**Age-dependent membrane release and degradation of full-length glycosylphosphatidylinositol-anchored proteins in rats**

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**A B S T R A C T**

Glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) are associated with the surface of eucaryotic cells only through a covalently coupled carboxy-terminal GPI glycolipid structure which is anchored at the outer leaflet of plasma membranes. This mode of membrane association may be responsible for the recent observations that full-length GPI-APs harbouring the complete GPI anchor are (i) released from isolated rat adipocytes *in vitro* and (ii) expressed in rat and human serum. The upregulation of the adipocyte release in response to increased cell size and blood glucose/insulin levels of the donor rats and downregulation of the expression in serum of insulin resistant and diabetic rats have been reconciled with enhanced degradation of the full-length GPI-APs released into micelle-like complexes together with (lyso)phospholipids and cholesterol by serum GPI-specific phospholipase D (GPI-PLD).

 Here by using a sensitive and reliable sensing method for full-length GPI-APs, which relies on surface acoustic waves propagating over microfluidic chips, the upregulation of (i) the release of the full-length GPI-APs CD73, alkaline phosphatase and CD55 from isolated adipocyte plasma membranes monitored in a “lab-on-the-chip” configuration, (ii) their release from isolated rat adipocytes into the incubation medium and (iii) the lipolytic cleavage of their GPI anchors in serum was demonstrated to increase with age (3-16 weeks) and body weight (87-477 g) of (healthy) donor rats. In contrast, the amount of full-length GPI-APs in rat serum, as determined by chip-based sensing, turned out to decline with age/body weight. These correlations suggest that age-/weight-induced alterations (in certain biophysical/biochemical characteristics) of plasma membranes are responsible for the release of full-length GPI-APs which becomes counteracted by elevated GPI-PLD activity in serum. Thus, sensitive and specific measurement of these GPI-AP-relevant parameters may be useful for monitoring of age-related cell surface changes, in general, and diseases, in particular.

**1. Introduction**

 Ageing has been linked to changes in the composition, structure and function of cell membranes on the basis of a multitude of biophysical and biochemical investigations. An increase in the curvature elastic constant was detected for erythrocytes during ageing in medium (Fricke and Sackmann, 1984). A decrease in outer leaflet fluidity of plasma membranes was correlated with ageing of erythrocytes and platelets (Sarmento et al., 1991; Hasan et al., 1995). Altered transbilayer fluidity and cholesterol asymmetry was observed in synaptic membranes of mice (Igbavboa et al., 1996). A significant positive correlation according to a second-degree equation was found between plasma membrane structural order and normal brain ageing and correlated to a decrease in fluidity of the outer membrane leaflet (Marinho et al., 1997). Increments in the cholesterol / phospholipid molar ratio, decreases in the lipid / protein molar ratio and elevations in the lipid peroxidation of membranes with resulting reductions in their fluidity were demonstrated for several animal models of ageing (Sawada et al., 1992; Yu et al., 1992) and vascular endothelial cells (Hashimoto et al., 1999) as well as jejunal brush border membrane vesicles from developing rats (Vazquez et al., 1997). Measurements of lateral diffusion coefficients for typical transmembrane proteins relying on fluorescence recovery after photobleaching revealed that in hepatocytes from all animal strains and species examined protein lateral diffusion declines with age in linear fashion (Kitani, 1999). Reduced fluidity of brain membranes, which is usually explained by increased cholesterol / phospholipid ratios and enhanced lipid peroxidation ultimately leading to higher brain membrane concentrations of saturated fatty acids, was correlated to many functional alterations of the aged brain for many species (Sun and Sun, 1979; Viani et al., 1991; Tacconi et al., 1991; Scheuer et al., 1995; Müller et al., 1997b). A decrease in the lipid dynamics and membrane lipid fluidity of the cell membranes as determined by generalized polarization of the phase-sensitive lipid probe 2-dimethylamino-6-lauroylnaphthalene in parallel to an increase in cholesterol concentration with age was demonstrated in two cell culture models of ageing, proerythropoetic K562 and lymphoblastoid HL60, which was reversible within 24 h after culture medium renewal (Parasassi et al., 1992; Levi et al., 1997). Finally, ageing was reported to be associated with typical "membrane defects" (Vigh et al., 2007). These and other findings prompted creation of the concept that molecular mechanisms coupled to certain biophysical states and biochemical compositions of cellular membranes, such as membrane fluidity, phase state, microheterogeneity (e.g. lipid rafts), operate as cellular sensors of ageing (Vigh et al., 2007).

 This concept may be extended to mechanisms which couple the stability of membrane proteins in membranes, in particular the strength of association of cell surface proteins with plasma membrane lipids, to ageing. Importantly, putative mechanisms have to be discriminated from the removal of transmembrane proteins from plasma membranes in course of the regulated formation and shedding of small vesicles with the cargo proteins remaining inserted in vesicular membranes without change of the membrane phospholipid-protein interaction. These so-called microvesicles are aimed to get rid of unwanted or unnecessary components (e.g. transferrin receptor during the differentiation of reticulocytes into erythrocytes) (Pan et al., 1983) or to transfer materials or information from donor to acceptor cells (e.g. epidermal growth factor receptor during tumor metastasis)(Al-Nedawi et al., 2008). The biogenesis of microvesicles is highly controlled and involves vertical trafficking of molecular cargo to plasma membranes, redistribution of membrane lipids and proteins, and use of the contractile machinery underneath the surface to allow for vesicle pinching (Cocucci et al., 2009; Tricarico et al., 2017). However, age dependence of microvesicle biogenesis and release of transmembrane proteins *via* vesicles from the cell surface into extracellular spaces has not been reported so far.

