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Glycosylphosphatidylinositol-anchored proteins coordinate lipolysis inhibition between large and small adipocytes

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ABSTRACT

In response to palmitate, the antidiabetic sulfonylurea drug glimepiride, phosphoinositoglycans, or H_2O_2, the release of the glycosylphosphatidylinositol-anchored and cyclic adenosine monophosphate–degrading phosphodiesterase Gce1 from adipocytes into small vesicles (adiposomes) and its translocation from adiposomes to cytoplasmic lipid droplets (LD) of adipocytes have been reported. Here the role of Gce1-harboring adiposomes in coordinating lipolysis between differently sized adipocytes was studied. Separate or mixed populations of isolated epididymal rat adipocytes of small and large size and native adipose tissue pieces from young and old rats were incubated with exogenous adiposomes or depleted of endogenous adiposomes and then analyzed for translocation of Gce1 and lipolysis in response to above antilipolytic stimuli. Large compared with small adipocytes are more efficient in releasing Gce1 into adiposomes but less efficient in translocating Gce1 from adiposomes to LDs. Maximal lipolysis inhibition by above antilipolytic stimuli, but not by insulin, was observed with mixed populations of small and large adipocytes (1:1 to 1:2) rather than with separate populations. In mixed adipocyte populations and adipose tissue pieces from young, but not old, rats, lipolysis inhibition by above antilipolytic stimuli, but not by insulin, was dependent on the function of Gce1-harboring adiposomes. Inhibition of lipolysis in rat adipose tissue in response to palmitate, glimepiride, and H_2O_2 is coordinated via the release of adiposome-associated and glycosylphosphatidylinositol-anchored Gce1 from large "donor" adipocytes and their subsequent translocation to the LDs of small "acceptor" adipocytes. This transfer of antilipolytic information may be of pathophysiologic relevance.

1. Introduction

For more than 4 decades, it has been known that the metabolic activity of mammalian adipocytes critically depends on their position within the life cycle and thus on their size. Studies in a variety of species, including rats and humans, have demonstrated that, as adipocytes increase in size, the release of fatty acids from triacylglycerol (TAG) becomes up-regulated in parallel. As a result, larger adipocytes exhibit higher turnover rates of their lipid droplets (LDs) than smaller ones [1-4]. The underlying molecular mechanisms could involve an increase in the mass and/or activity of key enzymes and transporters engaged in lipolysis as the cell continues to increase in size [5,6]. According to this "mass action" model, the lipolytic rate depends on intrinsic properties of the adipocyte, that is, size and age, exclusively, and is not affected by neighboring adipocytes of the same or distinct adipose tissue depots, for example, via cell-to-cell contacts or adipokines. However, the "mass action" model does not consider the possibility of a physiologic control for shifting the burden

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of net fatty acid and TAG storage from large adipocytes with their exhausted capacity in LD biogenesis to small adipocytes with their fully developed capacity. Accordingly, younger and smaller adipocytes are more potent in the relative suppression of the basal and stimulated release of fatty acids from TAG compared with older and larger ones [5,6].

However, the causal relationship between lipolysis and cell size that, in addition to intracellular mechanisms, may also involve interactions between small and large adipocytes via cell adhesion molecules and/or humoral factors within the same or different adipose tissue depots [7,8] remains poorly understood so far. Metabolites, such as adenosine; nutrients, such as fatty acids; and adipo(cyto)kines, such as leptin and tumor necrosis factor (TNF)–α, produced and secreted by adipose tissue cells have been considered as candidates for the paracrine and endocrine signaling between adipocytes of the same and distinct adipose tissue depots, respectively [9-11]. In fact, the expression and secretion of numerous adipokines are altered in different obesity states and, in the few cases studied, depend on the size of the adipocytes [12,13]. Thus, adipokines alone or in combination with metabolites or nutrients released from adipocytes have been recognized to profoundly contribute to the control of lipolysis and cell size. Ultimately, the signaling cascades thereby initiated regulate the total cytosolic cyclic adenosine monophosphate (cAMP) levels [14,15] with resulting modulation of the cAMP-dependent phosphorylation and activities of key enzymes of lipolysis, such as hormone-sensitive lipase and adipocyte triglyceride lipase [16,17], which may be sufficient for the size control of adipocytes.

A novel molecular mechanism for the control of lipolysis has recently been elucidated in primary rat adipocytes. The physiologic stimuli palmitate and H_2O_2, the antidiabetic sulfonylurea drug glimepiride, and the insulin-mimetic phosphoinositolglycans (PIGs), which represent the polar core glycan head groups of the glycosylphosphatidylinositol (GPI) anchor of GPI-anchored proteins (GPI proteins), have been demonstrated to inhibit lipolysis via engagement of the following components and mechanisms [18-21]: (1) Lipid droplets act as the major storage compartment of TAG in the cytoplasm of almost each mammalian cell type and consist of a central core of TAG surrounded by a phospholipid monolayer with embedded cholesterol and specific proteins [22,23]. 2) The GPI proteins are modified by a highly conserved GPI glycolipid structure that is added posttranslationally to the carboxy-terminus of many eukaryotic proteins equipped with an additional transient carboxy-terminal signal sequence [24,25]. (3) Detergent-insoluble glycolipid-enriched membrane microdomains (DIGs) of the plasma membrane resist solubilization by nonionic detergents in the cold, float at high buoyant density during sucrose gradient centrifugation, and are the residence of a subset of GPI proteins with their protein moieties facing the cell surface [26]. (4) Small microvesicles and exosomes are released by plasma membrane shedding and exocytosis of multivesicular bodies, respectively, from many mammalian cell types in response to cholesterol-binding drugs, calcium, or lipids [27-30], often with unclear functional relevance. Their equivalents in cultured and primary mouse and rat adipocytes, the so-called adiposomes [31], harbor GPI proteins, among them the cAMP-degrading phosphodiesterase Gce1, and are released in response of palmitate, H_2O_2, glimepiride, and PIGs [32-34]. (5) Adiposomes released from "donor" adipocytes interact with "acceptor" adipocytes and thereby transfer Gce1 to DIGs of the "acceptor" adipocytes [34,35]. (6) Gce1 is translocated from the DIGs to intracellular LDs of the "acceptor" adipocytes [36]. (7) Gce1 specifically degrades cAMP at the LD surface zone [21]. The cooperation of these components and mechanisms could lead to down-regulation of fatty acid release from TAG in the "acceptor" adipocytes [37,38], even in cases where only the "donor" adipocytes are directly exposed to palmitate, H_2O_2, glimepiride, and PIGs.

Lipolysis inhibition by palmitate, H_2O_2, and glimepiride is likely to be of physiologic/pharmacologic relevance on the basis of their antilipolytic effects reported for relevant primary and cultured adipose cells upon treatment with total (free and protein-bound) maximal plasma concentrations, which have been measured during starvation (0.5-2 mmol/L total fatty acids) [39], experimental induction of oxidative stress (10-100 μmol/L total reactive oxygen species) [40], and antidiabetic therapy (0.5-1.0 μmol/L glimepiride) [41] of healthy probands and type 2 diabetes mellitus patients, respectively. Moreover, excess of palmitate is thought to down-regulate its further lipolytic generation by a negative feedback mechanism [16], whereas H_2O_2 [42] and PIGs [37] have been implicated as intracellular second messengers for insulin signal transduction in relevant insulin target cells, among them lipolysis inhibition in adipocytes. Thus, elucidation of the molecular mechanism of lipolysis inhibition by palmitate, glimepiride, PIG37, and H_2O_2 may be useful for the identification of novel pharmacologic targets for diabetes therapy.

Here the possibility of the involvement of Gce1-harboring adiposomes in the communication between large and small adipocytes about their lipolytic states was studied. The data obtained with mixed populations of large and small adipocytes compared with separate populations of uniform size as well as with native adipose tissue pieces in the presence and absence of functional adiposomes argue for the transfer of information encoding lipolysis inhibition from large to small adipocytes via GPI protein–harboring adiposomes.

2. Materials and methods

2.1. *Materials*

Phosphoinositolglycan 37 and 1 [43] and glimepiride [44] were synthesized by the chemical synthesis department of Sanofi-Aventis Deutschland (Frankfurt, Germany). Glucose oxidase (GO) and complete protease and phosphatase inhibitor cocktails were provided by Roche Biochemicals (Mannheim, Germany). Annexin-V (free or coupled to biotin) was delivered by Calbiochem/Merck (Darmstadt, Germany). All other materials were purchased from sources as described previously [18-21,32-35,45-49].

2.2. *Preparation of large and small adipocytes*

Adipocytes were isolated by collagenase digestion of epididymal fat pads from male Sprague-Dawley rats (fed ad libitum)

and washed according to published procedures [45]. After determination of the cell number on basis of the lipocrit (see below), the adipocytes were adjusted with KRH buffer (140 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 20 mmol/L HEPES/KOH [pH 7.4]) supplemented with 0.5% bovine serum albumin (BSA), 100 μg/mL gentamicin, 50 U/mL penicillin, and 50 μg/mL streptomycin sulfate to a titer of 3.5×10^6 cells per milliliter. Adipocytes prepared from 1- (120-140 g) and 6-month-old (320-380 g) male rats were filtered through serial nylon mesh screens with pore sizes of 75, 150, and 400 μm to obtain small (diameter <75 μm) and large (diameter >400 μm) adipocytes, respectively. After fixation of aliquots of the adipocyte suspension with osmic acid, the cell number was determined using a Coulter counter. Total lipid content of the adipocyte suspension ("lipocrit") was measured as described previously [49]. The rats were killed in accordance with the German animal protection law.

2.3. Preparation of partially digested fat pads

Intact epididymal fat pads were excised from young (120-140 g) or old (320-380 g) male rats (Sprague-Dawley, fed ad libitum) under sterile conditions, liberated from all adherent blood vessels and connective tissue fragments, cut into pieces of similar size (~5 mm diameter), washed several times with buffer F (125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 2.5 mmol/L $MgCl_2$, 1 mmol/L KH_2PO_4, 2% BSA [type V, fatty acid-free], 5.5 mmol/L glucose, and 25 mmol/L Tris/HCl [pH 7.4]), and then incubated (20 minutes, 30°C) with 80 and 450 μg of collagenase type II (Sigma, Deisenhofen, Germany; 480 U/mg) in 2 mL of buffer F containing 8 and 4 adipose tissue pieces derived from fat pads of young and old rats, respectively. Thereafter, the adipose tissue pieces were washed with buffer F (3 times with 2 mL/mg of adipose tissue piece each).

