation methods for assay of metabolic $^{14}CO_2$ from HepG2 cells. HepG2 cells were seeded either in 12.5 cm^2 flasks or on 96-well tissue culture plates and grown to confluence. The flasks and wells were given equal amounts of [1-^{14}C]OA (20 μM) relative to their respective growth areas. Incubation was stopped after 2 h, and the $^{14}CO_2$ trapped by either system was monitored by liquid scintillation (B). Some flasks and wells were harvested for protein determination (C). Results represent means ± SEM of two separate experiments.

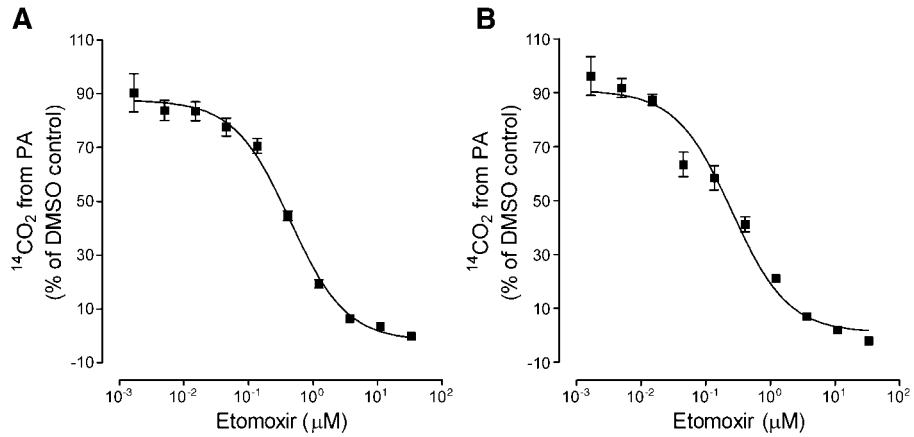

Fig. 6. Effects of a mitochondrial inhibitor on the oxidation of PA. [^{14}C]PA (20 μM) was incubated with cultured HepG2 cells or myotubes for 2 h with increasing concentrations of etomoxir (an irreversible inhibitor of mitochondrial β-oxidation). Etomoxir inhibited the oxidation of [^{14}C]PA in HepG2 cells ($IC_{50} \sim 0.4$ μM) (A) and in myotubes ($IC_{50} \sim 0.2$ μM) (B). Data shown represent a 10 point dose-response test (n = 3).

generates two-dimensional images of tracer distribution in the samples, but at the expense of low throughput and high cost.

Similar arguments presented as rationales for using SPA microplates to monitor nutrient accumulation in vitro also hold for our method for CO_2 trapping. We demonstrate the capture and quantification of $^{14}CO_2$ produced from cells grown on standard 96-well tissue culture plates. This method uses an inverted multiwell filter plate immersed with an alkaline solution (NaOH), placed with the wells aligned on top of the plate encompassing the cells of interest. This CO_2 trap generates similar results compared with a conventional method of capturing CO_2 and has a long linear range, covering the expected amount of CO_2 produced by cells grown on 96-well plates. In addition, the described method enables the cells to be washed and harvested after incubation, thereby increasing the yield of data that can be obtained from the experiment. Our constructed CO_2 trap also allows the exchange of filter plates during experiments, making time-course measurements very feasible. To validate the appliance for detecting CO_2 trapping, myotubes and HepG2 cells were incubated with the irreversible CPT1 inhibitor etomoxir, which markedly inhibited fatty acid oxidation. Myotubes were also preincubated with the selective peroxisome proliferator-activated receptor δ agonist GW501516, promoting a dose-dependent induction in $^{14}CO_2$ produced from oxidized [^{14}C]OA.

Comparison between markers of disease in vivo and defects/changes in cellular metabolism in vitro has the potential to be a fruitful strategy for studies of metabolic abnormalities. The exploration of this field of research has to a large extent been slowed by time-consuming and cumbersome methods for studying nutrient handling. In this article, we have described two principles for the detection of substrate accumulation and oxidation that may help to overcome some of these obstacles.

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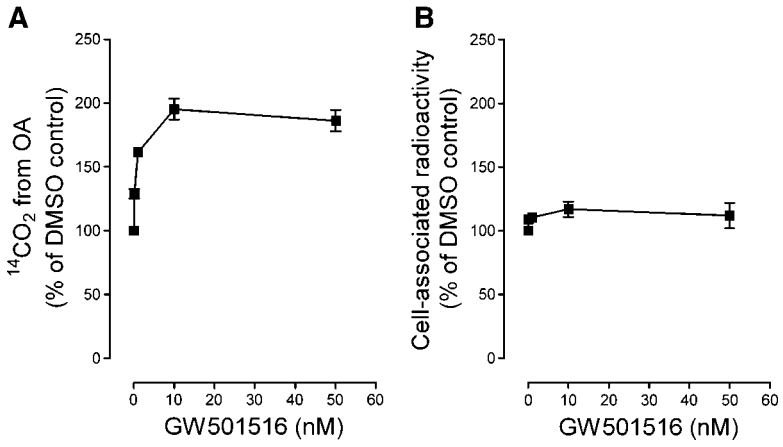

Fig. 7. Effects of an activator of peroxisome proliferator-activated receptor δ on cellular fatty acid oxidation. Differentiated myotubes were added to the activator GW501516 (0.1, 1.0, 10, or 50 nM) or DMSO (0.1%). After 3 days, with a change of medium after 2 days, the myotubes were assayed for the oxidation of [^{14}C]OA to $^{14}CO_2$ in the 96-well trapping system. The medium included DPBS with 20 mM HEPES, glucose (5 mM), L-carnitine (1 mM), and OA/[1-^{14}C]OA (100 μM/1 μCi/ml). The incubation was stopped after 4 h, and the trapped $^{14}CO_2$ was counted by scintillation (A), whereas the cells were harvested for determination of cell-associated radioactivity (B). Results represent means ± SEM of two separate experiments.

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