 In comparison to typical transmembrane proteins, which seem to be released from cell surfaces *via* the above vesicular mechanism only, glycosylphosphatidylinositol-anchored proteins (GPI-APs), which represent about 2 % of the cell surface proteins in mammals, may be regarded as candidates for spontaneous membrane release due to relatively low stability of cell surface association. GPI-APs are anchored at the outer leaflet of the plasma membrane bilayer by means of a small GPI glycolipid moiety, which is covalently coupled *via* a phosphoethanolamine bridge to the carboxyl-terminus of the typically large hydrophilic protein moiety (Fig. 1; Nosjean et al., 1997; Eisenhaber et al., 2001). This type of membrane anchorage in combination with the exquisitely amphiphilic “overall” nature of full-length GPI-APs, i.e. of those harbouring the complete GPI anchor, could compromise the strength of their association with and make them susceptible for spontaneous non-enzymic release from plasma membranes. In fact, a number of biophysical studies *in vitro* during the past two decades, relying on atomic force microscopy, Langmuir film technique, fluorescence microscopy or Fourier transform-infrared reflection-absorption spectroscopy, are compatible with this hypothesis: (i) The binding / extraction forces to / from supported phospholipid bilayers are rather low for GPI-APs compared to transmembrane proteins (Cross et al., 2005), (ii) the interaction forces between typical GPI anchors and phospholipids or cholesterol are moderate compared to transmembrane proteins (Rieu et al., 2004), and (iii) the strength of membrane association of GPI-APs critically depends on the type of the GPI and membrane phospholipids and the level of membrane cholesterol (Caseli et al., 2005 and 2008; Kouzayha and Besson, 2005; Ronzon et al., 2006).

 Sound evidence for the (patho)physiological relevance of those biophysical data was deduced from the seminal finding that the GPI-AP acetylcholinesterase (AChE), but not the transmembrane protein band 3, becomes translocated from intact human erythrocytes to protein-free sealed liposomes upon incubation (Bouma et al., 1977). The spontaneous release of GPI-APs from mammalian plasma membranes in course of contact formation with "empty" phospholipid mono/bilayers has been subsequently confirmed by similar experimental set-ups (Sunamoto et al., 1992; Nakamura et al., 1994; Okumura et al., 1994; Kogure et al., 1997; Suzuki and Okumura, 2000) and in concert argues for a rather weak cell surface anchorage of GPI-APs.

 However, until recently full-length GPI-APs with the complete GPI anchor attached have been detected outside of cells only as constituents of extracellular vesicles (microvesicles, exosomes) (Rabesandratana et al., 1998; Clayton et al., 2003 and 2011; Robertson et al., 2006; Müller et al., 2011 and 2012) and lipoprotein-like particles (lipoproteins, milk fat globules, surfactant-like particles, nodal vesicular particles) (Ceriani et al., 1988; Mahmood et al., 1994; Olofsson et al., 1999). In those extracellular GPI-AP-harbouring structures the GPI-APs become embedded in the outer leaflet of the vesicular phospholipid bilayer and the particle phospholipid monolayer, respectively, in course of their controlled biogenesis rather than spontaneous release from the cell surface in response of the donor cell to an extrinsic/intrinsic cue. The expression of GPI-APs in multimeric high-molecular aggregates in body fluids, such as seminal plasma (Feng et al., 2004), has been described, but it remains to be clarified whether those represent vesicles or particles or resemble membrane-/phospholipid-free assemblies as found for acylated morphogens (Rooney et al., 1996). Only very recently, the presence of full-length GPI-APs in extracellular structures different from vesicles, particles and lipid-free aggregates has been demonstrated in the incubation medium of rat adipocyte plasma membranes or isolated rat adipocytes as well as in rat and human serum (Müller et al., 2019). Subsequent biochemical analysis provided strong evidence for the expression of full-length GPI-APs together with lysophospholipids, phospholipids and cholesterol in micelle-like complexes, which is upregulated in response to metabolic derangement, such as obesity and diabetes (Müller et al., 2020). Strikingly, the increased release of full-length GPI-APs into these complexes seems to be counteracted by their elevated lipolytic degradation in the circulation through GPI-specific phospholipase D (GPI-PLD) (Fig. 1; Müller et al., 2020). Together these findings are compatible with GPI-APs being susceptible to release from the surface of cells and tissues upon exposure to "membrane-relevant" stressors on basis of the relatively weak GPI anchor-membrane phospholipid interaction.