2.4. Release of Gce1 into adiposomes

Rat adipocytes (3.5×10^6) metabolically labeled with *myo*-[^{14}C] inositol were incubated (120 minutes, 37°C) in the absence or presence of stimuli as described previously [32,33]. Three-hundred-fifty–microliter portions of the incubation mixtures were transferred to microfuge tubes prefilled with 100 μL of dinonylphtalate and then centrifuged (2000 rpm, 1 minute, 20°C). The incubation medium below the dinonylphtalate layer was removed and centrifuged (3000*g*, 20 minutes, 4°C). The adiposomes were collected from the supernatant by centrifugation (Beckman Airfuge, Beckman-Coulter, Krefeld, Germany; A-110 fixed angle rotor, 110 000 rpm, 30 minutes, 4°C) and then suspended in 100 μL of 10 mmol/L Tris/HCl (pH 7.4), 1 mmol/L EDTA, 0.5 mmol/L EGTA, 140 mmol/L NaCl, 10 mmol/L $MgCl_2$, 2 mmol/L $MnCl_2$, 1 mmol/L dithiothreitol (DTT), and protease inhibitor mix for affinity purification. For this, the adiposomes were solubilized by addition of 100 μL of 50 mmol/L MOPS/KOH (pH 7.0), 1 mol/L NaCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 100 μmol/L isobutylmethylxanthine, 120 mmol/L octyl glucoside, and 1% ß-amidotaurocholate and then incubated in the absence or presence of 1 mmol/L cAMP (as control) with 0.2 mL of N^6-(2-aminoethyl)-cAMP-Sepharose (GE Healthcare, Freiburg, Germany), which had been equilibrated with the same buffer supplemented with 0.5 mg/mL BSA. After centrifugation (12 000*g*, 2 minutes, 4°C), the pelleted Sepharose beads were washed 2 times with 1 mL of the same buffer each and then suspended in 0.2 mL of the same buffer containing 1 mmol/L cAMP. After incubation (30 minutes, 4°C) and subsequent centrifugation (12 000*g*, 2 minutes, 4°C), the supernatant was precipitated by incubation (1 hour, on ice) with 0.2 mL of 20% trichloroacetic acid and centrifugation (12 000*g*, 10 minutes, 4°C). Precipitated proteins were washed once with ice-cold acetone, dried, solubilized in 50 μL of 2-fold Laemmli sample buffer, and finally analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and phosphorimaging as described previously [18].

2.5. Translocation of Gce1 from adiposomes to LDs

For preparation of the adiposomes, untreated rat adipocytes (25 mL, 3.5×10^5 cells per milliliter) were washed twice with 50 mL of labeling medium (buffer F supplemented with 1 mmol/L sodium pyruvate, 0.2% BSA, 25 μg/mL gentamicin, 20 U/mL penicillin, and 20 μg/mL streptomycin sulfate) each and then incubated (2 hours, 37°C) in 44 mL of labeling medium in 150-mL culture flasks under 95% O_2/5% CO_2. Labeling was started by the addition of *myo*-[U-^{14}C]inositol (25 μCi in 1 mL of labeling medium, 0.1 mmol/L final concentration). After incubation (5 hours, 37°C), the adipocytes were induced by the addition of GO (0.5 U/mL). Following incubation (12 hours, 37°C) and subsequent addition of 5 mL of labeling medium supplemented with 100 mmol/L *myo*-inositol, the adiposomes were prepared by transfer of the adipocyte suspension (50 mL, continuous shaking) into polypropylene tubes and subsequent centrifugation (2000*g*, 1 minute, 20°C). The infranatant below the floating adipocyte layer was removed by suction taking care to avoid contamination with the adipocytes. After transfer into new tubes, the procedure was repeated 2 times. The final infranatant was passed through a 5-μm mesh to remove residual cells. The effluent was supplemented with DTT (final concentration 0.5 mmol/L) and protease inhibitor mix "complete" (10 mmol/L Tris/HCl [pH 7.4], 250 mmol/L sucrose, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 140 mmol/L NaCl, 10 mmol/L $MgCl_2$, 2 mmol/L $MnCl_2$, 1 mmol/L isobutylmethylxanthine, 1 mmol/L DTT, 20 mmol/L sodium fluoride, 25 mmol/L glycerol-3-phosphate, 10 mmol/L sodium pyrophosphate, and 1 tablet per 2 mL of protease inhibitor mix "complete") and then centrifuged (3000*g*, 20 minutes, 4°C) to remove residual cell debris. The supernatant was centrifuged (150 000*g*, 60 minutes, 4°C, Ti-40 rotor, Beckman). After careful aspiration of the supernatants, the pellets were suspended in 30 mL of ADIP buffer and recentrifuged as above. The pellet (adiposomes) was washed (vortexing) 2 times with 15 mL of ADIP buffer each and finally suspended in ADIP buffer at 1 μg phosphatidylcholine per microliter.

For studying the translocation, isolated rat adipocytes (3.5×10^6 cells) were incubated (37°C, 2 hours) with identical amounts (ie, radioactivity) of adiposomes (20-50 μg phosphatidylcholine) harboring metabolically labeled Gce1 in 1 mL of ADIP buffer and then recovered from the incubation mixture by flotation (500*g*, 1 minute, 20 °C), washed once with 15 mL of adipocyte buffer, and then suspended in 1.5 mL of adipocyte buffer (final titer, 1.4×10^6 cells per milliliter). Two-hundred-

microliter portions of the adipocyte suspension were transferred into microfuge tubes prefilled with 100 μL of dinonylphtalate and then centrifuged (2000 rpm, 1 minute, 20°C). The tubes were cut through the dinonylphtalate layer. The adipocytes at the top of the upper part were transferred into 1-mL polystyrene tubes containing 0.5 mL of adipocyte buffer and then centrifuged (500*g*, 2 minutes, 20°C). The adipocytes floating at the top (~0.2 mL) were suspended in 0.5 mL of buffer L (25 mmol/L Tris/HCl [pH 7.4], 250 mmol/L sucrose, 5 mmol/L NaF, 10 mmol/L $NaPP_i$, 1 mmol/L Na_3VO_4, 1 mmol/L EDTA, 5 μg/mL pepstatin, 20 μg/mL leupeptin, 1 mmol/L benzamidine, 0.5 mmol/L phenylmethylsulfonyl fluoride supplemented with complete protease and phosphatase inhibitor cocktail tablets (Roche).

For preparation of the LDs, the adipocytes were homogenized (1-mL Teflon-in-glass homogenizer, 10 strokes, 500 rpm, 15°C). The homogenate was centrifuged (500*g*, 5 minutes, 15°C); 0.3-mL portions of the supernatant were combined with 0.5 mL of 65% sucrose (wt/vol) each and poured into 2-mL centrifuge tubes; 0.5 mL of 10% sucrose (wt/vol) was then layered on top of the sucrose cushion. The tube was filled to capacity with buffer L lacking sucrose. The gradients were centrifuged (172 000*g*, 1 hour, 15°C) and then allowed to coast to rest. The floating fraction of LDs was visualized as the upper white layer of the gradient, isolated by suction with a syringe (0.1-0.2 mL), and washed 2 times with 1 mL of buffer L each. Lipid droplets were reisolated from incubation mixtures by 10-fold dilution with buffer L, subsequent centrifugation (12 000*g*, 2 minutes, 15°C), and final recovery of the upper LD layer by suction.

For analysis of Gce1, LDs were suspended in 4-fold Laemmli sample buffer containing 20% SDS (typically 50 μL/10 μL of LD) by incubation (10 minutes, 65°C). After centrifugation (10 000*g*, 5 minutes, 25°C), the infranatant was withdrawn by suction; and proteins were precipitated by incubation (1 hour, on ice) with the same volume of 20% trichloroacetic acid and centrifugation (12 000*g*, 10 minutes, 4°C). Precipitated proteins were washed once with ice-cold acetone, dried, solubilized in 2-fold Laemmli sample buffer, and finally analyzed by SDS-PAGE and phosphorimaging.

2.6. Lipolysis

Rat adipocytes (3.5×10^5) were incubated (120 minutes, 37°C) in 1 mL of KRH in the absence or presence of stimuli and then challenged (15 minutes, 37°C) with 1 μmol/L isoproterenol. After flotation of the adipocytes (200*g*, 2 minutes, 30°C), portions of the infranatant were used for glycerol determination [50].

Partially digested fat pads were incubated (2 hours, 30°C) with buffer F (8 pieces in 1 mL) in the absence or presence of annexin-V (25 μg/mL) or cAMP-Sepharose (100 μg/mL) in a shaking water bath (250 cycles per minute). After addition of palmitate, glimepiride, PIG37, GO, or insulin and incubation (30 minutes) under the same conditions, isoproterenol (0.2 μmol/L final concentration) and adenosine deaminase (0.5 U/mL final concentration, Merck) were added. Following incubation (4 hours) under the same conditions, the incubation mixtures were filtered over a nylon mesh (5 μm pore size). Portions of the flow through were used for glycerol determination.

2.7. Data analysis

Results are given as mean ± SD for independent experiments using separate cell or adiposome preparations as detailed in the figure legends. Relations between concentrations and activities were analyzed by using a 1-site model with fitting (GraphPad Prism 4.03 software, GraphPad Software, Inc., La Jolla, CA) of the function $B = 1/(1 + [A]/[EC_{50}/IC_{50}])^n$ with B representing activity; $[A]$, signal concentration; EC_{50}/IC_{50}, half-maximally effective/inhibitory concentration; and n, slope parameter (Hill coefficient). Significances were calculated by the 2-tailed U test of Wilcoxon. $P \leq .05$ was considered as significant.

2.8. Miscellaneous

Published procedures were used for protein extraction under native and denaturing conditions, protein precipitation under denaturing conditions, determination of protein concentration using the BCA method (Pierce) with BSA as standard, SDS-PAGE, and phosphorimaging [18,20,48]. Figures of phosphoimages were constructed using the Adobe Photoshop software (Adobe Systems, Mountain View, CA).

3. Results

3.1. Large and small adipocytes differ in their efficacies of releasing Gce1 into adiposomes and translocating Gce1 to LDs

The apparent differential regulation of lipolysis inhibition in older and larger adipocytes vs younger and smaller adipocytes was confirmed with primary adipocytes prepared from epididymal fat pads of male rats of different age and then separated according to large (>400 μm) and small (<75 μm) size by filtration through appropriately sized meshes. The release

Table 1 – Basal lipolysis in separate and mixed adipocyte populations

Population	Separate			Mixed	Mixed vs separate
Adipocytes	Large	Small	Mean	Large + small	% of mean
Fatty acids	921 ± 144	106 ± 19	514[a]	293 ± 77	57[b]
Glycerol	437 ± 64	65 ± 11	251[a]	159 ± 25	63[b]

Large or small adipocytes or mixed populations (1:1) prepared thereof (1.1×10^6 cells per milliliter) were incubated (30 minutes, 37°C). The adipocytes were recovered by flotation (500*g*, 2 minutes, 20°C) and then assayed for lipolysis (2 hours, 37°C) as described previously [27]. Quantitative evaluations are given as the amounts of fatty acids or glycerol released into the incubation medium (in micromoles per liter, mean ± SD, total) derived from 3 to 5 adipocyte incubations each with determinations in duplicate and triplicate, respectively.

[a] Significant differences between the arithmetic means calculated from the values of the small and large adipocytes (identical numbers) and the measured values of the mixed populations.

[b] Significant differences in lipolysis rates of the mixed populations given as percentages of the arithmetic means of the separate small and large adipocytes (set at 100% each).

of fatty acids and glycerol from TAG in the basal, that is, unstimulated, state was significantly higher (8.7- and 6.7-fold, respectively) from (identical numbers of) large compared with small adipocytes (Table 1). The differential metabolic activity of large and small adipocytes raised the possibility that the release of GPI protein–harboring adiposomes, which seem to be involved in the intercellular regulation of lipid metabolism [34,35], also depends on the apparent size of the adipocytes. This was investigated by metabolic labeling of the GPI anchor of Gce1 with *myo*-[^{14}C]inositol before induction of large or small adipocytes with fatty acids, GO (for the production of H_2O_2 in glucose-containing incubation medium), antidiabetic sulfonylurea drugs, or insulin-mimetic PIGs known to provoke the release of adiposomes from rat adipocytes with different efficacy [32-34]. Analysis of the adiposomes recovered from the incubation medium for the presence of Gce1 by affinity purification, SDS-PAGE, and phosphorimaging revealed that, compared with the basal state, the sulfonylureas of the third-generation glimepiride and the second generation glibenclamide, but not of the first-generation tolbutamide; the saturated fatty acids palmitate and stearate, but not the unsaturated fatty acid oleate; the insulin-mimetic PIG37, but not the inactive PIG1; and GO increased in concentration-dependent fashion the amount of Gce1 that was recovered with adiposomes from the incubation medium of both large and small adipocytes (Fig. 1). Importantly, the release of Gce1 in response to each stimulus was significantly (by 2.2- to 2.9-fold) more efficient with large compared with small adipocytes. The ranking order between the stimuli in their efficacy of Gce1 release was identical for large and small adipocytes with GO > PIG37 > glimepiride > glibenclamide > palmitate > stearate (Fig. 1).

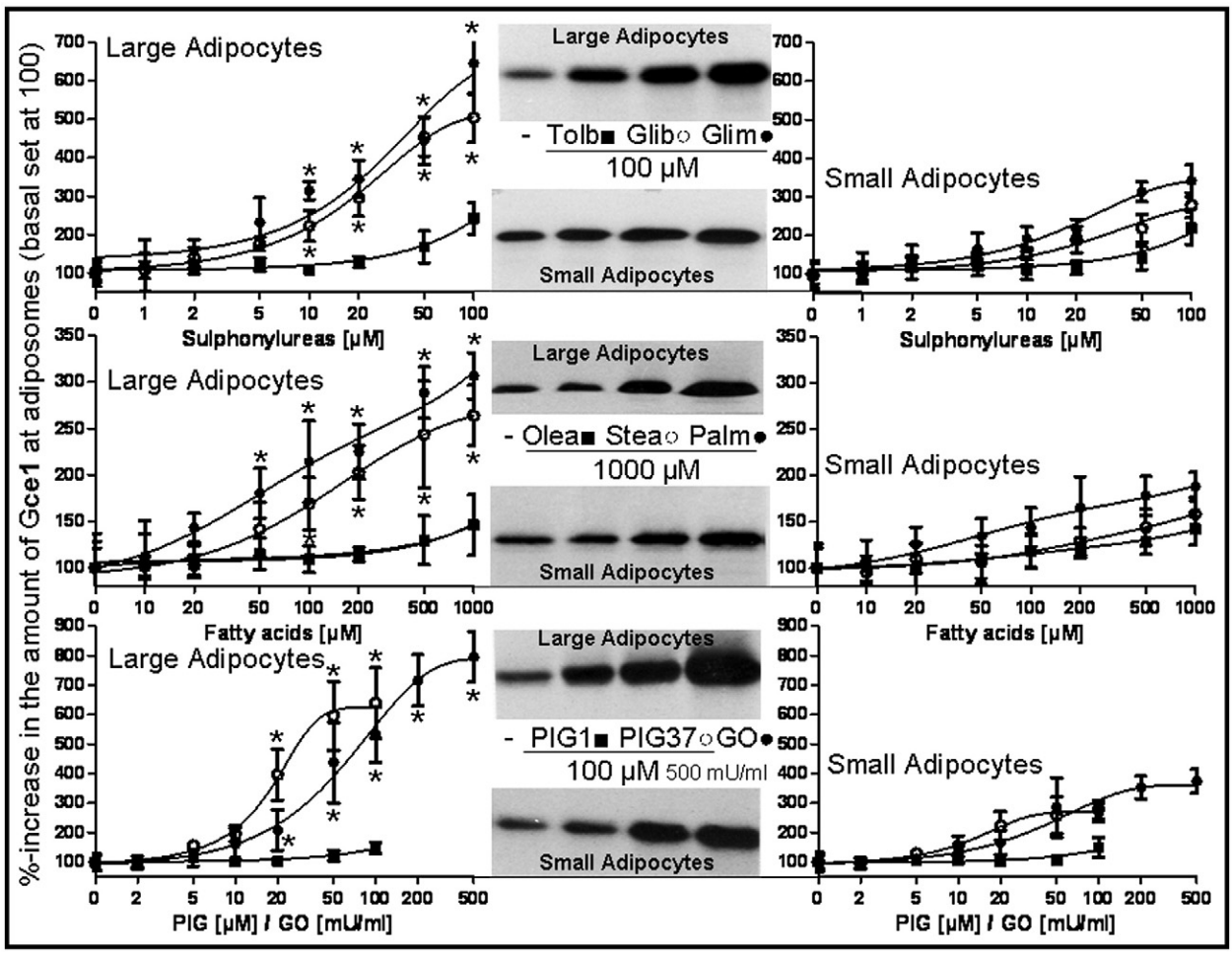

Fig. 1 – Differential release of Gce1 into adiposomes from large and small adipocytes. Large and small rat adipocytes (identical numbers) were metabolically labeled with *myo*-[^{14}C]inositol and then incubated (3 hours, 37°C) in the absence (basal state) or presence of increasing concentrations of palmitate (Palm), stearate (Stea), oleate (Olea), glimepiride (Glim), glibenclamide (Glib), tolbutamide (Tolb), GO, PIG37, or PIG1. After flotation (500*g*, 2 minutes, 20°C), adiposomes were prepared from the incubation medium and then extracted for proteins under native conditions. Gce1 was affinity purified from the extract; precipitated under denaturing conditions; and, after solubilization, analyzed by SDS-PAGE and phosphorimaging. Representative images for each stimulus at the highest effective concentration each are shown. Quantitative evaluations of the amounts of Gce1 recovered with adiposomes are given as percentage of increases set at 100 in the basal state. Data (mean ± SD) were derived from 3 adipocyte incubations each with determinations in duplicate. *Significant differences between large and small adipocytes in response to the same stimulus.

Adiposomes harboring the GPI-protein Gce1 and released from donor adipocytes are known to interact with acceptor adipocytes that trigger the translocation of Gce1 from plasma membrane DIGs to cytoplasmic LDs [34,35]. This raised the possibility that the differential release of Gce1 into adiposomes by large and small "donor" adipocytes is also reflected in its subsequent translocation to the cytoplasmic LDs of large and small "acceptor" adipocytes. To test for this, large and small adipocytes were incubated with adiposomes harboring metabolically labeled Gce1. Analysis of the LDs prepared from the adipocytes for the presence of Gce1 by affinity purification, SDS-PAGE, and phosphorimaging revealed that, compared with the basal state, glimepiride and glibenclamide rather than tolbutamide, palmitate and stearate rather than oleate, PIG37 rather than PIG1, and GO increased in concentration-dependent fashion the amount of Gce1 that was recovered with LDs from both small and large adipocytes (Fig. 2). Importantly, this apparent translocation of Gce1 to LD in response to each stimulus was significantly (by 1.8- to 5.1-fold) more efficient in small compared with large adipocytes. The ranking order between the stimuli in their efficacy of Gce1 translocation was identical for small and large adipocytes with GO > PIG37 > glimepiride > glibenclamide > palmitate > stearate (Fig. 2). The EC_{50} values for the induction of the release of Gce1 into adiposomes and its translocation to LD were similar for each stimulus and not affected by the adipocyte size (Figs. 1 and 2). In conclusion, large and small adipocytes apparently differ in the regulation of lipolysis inhibition as well as release of Gce1 into adiposomes and its translocation to LD. Large and small adipocytes operate as the more efficient donor and acceptor cells, respectively, for both fatty acids and Gce1.