 To test for the hypothesis that the cell surface association of GPI-APs becomes weakened during ageing in course of biophysical/biochemical alterations of plasma membranes, the spontaneous release of full-length GPI-APs harbouring the complete GPI anchor from plasma membranes of isolated epididymal adipocytes and livers into the incubation medium as well as their presence in serum were studied for rats (healthy, chow diet) encompassing a broad range of age. For this, a recently developed homogenous assay design based on a microfluidic chip-based sensor system was used. It relies on the propagation of horizontal surface acoustic waves (SAW) along a chip gold surface, which is affected by binding (capturing) of any molecules or complexes of interacting molecules with the surface (Gronewold 2007). The resulting amplitude reductions and right-ward phase shifts of the SAW represent a measure for the increased viscosity (i.e. the biophysical properties) and the loaded mass (i.e. the presence and amount), respectively, of the contacting molecules or complexes as the sample analyte. Specific capturing of GPI-APs by the chip surface is guaranteed by its coating with α-toxin, which interacts with the core glycan of GPI-APs with high specificity (Gordon et al., 1999). The presence of (lyso)phospholipids in complex with the GPI-APs was then detected by binding “in sandwich” of the phosphatidylserine-interacting protein annexin-V to the captured GPI-APs. The major advantages of SAW compared to optical sensing are its compatibility with turbid and complex matrices and its exquisite sensitivity (Gronewold et al., 2005b; Müller et al., 2019; see also the Supplemental material herein for further theoretical and experimental details and references). Using this technology it was found that the release from adipocyte/liver plasma membranes as well as from isolated adipocytes, the degradation in serum and the serum expression of full-length GPI-APs are correlated to the age of the donor rats in positive and negative fashion, respectively Analysis of these processes may be useful for monitoring and stratification of age-related phenomena and diseases.

**2. Materials and methods**

*2.1. Materials*

 Collagenase (Worthington, CLS type I, 190-250 units/mg) was provided by Biochrom (Berlin, Germany). Bovine serum albumin (BSA; fraction V, defatted), phenylisopropyladensine (PIA), adenosine deaminase (ADA), leupeptin, aprotinin, pepstatin A, benzamidine, 1,10 phenanthroline and PMSF were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). Anti-CD73 antibodies (rabbit polyclonal, affinity-purified, IgG isotype, prepared against recombinant full-size human CD73 with reactivity against rat CD73) were obtained from Genetex/Biozol (Eching, Germany). Anti-CD55 antibodies (ab231061, rabbit polyclonal, affinity-purified, IgG isotype, prepared against the unconjugated His-tagged recombinant [E. coli] peptide corresponding to rat CD55 aa 254-372) and alkaline phosphatase (aP) (ab95462, rabbit polyclonal, affinity-purified, IgG isotype, prepared against an unconjugated synthetic peptide corresponding to tissue-nonspecific alkaline phosphatase [TNAP]) were delivered by Abcam (Oxford, UK). Annexin-V (human, recombinant) was purchased from ProSpec (East Brunswick, NJ, USA). 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS, premium grade) were bought from Pierce/Thermo Scientific (Rockford, IL, USA). Egg york L-α-phosphatidylcholine (PC), bovine brain L-α-phosphatidylserine (PS), bovine brain L-α-lysophosphatidylcholine (lysoPC) and cholesterol (all stored in chloroform) for reconstitution of the the micelle-like AChE complexes were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Polystyrene Bio-Beads SM-2 (20-50 mesh) was bought from Bio-Rad Laboratories (Munich, Germany). All other reagents (highest purity available) were from Calbiochem/Merck (Darmstadt, Germany). Sera from male Wistar rats (Crl:WI(WU)) (Charles River, Sulzfeld, Germany) including characterization of the donor rats were made available by Sanofi Pharma Deutschland GmbH.

*2.2. Animal handling*

 Rats were housed two *per* cage in an environmentally controlled room with a 12:12-h light–dark circle (light on at 06:00) and *ad libitum* access to water and standard rat chow (17.7 kJ/g, Ssniff diet R/M-H, V1535 with 18 % crude protein, 4.7 % sugar, and 3.5 % crude fat) (Ssniff, Soest, Germany). All experimental procedures (housing and serum collection) were conducted in accordance with the German Animal Protection Law (paragraph 6) and corresponded to international animal welfare legislation and rules.

*2.3. Serum collection*

 Blood was collected from the tail vein of conscious rats under terminal isoflurane, placed in serum gel tubes and centrifuged (1,000 x *g*, 5 min, 4 °C) to yield serum, which was immediately frozen in liquid N2 and then stored at -20 °C. The samples were thawed by incubation at 37 °C and then immediately used for measurement unless indicated otherwise. Blood metabolic parameters were determined enzymatically according to standard procedures using commercially available kits and the Hitachi 912 instrument. For glucose, the Gluco-quant glucose/HK kit was used (Roche, Mannheim, Germany). For insulin, 15 µl of sample volume was assayed with an enzyme immunoassay (ultrasensitive rat insulin ELISA; Mercodia 10-1248-10, Uppsala, Sweden) covering a sensitivity range of 0.5-100 µg/l and displaying the following crossreactivities: Human insulin 167 %, porcine insulin 476 %, sheep insulin 179 %, bovine insulin 78 %. All assays were performed according to the instructions of the manufacturers.