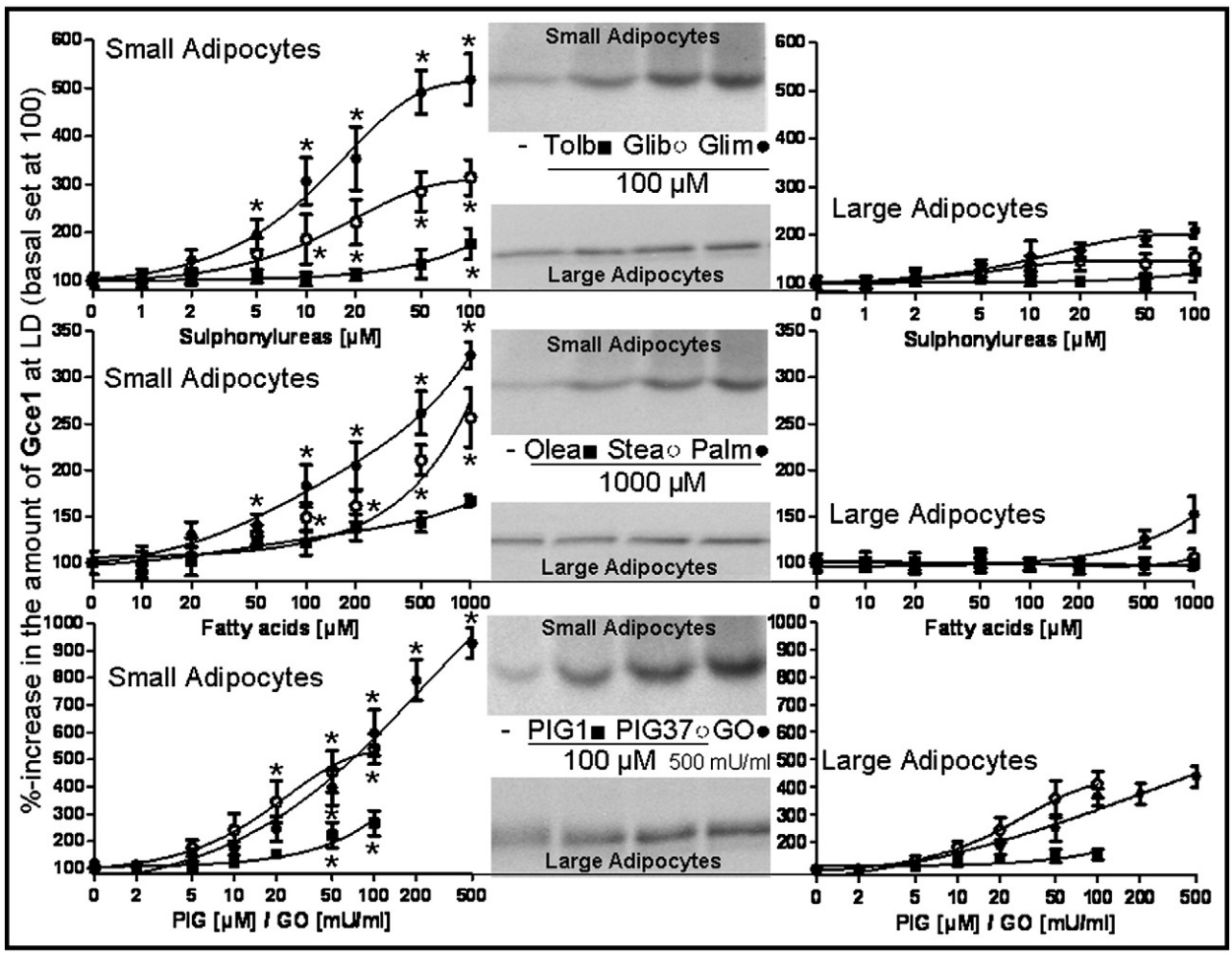

Fig. 2 – Differential translocation of Gce1 from adiposomes to LDs in large and small adipocytes. Adiposomes (identical amounts according to radioactivity) harboring metabolically labeled Gce1 from GO-induced (0.5 U/mL) adipocytes were incubated (2 hours, 37°C) with small adipocytes or large adipocytes (identical numbers) in the absence (basal state) or presence of increasing concentrations of palmitate (Palm), stearate (Stea), oleate (Olea), glimepiride (Glim), glibenclamide (Glib), tolbutamide (Tolb), GO, PIG37, or PIG1. After recovery of the adipocytes by flotation, LDs were prepared and extracted for proteins under native conditions. Gce1 was affinity purified from the extract; precipitated under denaturing conditions; and, after solubilization, analyzed by SDS-PAGE and phosphorimaging. Representative images for each stimulus at the highest effective concentration each are shown. Quantitative evaluations of the amounts of Gce1 recovered with the LDs are given as the percentage of increases set at 100 in the basal state. Data (mean ± SD) were derived from 2 adipocyte incubations each with determinations in quadruplicate. *Significant differences between large and small adipocytes in response to the same stimulus.

3.2. *Mixed populations of large and small adipocytes exhibit the highest responsiveness and sensitivity for adiposome-mediated inhibition of lipolysis*

To recognize putative interactions between adipocytes of different size during lipolysis inhibition, large and small adipocytes were mixed at varying ratios ranging from 20:1 to 1:20 and then tested for lipolysis at identical total cell number and increasing lipocrit (= percentage of total lipid in the incubation mixture). In case of interaction, the observed rates of lipolysis should deviate from the arithmetic means that are calculated for the same numbers of small and large adipocytes on the basis of separately performed assays (Fig. 3). In fact, in the basal state, the release of glycerol into the incubation medium was significantly diminished by 37% to 43% in the mixed (1:1) population compared with the arithmetic means of the lipolysis rates of small and large adipocytes. In the stimulated state (provoked by palmitate, glimepiride, PIG37, and GO, in that order of increasing potency), the release of glycerol into the incubation medium was most pronounced with 2:1 to 1:2 mixed populations of small and large adipocytes and steadily declined with higher portions of either small or large cells. Significant deviations from the incubations with either large or small adipocytes were already observed with mixed populations constituted by relative portions of large and small cells as low as 1:10 to 1:20 and 1:5 to 1:20 (Fig. 3). As expected, in the basal state, lipolysis steadily increased with increasing portions of large adipocytes. Furthermore, inhibition of lipolysis by insulin was most pronounced for populations of adipocytes of uniform small size and steadily declined in mixed populations with increasing portions of large adipocytes (Fig. 3). In conclusion, inhibition of lipolysis by palmitate, glimepiride, PIG37, and H_2O_2, but not by insulin, critically depends on the ratio of large and small adipocytes, with mixed populations displaying higher maximal responsiveness compared with populations with adipocytes of uniform large or small size.

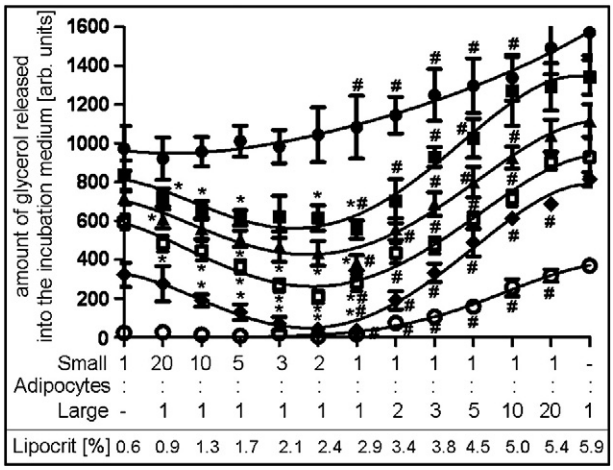

Fig. 3 – Dependence of the maximal lipolysis inhibition by fatty acids, sulfonylureas, PIGs, and H_2O_2 on the relative portions of large and small adipocytes. Populations of either large or small adipocytes and mixed populations derived thereof with varying ratios as indicated (1.1×10^6 cells per milliliter each) were incubated (30 minutes, 37°C) in the absence (basal state, •) or presence of palmitate (1 mmol/L, ■), glimepiride (30 μmol/L, ▲), PIG37 (50 μmol/L, □), GO (1 U/mL, ♦), or insulin (5 nmol/L, ○). The adipocytes were recovered by flotation and then assayed for isoproterenol-induced lipolysis (presence of 0.1 μmol/L isoproterenol, 2 hours, 37°C). Quantitative evaluations are given as the amounts of glycerol released into the incubation medium. Data (mean ± SD) were derived from 3 adipocyte incubations each with determinations in duplicate. Significant differences between the populations consisting of either *only small (extreme left values) or #only large (extreme right values) adipocytes and the mixed populations in response to the same stimulus.

Putative alterations in the sensitivity of large and small adipocytes toward lipolysis inhibition by these stimuli upon incubation in the mixed (1:1) compared with the uniform populations were studied by determination of the corresponding IC_{50} values. The concentration-response curves for palmitate, glimepiride, PIG37, and GO for lipolysis inhibition were significantly shifted to the left for the mixed compared with the uniform populations of small and large adipocytes (Fig. 4). This resulted in significantly lower EC_{50} values for each stimulus in the mixed populations of small and large adipocytes vs populations consisting of either small or large adipocytes (mixed vs small vs large for palmitate: 0.17 vs 0.24 vs 0.42 mmol/L; for glimepiride: 6.8 vs 18.5 vs 27.4 μmol/L; for PIG37: 7.3 vs 19.9 vs 28.6 μmol/L; for GO: 0.06 vs 0.19 vs 0.43 U/mL). In contrast, the IC_{50} values for insulin inhibition of lipolysis were lowest for small adipocytes (0.05 nmol/L), followed by the mixed (1:1) population (0.18 nmol/L) and, lastly, large adipocytes (0.45 nmol/L) (Fig. 4). In conclusion, the significantly higher sensitivity of mixed adipocyte populations compared with populations consisting of either small or large adipocytes for inhibition of lipolysis by palmitate, glimepiride, PIG37, and H_2O_2 argues for communication and interaction between the differently sized adipocytes under the incubation conditions used that may operate via diffusible factors, such as adiposomes, rather than cell-to-cell contacts. At variance, the insulin sensitivity of lipolysis inhibition simply declines with increasing mean adipocyte size, that is, small > mixed > large. This is compatible with insulin signaling to the antilipolytic effector system being independent of and unaffected by communication between large and small adipocytes.

The above findings of the inverse relationship in the efficacies in releasing Gce1 into adiposomes and translocating Gce1 to LD between large and small adipocytes (Figs. 1 and 2) and of the maximal stimulus-induced inhibition of lipolysis with mixed populations of large and small adipocytes (Figs. 3 and 4) argued for the involvement of Gce1-harboring adiposomes in the regulation of lipolysis inhibition between adipocytes of different size by palmitate, glimepiride, PIG37, and H_2O_2. To test for this possibility, populations consisting of either small or large adipocytes or mixed (1:1) populations of large and small adipocytes were incubated with these stimuli in the presence of cAMP-Sepharose beads that bind to Gce1-harboring adiposomes. After removal of the beads together with the bound adiposomes by centrifugation, the adipocyte populations were assayed for lipolysis. Alternatively, the adipocyte populations were incubated with annexin-V