*2.4. Preparation of isolated rat adipocytes*

 Isolated rat adipocytes were prepared as described previously (Müller et al., 1997a) with the following modifications. Rats were killed by cervical dislocation. Epididymal fat pads from male Wistar rats (weight see below, fed *ad libitum*) were washed several times in Krebs-Ringer-Henseleit buffer (KRH; 25 mM HEPES free acid, 25 mM HEPES sodium salt, 1.2 mM KH2PO4, 140 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2) supplemented with 0.75 % (w/v) BSA and adjusted to pH 7.2 and then cut into two to three pieces. Two pieces each were incubated with 1.5 ml of digestion buffer (10 mg collagenase and 9 mg glucose in 10 ml of KRH containing 5 % BSA, 1 mM sodium pyruvate, 5.5 mM glucose, 0.5 U/ml ADA, 200 nM PIA, 100 µg/ml gentamycin, 50 units/ml penicillin and 50 µg/ml streptomycin sulfate) for 15–20 min at 37 °C in a shaking water bath (240 cycles/min) at constant bubbling with 95 % O2/5 % CO2. Released adipocytes were passed through a nylon web (mesh size 750 μm) with no pressure other than gentle teasing with a plastic spatula for passing of the cells through and then centrifuged (500 x g, 1 min, swing-out rotor). The supernatant layer of cells was washed three times with 25 ml each of KRH by centrifugation. After final aspiration of the infranatant, the adipocytes were suspended in KRH containing 2 % BSA at a lipocrit of 10 % corresponding to 100 µl of packed cell volume *per* ml incubation volume (determined by aspiration of small aliquots into capillary hematocrit tubes and centrifugation for 60 sec in a microhematocrit centrifuge in order to determine the fractional occupation of the suspension by the adipocytes). 10 % lipocrit corresponds to about 9.9, 6.8, 1.1 and 0.2 x 106 adipocytes *per* ml from rats of age classes I, II, III and IV, respectively.

*2.5. Preparation of incubation medium from rat adipocytes*

 Adipocytes were suspended in 10 ml of adipocyte buffer (20 mM HEPES/KOH, pH 7.4, 140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2 % (w/v) BSA, 100 μg/ml gentamycin, 1 mM sodium pyruvate and 5.5 mM glucose) at a lipocrit of 0.2 % and then incubated (20 h, 37 °C) in a shaking water bath (100 cycles/min) under constant bubbling with 5 % CO2/95 % O2 at a lipocrit of 3.3 %. Thereafter, twelve 350-µl portions of the total mixtures were transferred into microfuge tubes (Beckman) pre-filled with 100 μl of dinonylphtalate and then centrifuged (1,000 x *g*, 1 min, 20 °C). The tubes were cut through the dinonylphtalate layer separating the adipocytes at the top from the incubation medium in the bottom part of the tubes which was rescued. Care was taken to minimize the volume of dinonylphtalate taken along with the incubation medium in the bottom part. After transfer of the incubation medium (300 μl) into 1-ml Eppendorf cups and washing the bottom part of the tube once with 300 μl of adipocyte buffer containing 0.5 mM DTT and protease inhibitor mix, the combined medium and washing fluid were centrifuged (1,000 x g, 5 min, 4°C) for the removal of cell debris.

*2.6. Preparation of plasma membranes from rat adipocytes*

 Plasma membranes were prepared from isolated rat adipocytes as described previously (Müller et al., 1997a) with the following modifications: Adipocytes (107 cells) prepared from epididymal adipose tissue depots were washed and immediately homogenized in 2 ml of lysis buffer (25 mM TRIS/HCl, pH 7.4, 0.5 mM EDTA, 0.25 mM EGTA and 0.25 M sucrose, supplemented with 10 μg/ml leupeptin, 2 μM pepstatin, 10 μg/ml aprotinin, 5 μM antipain and 200 μM PMSF) using a motor-driven Teflon-in-glass homogenizer (10 strokes with a loosely fitting pestle) at 22 °C. The defatted postnuclear infranatant (1,500 x *g*, 5 min) was centrifuged (12,000 x *g*, 15 min). The pellet was suspended in 1 ml of lysis buffer and layered on top of an 8-ml cushion of 28 % Percoll, 0.25 mM sucrose, 25 mM TRIS/HCl (pH 7.0) and 1 mM EDTA. After centrifugation (45,000 x *g*, 60 min), the purified plasma membranes were withdrawn from the lower third of the gradient (0.5 ml), then pelleted (200,000 x *g*, 90 min) and finally suspended in 10 mM HEPES/KOH (pH 7.5), 150 mM NaCl and 100 mM sucrose at 50 μg protein/ml.

*2.7. Preparation of plasma membranes from rat liver*

 Plasma membranes were prepared from isolated rat liver as described previously (Cuatrecasas, 1972) with the following modifications. After removal from the animal, the livers were immediately rinsed with PBS in order to get rid of residual blood, then minced finely with 5 ml/g of homogenization buffer (10 mM TRIS/HCl, pH 8.0, 2 mM EDTA, 250 mM sucrose, 10 µM leupeptin, 0.05 U/ml aprotinin, 1 µM pepstatin A, 25 mM benzamidine, 1 mM 1,10 phenanthroline, 1 mM PMSF) and subsequently homogenized using a Potter-Elvehjem apparatus (10 passes at maximal speed, on ice). The homogenate was centrifuged (600 x *g*, 10 min, 4 °C). The supernatant was collected and the centrifugation repeated. The supernatant was collected and adjusted to a final concentration of 0.1 M NaCl and 0.2 mM MgSO4 by addition of 4 M NaCl and 0.5 M MgSO4, respectively. The mixture was centrifuged (6000 x *g*, 2 h, 4 °C). The supernatant was removed by aspiration from the loosely packed pellet containing the plasma membranes which was then resuspended in 10 volumes of homogenization buffer using a Potter-Elvehjem apparatus. The mixture was centrifuged (9000 x *g*, 1 h, 4 °C). The pellet was resuspended, homogenized and centrifuged as above. The final pellet was resuspended with homogenization buffer to give a concentration of 100 µg protein/ml.