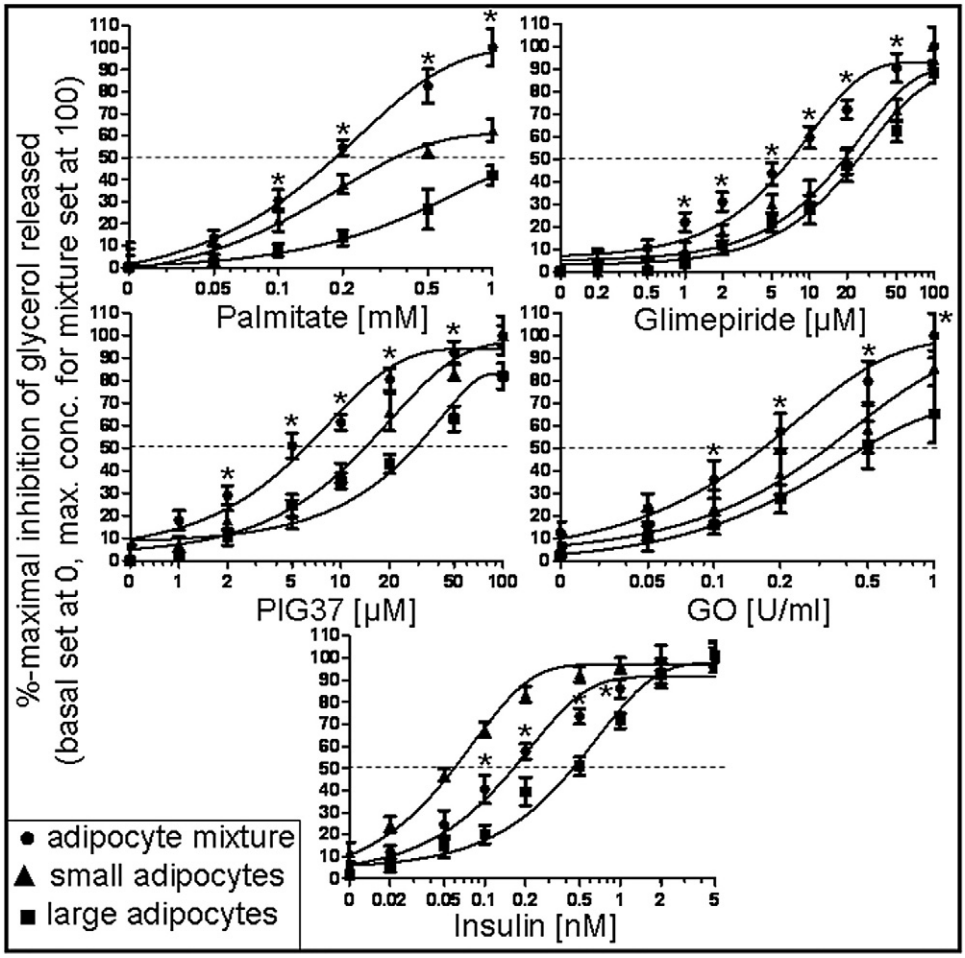

Fig. 4 – Dependence of the sensitivity of lipolysis inhibition by fatty acids, sulfonylureas, PIGs, and H_2O_2 on the relative portions of large and small adipocytes. Populations of either small or large adipocytes and mixed populations (2:1) derived thereof (1.1 × 10^6 cells per milliliter each) were incubated (30 minutes, 37°C) in the absence (basal state) or presence of increasing concentrations of palmitate, glimepiride, PIG37, GO, or insulin. The adipocytes were recovered by flotation and then assayed for isoproterenol-induced lipolysis (presence of 0.1 µmol/L isoproterenol, 2 hours, 37°C). Quantitative evaluations are given as percentage of the reductions in the amounts of glycerol released into the incubation medium by the populations consisting of either small (▲) or large (■) adipocytes and the mixed populations (●) derived thereof (basal set at 0) in response to each stimulus in comparison to its maximal effect in the mixed population (set at 100). Data were derived from 2 to 4 adipocyte incubations each with determinations in triplicate (mean ± SD). *Significant differences between the mixed populations and the separate populations of either small or large adipocytes.

coupled to biotin before the lipolysis assay. This was based on recent findings that the transfer of microvesicle cargo between donor and acceptor cells often involves interaction between phosphatidylserine residues exposed on the microvesicle surface and the acceptor cell plasma membrane. The interaction can be blocked by the phosphatidylserine-binding protein, annexin-V [51,52], or biotinylated annexin-V (biotinylation of annexin-V does not significantly affect its binding affinity to phosphatidylserine; Müller G, unpublished observation). The efficient binding of biotinylated annexin-V to GPI protein–harboring adiposomes was recently demonstrated by addition of excess of streptavidin-agarose and biotinylated annexin-V to adiposomes and subsequent identification of Gce1 in the pelleted and washed agarose complexes (Müller G, Dearey E-A, unpublished results).

In the mixed adipocyte populations, maximal down-regulation of glycerol release into the incubation medium by GO, PIG37, glimepiride, and palmitate (in that order of declining potency) was significantly diminished upon removal of the Gce1-harboring adiposomes before assaying lipolysis (Fig. 5) or the presence of biotinylated annexin-V (annexin-V–biotin) during the assay (Fig. 6, not shown for PIG37). In contrast, in populations consisting of either small or large adipocytes, the less pronounced (compared with the mixed population) stimulus-induced effects were not significantly affected by either removal of the adiposomes (Fig. 5) or presence of annexin-V–biotin (Fig. 6). Strikingly, in both mixed and uniform adipocyte populations, depletion of adiposomes (Fig. 5) as well as presence of annexin-V–biotin (Fig. 6) did not impair insulin inhibition of lipolysis. In conclusion,

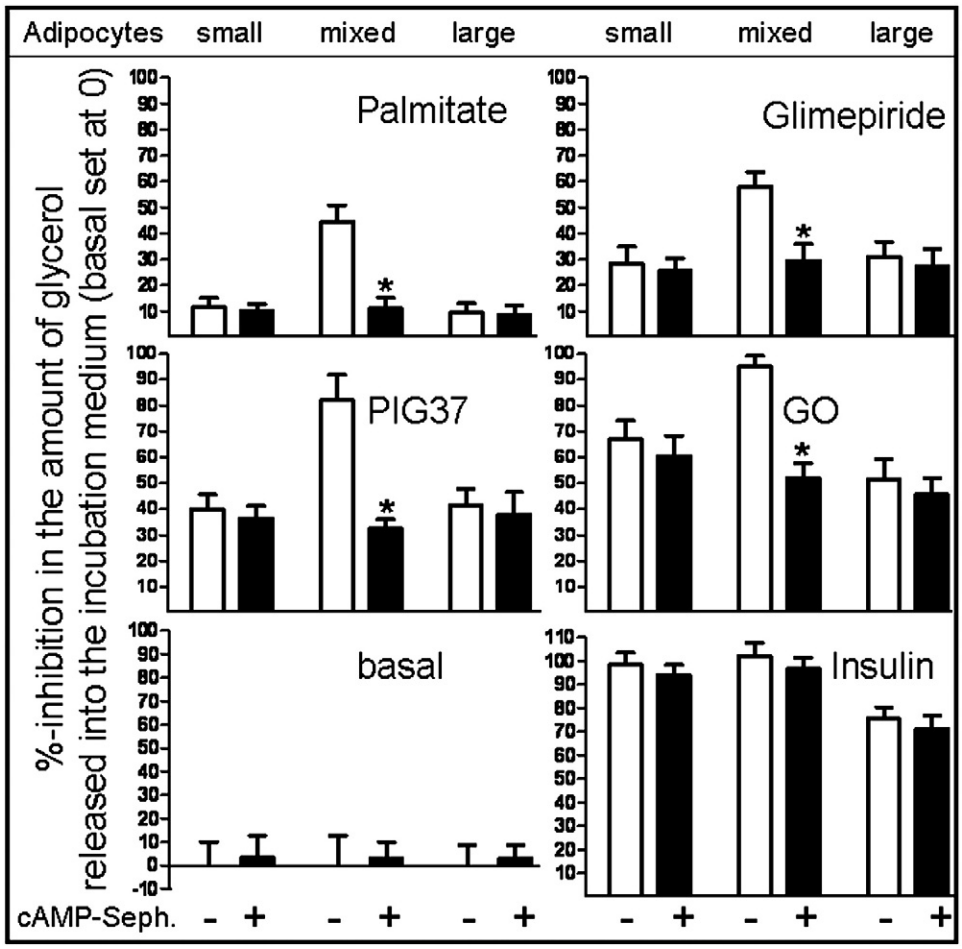

Fig. 5 – Dependence of lipolysis inhibition by fatty acids, sulfonylureas, PIGs and H_2O_2 in the mixed adipocyte populations on adiposomes harboring Gce1. Populations consisting either small or large adipocytes or mixed populations (2:1) derived thereof (1.1×10^6 cells per milliliter each) were incubated (30 min, 37°C) in the absence (basal state) or presence of palmitate (1 mmol/L), glimepiride (30 μmol/L), PIG37 (50 μmol/L), GO (1 U/mL) or insulin (5 nmol/L). After further incubation (2 hours, 37°C) in the absence or presence of cAMP-Sepharose, the adipocytes were recovered by flotation and then assayed for isoproterenol-induced lipolysis (presence of 0.1 μmol/L isoproterenol; 2 hours, 37°C). Quantitative evaluations of the amounts of glycerol released into the incubation medium are given as percentage inhibition compared with basal (set at 0) and derived from 2 to 4 adipocyte incubations with determinations in duplicate (mean ± SD). *Significant differences between the absence and presence of cAMP-Sepharose for the populations of either small or large adipocytes and the mixed populations derived thereof.

adiposomes harboring Gce1 with phosphatidylserine residues accessible at their surface are required for maximal inhibition of lipolysis in mixed populations of large and small adipocytes by H_2O_2, PIG37, glimepiride, and palmitate, but not by insulin.

3.3. *Adipose tissue pieces from young rats exhibit adiposome-mediated inhibition of lipolysis*

The demonstration of an apparent role of adiposomes in the inhibition of lipolysis by various stimuli with the exception of insulin in incubation mixtures of isolated primary rat adipocytes of large and small size prompted the question of whether this holds true for the native adipose tissue. To test for this, epididymal fat pads were excised from male old and young rats, then cut into pieces of similar size, and finally partially digested with collagenase to a degree that is compatible with the access of annexin-V and cAMP-Sepharose beads to the interstitial spaces. Care was taken to avoid dissociation of the adipose tissue pieces and release of floating adipocytes that could interfere with the transfer of adiposomes and/or the translocation of Gce1 via adiposomes between neighboring adipocytes within the adipose tissue pieces in positive or negative fashion. After incubation with annexin-V or cAMP-Sepharose and extensive washing, the adipose tissue pieces were challenged with increasing concentrations of palmitate, glimepiride, PIG37, GO, and insulin and subsequently assayed for isoproterenol-induced glycerol release.

In partially digested adipose tissue pieces prepared from young rats, each of these antilipolytic stimuli blocked lipolysis in a concentration-dependent fashion with varying

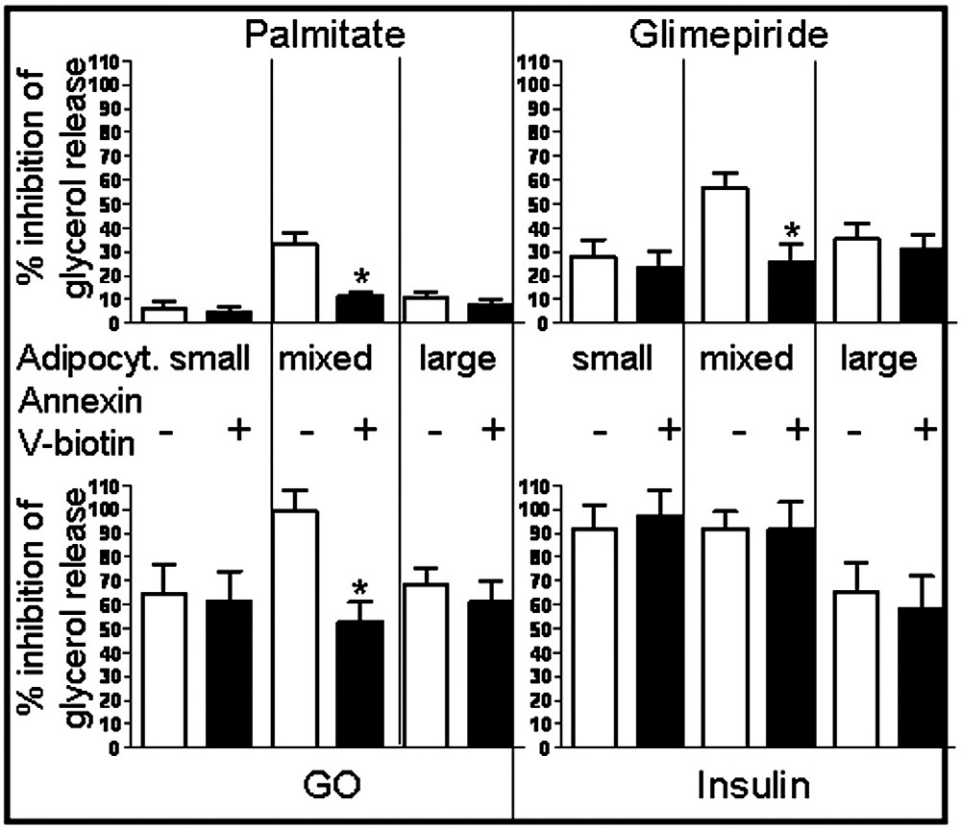

Fig. 6 – Impairment of lipolysis inhibition by fatty acids, sulfonylureas, and H_2O_2 in primary rat adipocytes in the presence of annexin-V. Populations of either small or large adipocytes and mixed populations (2:1) derived thereof (1.1×10^6 cells per milliliter each) were incubated (30 minutes, 37°C) in the absence (basal state) or presence of palmitate (1 mmol/L), glimepiride (30 μmol/L), GO (1 U/mL), or insulin (5 nmol/L). After further incubation (2 hours, 37°C) in the absence or presence of annexin-V coupled to biotin (annexin-V–biotin; 2 μg/mL), the adipocytes were separated from the incubation medium by flotation. Subsequently, the adipocytes were assayed for isoproterenol-induced lipolysis (presence of 0.1 μmol/L isoproterenol; 2 hours, 37°C). Quantitative evaluations of the amounts of glycerol released into the incubation medium are given as percentage inhibition compared with basal (set at 0). Data were derived from 3 to 5 adipocyte incubations each with determinations in triplicate and duplicate, respectively (mean ± SD). *Significant differences between the absence and presence of annexin-V–biotin for the populations of either small or large adipocytes and mixed populations derived thereof.

maximal potency (Fig. 7A), the ranking order of which (insulin > GO > PIG37 > glimepiride > palmitate) being identical to that observed with isolated rat adipocytes (Fig. 4). Pretreatment of the adipose tissue pieces with annexin-V or cAMP-Sepharose before assaying lipolysis inhibition revealed considerable rightward shifts of the concentration-response curves in parallel with significantly diminished responses at half-maximally and maximally effective concentrations for palmitate, glimepiride, PIG37, and GO (Fig. 7A). In contrast, the concentration-response curves for insulin did not differ significantly between the absence and presence of annexin-V or cAMP-Sepharose. Thus, the reduced maximal responsiveness and sensitivity of adipose tissue pieces toward lipolysis inhibition by palmitate, glimepiride, PIG37, and GO upon exposure to annexin-V or cAMP-Sepharose suggest the involvement of Gce1-harboring adiposomes in mediating their antilipolytic activity in adipose tissue pieces from young rats.

As expected, in partially digested adipose tissue pieces prepared from old rats, the maximal inhibition of lipolysis by palmitate, glimepiride, PIG37, GO, and insulin under control conditions approached 50% or even less, only, of that for young rats (Fig. 7B), with considerable increases in the corresponding IC_{50} values for old rats (1 mmol/L, 100 μmol/L, >100 μmol/L, >2 U/mL, and 3.9 nmol/L; Fig. 7B) compared with those for young rats (0.3 mmol/L, 3.6 μmol/L, 11.9 mmol/L, 0.5 U/mL, and 1.2 nmol/L; Fig. 7A). This reflects resistance/lower sensitivity for lipolysis inhibition by the various stimuli, including insulin, of adipose tissue pieces from old vs young rats. Strikingly, pretreatment of the adipose tissue pieces from old rats with annexin-V or cAMP-Sepharose did not further impair lipolysis inhibition compared with control (Fig. 7B). In fact, in the presence of either annexin-V or cAMP-Sepharose, the IC_{50} values for palmitate, glimepiride, PIG37, and GO were found to approach those determined for the adipose tissue pieces from young rats (0.6-0.7 mmol/L, 21 μmol/L, 25-35 μmol/L,

and 0.7-1 U/mL). This missing effect of annexin-V and cAMP-Sepharose on lipolysis inhibition by the various stimuli in adipose tissue pieces of old rats and the similar lipolysis inhibition in adipose tissue pieces of old rats and young rats in the presence of annexin-V and cAMP-Sepharose are compatible with either the lack of a sufficient number of small acceptor adipocytes or the functional impairment of the translocation of Gce1 from adiposomes to LDs of adipocytes. Furthermore, potent lipolysis inhibition by insulin does not depend on Gce1-harboring adiposomes in adipose tissue pieces from both young and old rats.

4. Discussion

4.1. *Differential inhibition of lipolysis in small and large adipocytes by adiposomes*

The following experimental evidence presented here suggests that there is communication between adipocytes of different size about their lipolytic states both in the absence and, considerably more pronounced, in the presence of the antilipolytic stimuli palmitate, glimepiride, PIG37, and H_2O_2

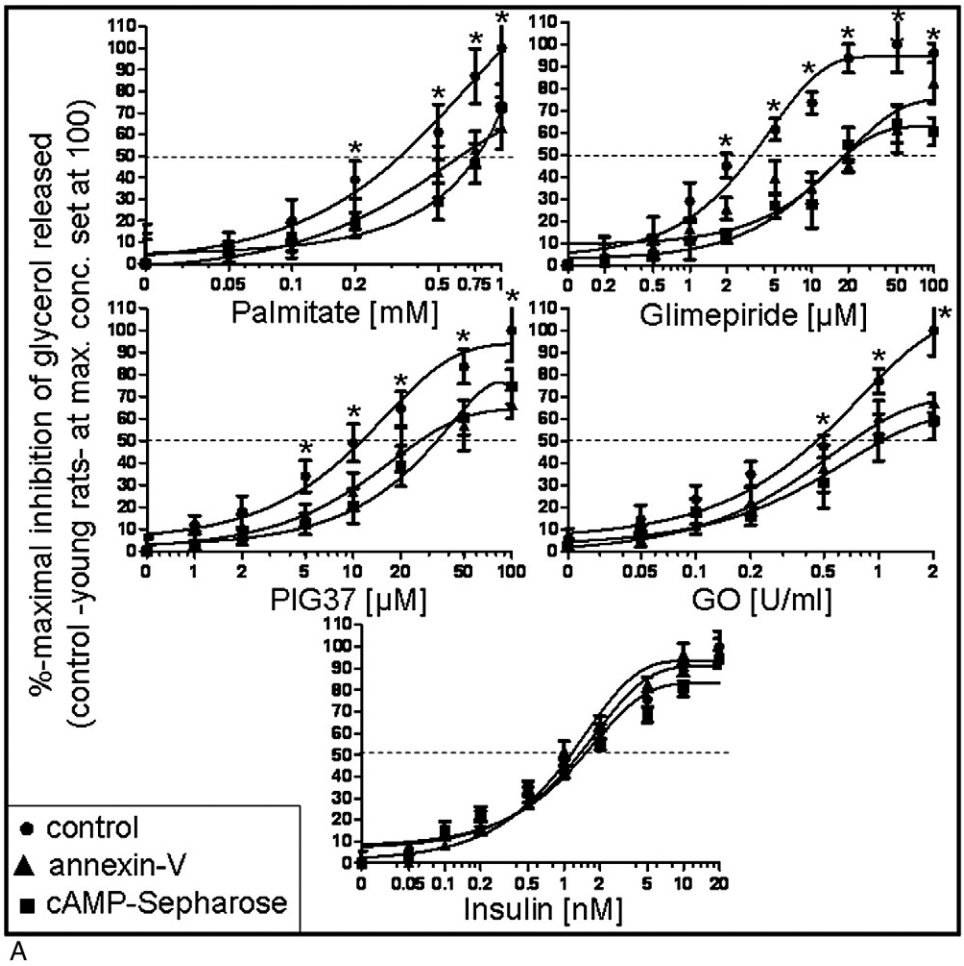

Fig. 7 – Impairment of lipolysis inhibition by fatty acids, sulfonylureas, PIG37, and H_2O_2 in rat adipose tissue pieces in the presence of annexin-V and cAMP-Sepharose beads. Epididymal fat pads prepared from male young or old rats were partially digested with collagenase, washed, and then incubated (2 hours, 30°C) in the absence (control; •), or presence of annexin-V (▲) or cAMP-Sepharose beads (■) under vigorous shaking. After addition of palmitate, glimepiride, PIG37, GO, or insulin at increasing concentrations and further incubation (30 minutes, 30°C), the incubation mixtures were supplemented with isoproterenol (0.2 μmol/L final concentration). After incubation (4 hours, 30°C), the mixtures were subjected to filtration. The flow throughs were centrifuged and then analyzed for glycerol released into the incubation medium (absence of annexin-V/cAMP and stimulus: 376 ± 139 and 1466 ± 159 μmol glycerol per milligram wet weight of fat pads from young and old rats, respectively). Quantitative evaluations are given as percentage of the reductions in the isoproterenol-induced glycerol release (difference between absence and presence of isoproterenol) by the fad pads from young (A) or old (B) rats (absence of stimulus set at 0) in response to each stimulus in the absence compared with the presence of annexin-V/cAMP-Sepharose in the incubation mixtures. The percentage lipolysis inhibition provoked by maximally effective stimulus concentrations in fad pads from young rats, which had been incubated in the absence of annexin-V/cAMP, was set at 100 for each stimulus and used for normalization of the relative inhibition values at each concentration for fat pads from young and old rats. Data were derived from 3 fat pad incubations each with determinations in triplicate (mean ± SD). *Significant differences between the absence and presence of annexin-V or cAMP-Sepharose.