*2.8. Chip-based SAW sensing of serum*

 Serum samples diluted five-fold were supplemented with 20 mM PIPES (pH 7.2), 100 mM K2SO4, 250 mM sucrose to 28 µl and then injected into α-toxin-coated or "blank" (uncoated) channels at a flow rate of 13 µl/min (capture). After injection of 120 µl of 20 mM PIPES (pH 7.2), 100 mM K2SO4, 150 mM NaCl, 250 mM sucrose at a flow rate of 180 µl/min, 2 x 65 µl of 20 mM TRIS/HCl (pH 8.0), 150 mM NaCl, 250 mM sucrose containing 400 nM annexin-V and 40 µM Ca2+ at a flow rate of 60 µl/min (detection) and then 160 µl of 20 mM PIPES/KOH (pH 7.2), 100 mM K2SO4, 150 mM NaCl, 250 mM sucrose at a flow rate of 180 µl/min were injected. Subsequently, 120 µl of 20 mM TRIS/HCl (pH 8.0), 150 mM NaCl, 250 mM sucrose were injected at a flow rate of 30 µl/min. After injection of 120 µl of 20 mM PIPES/KOH (pH 7.2), 100 mM K2SO4, 150 mM NaCl, 250 mM sucrose at a flow rate of 180 µl/min, 90 µl of 20 mM TRIS/HCl (pH 8.0), 150 mM NaCl, 250 mM sucrose containing 200 µM PIG41 were injected at a flow rate of 30 µl/min. Phase shift (as °) is given after correction for the "blank" channels and "buffer alone" controls (to compensate for unspecific interaction of sample matrix components with the chip surface and altered viscosity of the sample fluid, respectively) and normalization (set at 0 and 1, respectively, at 0 sec).

*2.9. Chip-based sensing of full-length GPI-APs from plasma membranes of adipocytes using the "lab-on-the-chip" configuration*

 For immobilization of plasma membranes from isolated rat adipocytes, three 100-μl portions of plasma membrane were consecutively injected into α-toxin-coated channels in the presence of Ca2+. For demonstration of the presence of the insulin receptor α-chain as a specific marker for plasma membranes, anti-IRα antibodies were injected at the end, which resulted in a considerable phase shift (data not shown). For the putative generation of unprocessed GPI-APs from the immobilized plasma membranes and their capture by the α-toxin-coated chip, running buffer was injected into the chip channels which were then incubated (110 min, 30 °C). For subsequent release of the plasma membranes from the chip surface, running buffer containing EGTA was injected. For demonstration of capture of unprocessed GPI-APs, annexin-V *plus* Ca2+, anti-CD73 and anti-aP antibodies, 200 μM PIG41 and mannose were injected in that sequential fashion.

*2.10. GPI-PLD activity measurement using AChE complexed to lipid micelles and SAW sensing*

*2.10.1. Preparation of the GPI-PLD substrate*

 AChE complexed to lipid micelles consisting of PC, PS, lysoPC and cholesterol were prepared by mixing of these constituents at the “optimized” ratio (Müller et al., 2020) in the detergent-solubilized state and subsequent removal of the detergent by adsorption onto polystyrene Bio-Beads according to previously published protocols (Rigaud et al., 1988; Agrand et al., 1997) with modifications (Müller et al., 2020). For reconstitution of AChE complexed to lipid micelles, lysoPC, PC and cholesterol in chloroform (10 mg/ml each) together with invariant amounts of AChE (0.3 nmol) and PS (0.26 µmol) were dispensed into a glass test tube in a total volume of 1 ml at the “optimized” constituent ratio lysoPC : PC : cholesterol : AChE of 1000 : 25 : 25 : 1. After evaporation of the chloroform under a stream of N2 under atmospheric pressure and then in a speed vac under high vacuum (60 min), the dried phospholipids (lipid films) were dispersed in 250 µl HSA and then completely dissolved by gentle vortexing and incubation (20 °C, 30 min). The hydrated lipid dispersion was exposed to six freezing-thawing cycles (-180 °C/+25 °C) and then passed 40 times through a polycarbonate membrane (0.2 µm) with a mini-extruder (Avanti Polar Lipids). Incorporation of AChE was initiated by addition of 750 µl of 20 mM octyl glucoside and incubation (15 min, 25 °C), which just destabilises the lipid bilayer to promote protein insertion. Subsequently, 100 µl of 3 µM AChE (0.3 nmol) freshly prepared from the lyophilized materials (see above) was added to the mixture in an Eppendorf tube. Reconstitution was initiated by the addition of 50 mg damp Bio-Beads to the tube and rotation on a tube rotator (20 rpm, 90 min, 20 °C). After addition of another 350 mg (damp weight) of Bio-Beads and rotation (180 min), the Bio-Beads were allowed to settle (5 min). 200-µl portions of the supernatant habouring 300 nM AChE and 2.6 mM lipids in HSA (molar ratio = 8700:1) were carefully removed and centrifuged (400,000 x *g*, 1 h, 4 °C; Beckman TL-100 ultracentrifuge, TLA-100 rotor, 95,000 rpm). The supernatants containing AChE complexed to lipid micelles were subjected to a second cycle of detergent absorption with Bio-Beads (50 and 350 mg). The final clear supernatants were subsequently purified by discontinuous flotation gradients (Rigaud et al., 1988; Agrand et al., 1997; Morandat et al., 2002). For this, 3-ml portions were thoroughly mixed with 3 ml of sucrose (60 % w/v) in 20 mM PIPES/KOH (pH 7.2), 100 mM K2SO4 at the bottom of 10-ml centrifuge tubes. 1-ml cushions of 10 % and 2.5 % sucrose (w/v) were successively layered above, followed by 2 ml of buffer. After centrifugation of the density gradients (30,000 rpm, 2 h, 4°C, Beckman SW41 rotor), 0.5-ml fractions were removed from the 2.5 %-sucrose buffer interface resulting in about 5- to 6-fold concentration of the purified AChE complexed to lipid micelles.

*2.10.2. Preparation of AChE*

 Amphiphilic AChE was prepared from a (commercially available) detergent extract from bovine erythrocyte membranes by affinity chromatography using a Sepharose 4B-L-tyrosine-tacrine affinity resin column according to published protocols (Gnagey et al., 1987; Kaya et al., 2013) with modifications (Müller et al., 2020). This one-step purification procedure typically resulted in a 500- to 700-fold enrichment (to apparent homogeneity according to SDS-PAGE and Coomassie staining) and a yield of 20 to 30 % *vs*. the original octylglucoside extract. The presence of the GPI anchor was evidenced by non-denaturing SDS-PAGE or TX-114 partitioning following incubation of samples in the absence or presence of phosphatidylinositol-specific phospholipase C (PI-PLC) (*Bacillus cereus*), which both enabled the discrimination of anchor-containing and anchor-less AChE. About 60 and 85 % of the AChE activity of the crude extract and purified enzyme, respectively, typically partitioned into the TX-114-enriched phase, while a near quantitative activity recovery in the TX-114-depleted aqueous phase was obtained after lipolytic cleavage with PI-PLC (*Bacillus cereus*).

*2.10.3. Incubation of AChE complexed to lipid micelles with serum*

 2-25 µl of AChE complexed to lipid micelles and displaying identical AChE enzymic activity were supplemented with 200 mM TRIS/Maleate (pH 7.0), 150 mM NaCl to 50 µl. Sera were diluted 20-fold with 10 mM TRIS/HCl (pH 7.4), 150 mM NaCl to reduce the impact of potential inhibitors (Rhode et al., 2000) and then incubated (37 °C, 30 min) with 50 µl substrate solution in a total volume of 150 µl of 200 mM TRIS/Maleate (pH 7.0), 150 mM NaCl2, 1 mM MgCl2, 0.01 mM zinc acetate, 40 µM CaCl2. The reaction was terminated by the addition of 800 µl of ice-cold 10 mM HEPES/NaOH (pH 7.0), 150 mM NaCl, 0.25 mM 1,10 phenanthroline and placing on ice. Subsequently, 50-µl portions were subjected to TX-114 partitioning.

*2.10.4. TX-114 partitioning*

 For temperature-induced TX-114 phase separation of serum, the sample (max. vol. 50 µl) was diluted to 200 µl with 10 mM TRIS/HCl (pH 7.4), 150 mM NaCl, 1 % (v/v) TX-114, mixed and left on ice (5 min). Subsequently, the sample was layered on top of 300 µl of 6 % (w/v) sucrose, 10 mM TRIS/HCl (pH 7.4), 150 mM NaCl, 1 % (v/v) TX-114 in a microcentrifuge tube, then incubated (30 °C, 3 min) and centrifuged (3000 x *g*, 3 min, 30 °C, swing-out rotor). Thereafter, the upper aqueous phase (200 µl) was transferred to a new tube and supplemented with TX-114 to a final concentration of 0.5 % (v/v). After mixing and incubation (0 °C, 5 min), the solution was overlayed on the same sucrose cushion, again incubated (30 °C, 3 min) and then centrifuged (3000 x *g*, 3 min, 30 °C). Subsequently, the upper aqueous phase (200 µl) was transferred to a new tube and supplemented with TX-114 to a final concentration of 2.0 % (v/v). After mixing and sequential incubation (0 °C, 5 min; 30 °C, 3 min), the solution was centrifuged (3000 x *g*, 3 min). Thereafter, 150 µl of the supernatant was carefully transferred to a new tube avoiding any disturbance of the interface. This represents the final aqueous TX-114-depleted phase containing the lipolytically cleaved hydrophilic protein moieties of serum GPI-APs.

*2.10.5. Chip-based assay*

 120 µl of aqueous phase sample derived from incubations in the presence of serum were injected into α-toxin-coated or "blank" channels of the chip at a flow rate of 36 µl/min. Thereafter, 180 µl of 30 µM PIG41 in 10 mM TRIS/HCl (pH 7.4), 150 mM NaCl were injected at a flow rate of 120 µl/min. Phase shift is given (as °) upon corrections for a "blank" channel (unspecific interaction of sample components), “buffer alone” (altered viscosity of the matrix fluid) and a "blank" incubation (amphiphilic-to-hydrophilic conversion of AChE in the absence of serum) and normalization (set at 0 for 0 sec).