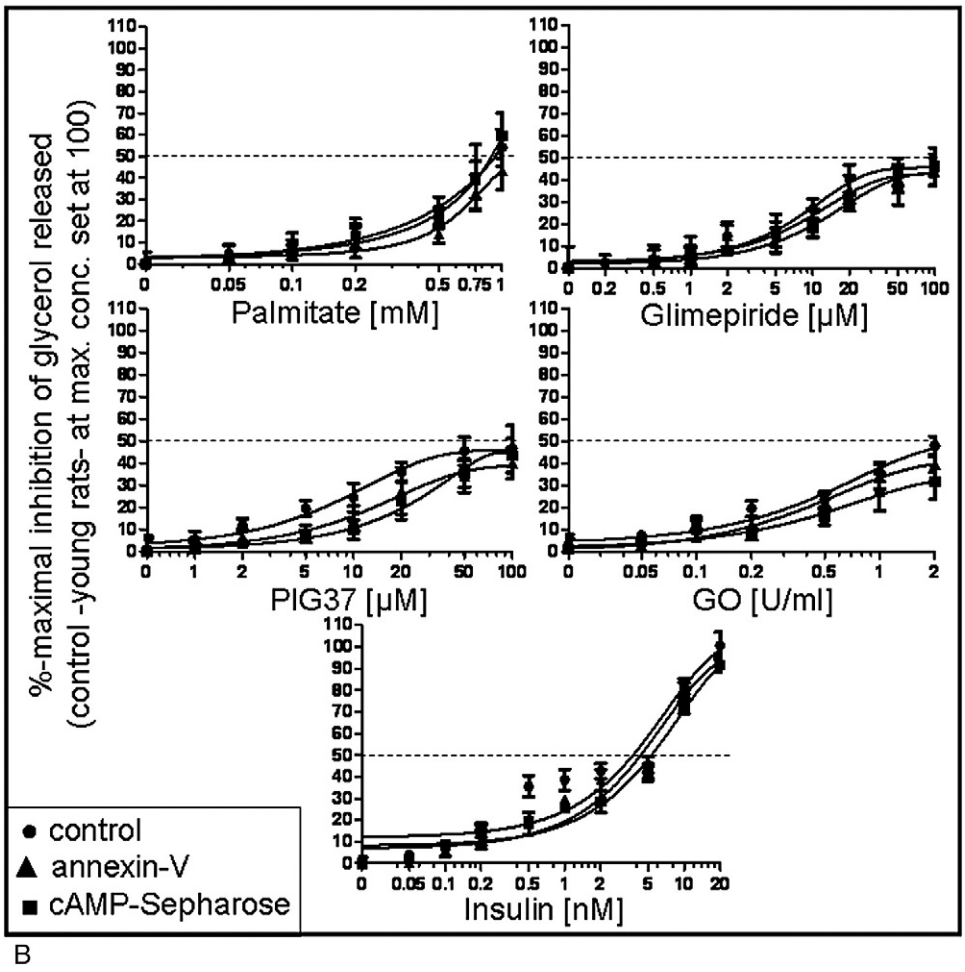

Fig. 7 – (*continued*).

via GPI protein–harboring adiposomes: (1) Older and larger adipocytes are more efficient in releasing the GPI protein Gce1 into adiposomes than younger and smaller ones (Fig. 1) and thus operate as the "better donors." (2) Younger and smaller adipocytes are more efficient in translocating Gce1 from adiposomes to LDs than older and larger ones (Fig. 2) and thus operate as the "better acceptors." (3) Mixed populations of identical numbers of large and small adipocytes are more susceptible to lipolysis inhibition by palmitate, glimepiride, PIG37, and H_2O_2 than populations consisting of the same total number of either large or small adipocytes (Fig. 3). This is true for both maximal responsiveness (ie, percentage of inhibition at maximally effective concentrations) (Fig. 3) and sensitivity (ie, IC_{50} values) (Fig. 4). (4) The responsiveness of the mixed populations of identical numbers of large and small adipocytes, but not of populations consisting of the same total number of either large or small adipocytes, for lipolysis inhibition by palmitate, glimepiride, PIG37, and H_2O_2 becomes significantly reduced upon removal of Gce1-harboring adiposomes (Fig. 5) or masking of surface-exposed phosphatidylserine residues (Fig. 6). (5) In partially digested adipose tissue, pieces derived from young rats lipolysis inhibition by palmitate, glimepiride, PIG37, and H_2O_2, but not by insulin, is significantly impaired upon removal of Gce1-harboring adiposomes or masking of surface-exposed phosphatidylserine residues (Fig. 7A).

It is reasonable to assume that the antilipolytic communication between large and small adipocytes via Gce1-harboring adiposomes is directly regulated by palmitate, glimepiride, PIG37, and H_2O_2 rather than represents an indirect consequence of elevated apoptosis on adiposome release in large vs small adipocytes. So far, there is only limited experimental evidence for a higher sensitivity of primary adipocytes from old compared with young rats, that is, of larger compared with smaller adipocytes, toward certain apoptotic signals, such as TNF-α and troglitazone, which accounted for up to 1.5- to 2-fold relative increases [53,54]. Previous findings with mixtures of large and small rat adipocytes have revealed that, under comparable short-term exposures, 2-fold increases in apoptosis (provoked by combinations of TNF-α and cycloheximide) did not provoke the release of Gce1-harboring adiposomes to any degree [32]. Thus, it seems unlikely that lipolysis and size regulation between large and small rat adipocytes by palmitate, glimepiride, and H_2O_2 relies on their differential age- and size-dependent susceptibility toward stimulus-induced apoptosis and accompanying vesicle release by plasma membrane shedding/blebbing. Furthermore, the protein composition of membrane vesicles released in response to cellular stress and

apoptosis has been analyzed for a number of mammalian cell types in the past, but did not reveal significant enrichments of GPI proteins vs total plasma membranes [28-31]. However, the specific incorporation of GPI proteins, among them Gce1, into microvesicles and exosomes, that is, adiposomes, is however typical for rat adipocytes, which are characterized by low rates of apoptosis, indicating the distinct nature of fat cell apoptotic vesicles and adiposomes.

4.2. *Roles of information transfer via microvesicles and exosomes*

Microvesicles and exosomes have been implicated in many different (patho)physiologic processes, such as secretion, immunomodulation, inflammation, coagulation, and intercellular communication [28-31]. These functions are usually attributed to the exchange of the microvesicle/exosome cargo between adjacent or remote cells, which encompasses lipids, nucleic acids [55], soluble proteins [56], or transmembrane proteins [52,57], such as chemokine receptors [58], transferrin receptors [59], tissue factor [57], and receptor tyrosine kinases [52,59]. Recently, a mechanism leading to endothelial cell expression of the epidermal growth factor (EGF) receptor by its microvesicle-mediated transfer from EGF receptor–transformed cancer cells has been reported [52,60]. Fusion of EGF receptor–containing microvesicles with endothelial cells led to incorporation of the receptor into the plasma membrane of the acceptor cell and triggered downstream signaling to angiogenesis. Thus, it was proposed that oncogene-containing tumor cell–derived microvesicles may act as information carrier for angiogenesis that are capable of switching endothelial cells. This concept has meanwhile been expanded to activated platelets during the development of lung cancers [61]. Furthermore, tumor cells have been shown to use shedding and uptake of microvesicles to evade destruction by immune cells [62,63]. However, these pathophysiologic mechanisms are actually only examples of the misuse of the release and transfer of constituent components by microvesicles from donor to acceptor cells, which developed in the course of evolution. In contrast, the transfer of antilipolytic information via adiposomes between adipocytes may be regarded as an example for a physiologic and evolutionary advantageous function of microvesicles and exosomes.

The down-regulation of lipolysis via the transfer of Gce1-harboring adiposomes to small adipocytes is compatible with

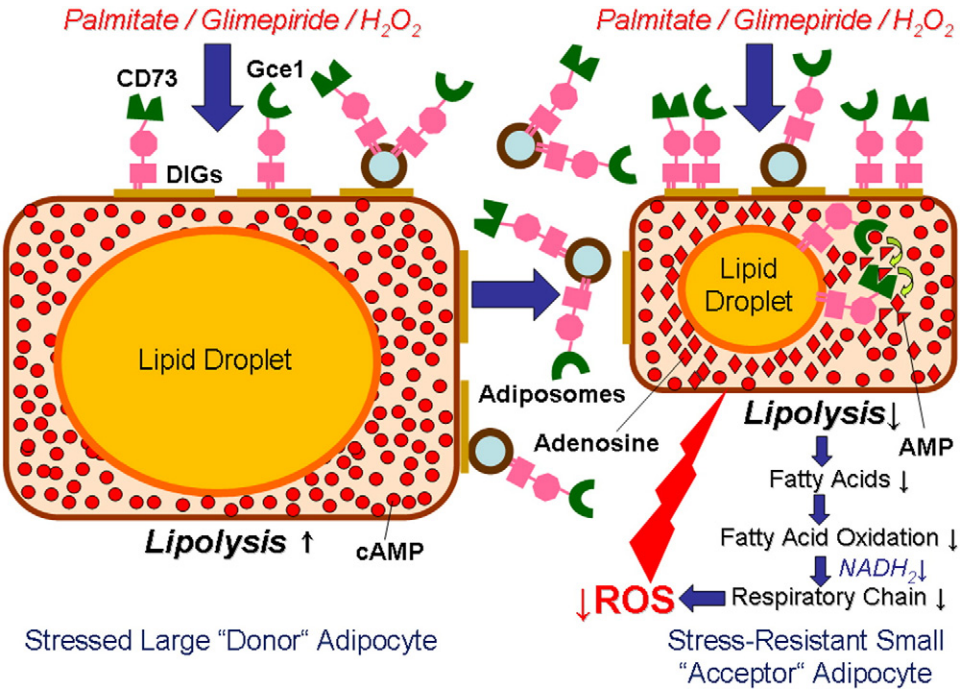

Fig. 8 – Hypothetical model for a physiologic function of the transfer of antilipolytic information between large and small adipocytes by GPI protein–harboring adiposomes in stress protection. Exposure of native adipose tissue consisting of both large and small adipocytes to physiologic concentrations of palmitate and H_2O_2 or pharmacologic concentrations of glimepiride induces the release of Gce1 and CD73 from plasma membrane DIGs of large "donor" adipocytes, which typically exert pronounced lipolysis due to high cytosolic cAMP concentrations, into adiposomes. Subsequently, Gce1 and CD73 transferred from the adiposomes to DIGs of small "acceptor" adipocytes are translocated to their LDs. The resulting degradation of cAMP through Gce1 and CD73 to AMP and adenosine, respectively, causes down-regulation of lipolysis; levels of free fatty acids and fatty acyl–coenzyme A; ß-oxidation and levels of $NADH_2$; and, ultimately, respiratory chain activity. The submaximal electron flow through the respiratory chain is accompanied by generation of reduced amounts of reactive oxygen species. Thereby, the extent of this kind of oxidative stress becomes restricted, preferably in the adipose tissues of young rats, which harbor a higher portion of smaller "acceptor" vs larger "donor" adipocytes than those of old rats. See text for details. ROS indicates reactive oxygen species.