*2.11. SAW Measurement*

 The Seismos NT.X Instrument for SAW chip-based sensing (NanoTemper Technologies, Munich, Germany) integrates a high-frequency unit, control and reader units and all fluid handling components required for a systematic buffer and analyte solution handling (S-sens K5). This enables fluidic and electrical contacting of the chip with four independent flow-through microfluidic channels at a stable temperature of 22 °C (Δ*T* = 0.05 °C by means of four peltier elements). Mass loading and loss of elasticity (gain of viscosity) resulting from biomolecular interaction processes on the channel surface will result in phase shift and amplitude reduction, respectively, of the SAW generated by the inverse piezoelectric effect. The instrument was run and the signals generated were recorded in real-time using a double-frequency measurement mode as described previously (Gronewold et al., 2005a; Ferreira et al., 2009; Onen et al., 2012) with the following modifications. Measurements were performed with a continuous buffer stream at the flow rates and temperatures as indicated. For each chip, the phase shift and amplitude generated by the α-toxin-coated channels were corrected for unspecific non-GPI-mediated interactions by subtraction of the values of the "blank" channel. In addition, in case of medium or serum samples, the values of the sample channels were corrected for a "medium" or "albumin" channel, respectively, which reflects the unspecific and non-covalent adsorption of medium components or albumin to α-toxin and is generated by injection of incubation medium or 1 % BSA in PBS, respectively, and further processing identical to the sample channels. To avoid the generation of air-bubbles (by spontaneous degassing, EDC/NHS reaction, pipetting or others), the buffers were degassed by applying vacuum (200 mbar for 30 min) and eventual air-bubbles removed immediately before injection (by gently tapping the vial). To avoid blockage of tubing, sterile-filtered buffers (0.2-μm sterile filters) and degassed H2Obidest were used only and visually inspected for lack of particles or other contaminants. To avoid blockages in the system (autosampler-needle, autosampler, fluidic cell, tubing), it was cleaned after each experiment and on a regular basis weekly and monthly according to the instructions of the manufacturer.

*2.12. Statistical analysis*

 All numerical data were presented as the means and standard deviations (S.D.). Statistical significance was calculated using GraphPad Prism6 software (version 6.0.2, GraphPad Software) on the basis of one-way ANOVA performed with *Tukey's* multiple comparison post test. p < 0.05 was considered to be statistically significant. Linear regression analysis of primary data was performed with FitMaster Origin-based software (OriginLab Inc., Northampton, USA).

*2.13. Micellaneous*

 Chemical synthesis of PIG41, protein determination, preparation of α-toxin from the culture supernatant of *Clostridium septicum*, coupling of α-toxin to the channel gold surface of long-chain 3D CM-dextran sam® 5 chips using conventional EDC/NHS-based protocol in a SamX instrument (SAW/Nanotemper, Bonn/Munich, Germany), SAW measurement, instrumentation and evaluation were performed as has been described in detail previously (Müller et al., 2019).

**3. Results**

*3.1. Design of the experiments and characteristics of the rats differing in age*

 For determination of the amounts of full-length GPI-APs harbouring the complete GPI anchor in the incubation medium of isolated adipocyte and liver plasma membranes or of isolated adipocytes as well as in serum from rats of varying age, the medium and serum samples were assayed for the presence of GPI-APs which exhibit the highly conserved GPI core glycan and are associated with phospholipid moieties. Signals would reflect release of GPI-APs from the cell surface due to age-related changes in the biophysical properties of plasma membranes rather than enzymic removal of the GPI anchor by an age-induced protease or phospholipase. Upon injection of the samples into the microfluidic α-toxin-coated channels of the chips, only proteins expressing the GPI core glycan (harbouring or lacking phospholipids) were captured through interaction with α-toxin, which is known to act as a specific receptor protein for GPI-APs (Gordon et al., 1999; Hong et al., 2002). The simultaneous expression of phospholipids (i.e. of phosphatidylserine) as constituents of micelle-like complexes and the nature of the GPI-AP protein moiety were elucidated by consecutive injection (with rinsing of the channels between injections) of the phosphatidylserine-binding protein annexin-V (in the presence of Ca2+) and antibodies against the GPI-APs CD73, tissue-nonspecific alkaline phosphatase (aP) and CD55, which all have been demonstrated to reside at the adipocyte surface and serum of rats (Okochi et al., 1987; Gronthos et al., 2001; Tariq Ali et al., 2005; Festy et al., 2005; Ardianto et al., 2010; Müller et al., 2011; Bajek et al., 2015; Hernandez-Mosqueira et al., 2015; Chirambo et al., 2017). Capturing *via* α-toxin and the subsequent interactions “in sandwich” with annexin-V and the antibodies were reflected in step-wise right-ward shifts of the phase (i.e. decreases in frequency) of the SAW propagating between gold electrodes along the chip surface (Gronewold, 2007; Ferreira et al., 2009). The specificity of capturing was revealed by the final injection of excess of the phosphoinositolglycan PIG41 (final conc. 200 μM), which mimics the structure of the GPI core glycan (Frick et al., 1998). This led to reduction of phase shift by typically 70 % demonstrating dissociation of the captured GPI-APs by competition between the core glycans of GPI-AP and PIG41 for binding to α-toxin. Thus the difference between maximal phase shift measured after injection of annexin-V or the corresponding antibodies and the phase shift left after injection of PIG41 (as indicated in the figures) upon correction for unspecific capturing of sample proteins to the channels (see curves ‘B’ obtained with uncoated channels) and normalization for the GPI-AP capturing capacity of different channels reflects the total amount of full-length GPI-APs in the sample and eventually hints to the presence of CD73, aP and CD55 in the sample.