the assumed physiologic role of young and small adipocytes in the net storage of fatty acids esterified into TAG rather than the net release of fatty acids from TAG. This postmaturation increase in adipose tissue mass, so characteristic of people of the Western world, is driven by the need to accommodate excess energy intake. It can be accomplished by hypertrophy, that is, the transition of small adipocytes into large adipocytes. On the basis of the finding of small adipocytes operating as better acceptors for adiposomes than large ones, it is tempting to speculate that the transfer to DIGs and the subsequent translocation to LD of Gce1 with the resulting degradation of cAMP at the LD surface zone make the functional difference between small and large adipocytes. It would guarantee coordinated and more efficient lipolysis inhibition in the course of dephosphorylation and inactivation of the relevant lipases in small adipocytes compared with that of large ones. In fact, the observed higher efficacy of large vs small adipocytes in operating as donors, that is, in releasing Gce1 into adiposomes following its translocation from DIGs to LDs, may indicate a shorter period of residence of Gce1 at the LDs of large compared with small adipocytes. Currently, the anticipated higher abundance of Gce1 at the LDs of small compared with large adipocytes in the basal and more pronouncedly in the palmitate-, PIG37-, glimepiride-, and H_2O_2-induced states is being investigated using confocal immunofluorescence microscopy of intact adipocytes.

The significant impairment of the palmitate-, glimepiride-, PIG37-, and H_2O_2-induced lipolysis inhibition provoked by annexin-V and cAMP-Sepharose in the mixtures consisting of large and small adipocytes (Figs. 5 and 6) was also demonstrated with adipose tissue pieces prepared from young rats (Fig. 7A), but only after limited digestion with collagenase (Müller G, Wied S, unpublished data). The findings suggest that annexin-V and cAMP-Sepharose upon penetration into the interstitial spaces interfere with the function of Gce1-harboring adiposomes, that is, the transfer of Gce1 from donor to acceptor and/or its translocation into acceptor adipocytes, within native rat adipose tissue. These data hint to the relevance of lipolysis regulation by GPI protein–harboring adiposomes in vivo.

Native adipose tissue pieces consist of a mixture of differently sized cells with the portion of large vs small adipocytes rapidly increasing with the age of the rats and with higher sensitivity toward lipolysis inhibition of smaller compared with larger adipocytes [53]. Thus, in adipose tissue of young rats, the transfer of the antilipolytic information via Gce1-harboring adiposomes from the few large to the many small adipocytes and, in consequence, the inhibition of lipolysis by palmitate, glimepiride, PIG37, and H_2O_2 will proceed very efficiently and critically depend on functional adiposomes (Fig. 7A). At variance, in adipose tissue of old rats, the number of small adipocytes acting as acceptors for the Gce1-harboring adiposomes may be insufficient for a major contribution to lipolysis inhibition. This would explain the missing effect of functional adiposomes in the interstitial tissue spaces of adipose tissue pieces from old rats (Fig. 7B). In addition, a molecular defect in the translocation of Gce1 from adiposomes to LDs in small acceptor adipocytes could prevent involvement of adiposomes in lipolysis inhibition in adipose tissue pieces of old rats. Interestingly, small adipocytes prepared from young rats have been shown to respond to adiposomes with higher efficacy compared with large cells (Fig. 2). The putative differential, that is, more and less efficient, functioning of small and large adipocytes as acceptors for adiposomes, which remains to be demonstrated for native adipose tissue pieces, could explain the apparent failure in shifting the burden of lipid loading from large to small adipocytes in old rats. In conclusion, on the basis of the combined evidence from the mixtures of isolated adipocytes and the adipose tissue pieces, it is reasonable to assume that, in the native adipose tissue, in response to palmitate, glimepiride, PIG37, and H_2O_2, but not insulin, Gce1 is transferred from (DIGs of) larger donor adipocytes to (LDs of) smaller acceptor adipocytes here resulting in degradation of cAMP at the LD surface zone and inhibition of lipolysis, preferentially in young rats (Fig. 8).

In addition to the regulation of cell size within native rat adipose tissues in response to various physiologic and pharmacologic stimuli, the adiposome-mediated transfer of antilipolytic information between neighboring large and small adipocytes may fulfill other physiologic functions, such as protection against oxidative stress (Fig. 8). It is thought that the palmitate-, glimepiride-, PIG37-, and H_2O_2-induced blockade of the release of free fatty acids from adipocyte LDs leads as a consequence of diminished fatty acid oxidation to submaximal fueling of the respiratory chain that thereby produces less reactive oxygen species as intrinsic "unwanted" side products. For H_2O_2 and palmitate, the adiposome-mediated transfer from donor to acceptor adipocytes and the subsequent translocation from their DIGs to LDs of Gce1 may be regarded as a physiologic negative feedback mechanism that restricts oxidative damage in the course of maximal fatty acid oxidation. However, this adiposome-dependent stress protection would be restricted to small adipocytes of young rats and explain a more pronounced sensitivity toward reactive oxygen species of adipocytes in old compared with young rats, compatible with the frequently reported increased inflammatory state of adipocytes from older rats [55,63,64].

4.3. *Molecular mechanisms of the adipocyte size-dependent regulation of lipolysis*

The communication of large and small adipocytes about their lipolytic states can be regarded as a component of a feedback loop that ultimately prevents overloading of adipocytes with LDs when their sizes have reached critical values. The following experimental evidences and theoretical considerations suggest that the sensor for the adipocyte and LD sizes may actually measure the efficacy of the translocation of cholesterol and GPI proteins, among them Gce1, from plasma membrane DIGs to LDs: (1) The adipocyte and LD sizes and the translocation efficacies are inversely correlated [33]. (2) During translocation, Gce1 is accompanied by another GPI protein, CD73; the cholesterol-binding protein caveolin-1; the LD-associated structural protein perilipin-A; and cholesterol. (3) Removal of cholesterol from plasma membrane DIGs by cholesterol-complexing agents blocks the induced translocation of Gce1 and CD73 to LDs [33,47,48]. (4) Plasma membrane (DIGs) and LDs represent the major

compartments of both cholesterol deposition and GPI protein expression in adipocytes [18,47,48]. (5) A continuous flux of cholesterol from the plasma membrane (DIGs) to the LDs, with the higher the LD content, the greater the need for cholesterol transport from the DIGs, has been hypothesized to participate in the sensing of TAG stores and LD size in adipocytes [64,65]. Taken together, it is tempting to speculate that the translocation of cholesterol in concert with Gce1, CD73, caveolin-1, and perilipin-A from plasma membrane DIGs to LDs might serve as intracellular signal for the increase in LD content, that is, LD size and number, during adipocyte maturation and enlargement. Consequently, defects in certain components of the cholesterol-dependent GPI protein translocation machinery could lead to failure of large adipocytes to recognize filling up of their TAG stores and accumulation of LDs of critical size and number. Those large adipocytes, which sense themselves as "small," should consequently fail to release Gce1-harboring adiposomes at amounts required to shift the burden of fatty acid and lipid loading to small adipocytes. As a result, small adipocytes would fail to gain size.

Interestingly, very small adipocytes were recently detected in moderately obese human subjects with the help of osmium tetroxide fixation and scanning electron microscopy [66,67]. These are apparently resistant toward further TAG accumulation and maturation, more efficient in secreting proinflammatory adipokines than larger adipocytes, and present at higher portions in insulin-resistant compared with insulin-sensitive obese subjects. Defective lipid synthesis and storage, LD biogenesis, adipogenesis, and control of cell size have been debated as the molecular mechanisms underlying the persistence of the very small adipocytes [66-68]. It is conceivable that (some of) these putative defects may be overcome by pharmacologic up-regulation of the transfer of antilipolytic information from large to these very small adipocytes, for example, with drugs stimulating the translocation of GPI protein–cholesterol complexes from DIGs to LDs in the small adipocytes. However, the physiologic relevance of the very small vs large adipocytes for the pathogenesis of insulin resistance and type 2 diabetes mellitus remains to be elucidated [13].

In any case, the proposed pathway of intercellular transfer of antilipolytic information via adiposome-associated Gce1 may provide novel drug targets to foster LD biogenesis in (initially very) small adipocytes by pharmacologic modulation of the release and/or translocation of adiposome-associated GPI-proteins. This possibility is reinforced by the present finding that annexin-V abrogated lipolysis inhibition by Gce1-harboring adiposomes in adipocytes presumably by blockade of the interaction between phosphatidylserine and adiposome-associated surface receptors specific for phosphatidylserine, such as Tim4, BAI1, and KIM1 [69-71]. In the future, it will be important to identify components and mechanisms that positively regulate the transfer of antilipolytic information from large to (very) small adipocytes. This analysis should also include intracellular components, such as the LD coat proteins, among them the PAT proteins, perilipin, adipophilin, and TIP47 [72-74]. They could be involved in targeting to and anchorage at the LD surface phospholipid monolayer of Gce1 upon its transfer from adiposomes to plasma membrane DIGs of (very) small "acceptor" adipocytes. This knowledge will help to shift the emphasis on the reduction of the total amount of adipose tissue as prevalent during the past decades [75] toward the need for the identification of specific mechanisms and factors that allow control of the optimal size distribution of adipocytes.

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