 Importantly, previous studies have demonstrated that the phase shifts exerted by capturing through α-toxin and “sandwich binding” of annexin-V and antibodies are caused by micelle-like complexes consisting of full-length GPI-APs, lysophospholipids, phospholipids and cholesterol at an “optimized” ratio rather than by full-length GPI-APs in a monomeric state (Müller et al., 2019 and 2020). At the end of each SAW measurement cycle the chip channels were regenerated for re-use by injection of mannose and then glycine at low pH. This led to baseline phase shift indicating complete removal of sample proteins from the chip. Consequently, SAW sensing relying on this configuration was used for determination of the release of full-length GPI-APs from adipocyte (Fig. 2) or liver (Fig. 3) plasma membranes *in vitro* and from isolated adipocytes (Figs. 4, 6) as well as of their presence in serum (Figs. 5 and 6).

 Male wildtype Wistar rats fed a chow diet for 3, 6, 9 and 16 weeks with a mean body weight of 87.0 + 3.5, 171.4 + 6.8, 325.1 + 12.9 and 477.3 + 18.0 g (corresponding to age classes I, II, III and IV, respectively) were used as donors for the adipocytes and serum samples (Table 1). Fasting plasma glucose levels did not differ significantly between the four age classes. Fasting plasma insulin levels tended to increase slightly with increasing body weight. Thisis explained best by compensatory insulin secretion in response to the well-known moderate impairment of insulin sensitivity with age and/or body weight in wildtype rats as well as in human beings (Bogardus et al., 1987; Lissner et al., 1990; Wing, 1997; Weyer et al., 2000). Thus the rats of the four age classes do not exhibit metabolic derangements on the basis of their normoglycemic and –insulinemic state and differ only in age and body weight.

*3.2. Full-length GPI-APs are released from adipocyte and liver plasma membranes in vitro in dependence on the age of the donor rats*

The release of full-length GPI-APs from adipocyte and liver plasma membranes was studied *in vitro* using a "lab-on-the-chip" configuration. For this, plasma membranes were immobilized by hydrophobic interactions on the chip gold surface (Andrä et al., 2008), which was reflected in the stepwise increase in phase shift with each injection (but only in the presence of Ca2+ for neutralization of negative surface charge; data not shown, but see Müller et al., 2019). Saturating amounts of plasma membranes according to maximal phase shift were used for capture, which ensured the comparison of roughly identical amounts of immobilized plasma membranes as putative source for full-length GPI-APs. The pronounced phase shift upon injection of anti-insulin receptor-α antibodies into the chip confirmed the immobilization of the adipocyte and liver plasma membranes (data not shown, but see Müller et al., 2019). Full-length GPI-APs released from the immobilized plasma membranes during the subsequent injection of buffer were captured by the α-toxin-coated chip. This can be monitored only after almost complete elimination of the initial phase shift provoked by the immobilized plasma membranes which is achieved by their detachment from the chip through deprotonization (pH 10), chelating of Ca2+ (EGTA) and final washing with buffer. This “lab-on-the-chip” configuration was used for the detection of CD73 and aP and presumably other GPI-APs together with phospholipids in micelle-like complexes released from the immobilized plasma membranes during the previous injection of running buffer at defined assay conditions (i.e. time, flow rate, buffer composition; see Müller et al., 2019), subsequently captured by the α-toxin-coated channels and finally monitored upon depletion of the plasma membranes.

 Analysis of the release of GPI-APs from plasma membranes using the “lab-on-the-chip” configuration and membranes, which had been prepared from together 16 pools of adipocytes and livers, respectively, derived from four rats each for each of the four age classes, revealed deviations in the realtime courses for the annexin-V- and antibody-dependent increases in phase shift difference between the age classes IV, III and II/I for both adipocytes (Fig. 2A) and liver (Fig. 3A). Age class IV displayed the highest phase shift difference and age classes II and I overlapping phase shift differences. Quantitative evaluation of the phase shift differences confirmed that micelle-like complexes consisting of phosphatidylserine, CD73 and aP were released from isolated adipocyte plasma membranes from rats of age class IV with significantly higher efficacy compared to class III and then classes II/I (with no significant difference between the latter (Fig. 2B). With isolated liver plasma membranes, significantly higher efficacy was found for age class IV compared to class II/I (Fig. 3B). The specific phase shift differences revealed considerably lower (by about 50 %) releases of CD73 and aP into micelle-like complexes from liver compared to adipocyte plasma membranes for each of the four age classes with identical ranking order (IV > III > II > I) for the two sources (Figs. 2 and 3). In consequence, the resolving power seems to be less pronounced for liver compared to adipocyte plasma membranes.

*3.3. Full-length GPI-APs are released from intact adipocytes during primary culture in dependence on the age of the donor rats*

 Analysis of the release of GPI-APs from the surface of isolated adipocytes (together 16 pools consisting of four rats each with four pools for each of the four age classes) into the incubation medium revealed deviations in the realtime courses for the annexin-V- and antibody-dependent increases in phase shift difference between the age classes IV, III and II/I (Fig. 4A). Age class IV displayed the highest phase shift difference and age classes II and I overlapping phase shift differences. Quantitative evaluation of the phase shift differences confirmed that micelle-like complexes consisting of phosphatidylserine, CD73, aP and CD55 were released from isolated adipocytes from rats of age class IV with significantly higher efficacy compared to class III and then classes II/I (with no significant difference between the latter (Fig. 4B).

*3.4. Full-length GPI-APs are expressed in serum in dependence on the age of the rats*

 Analysis of the expression of GPI-APs in the serum of rats (shown are four representatives for each of the four age classes) revealed deviations in the realtime courses for the annexin-V- and anti-CD55 antibody-dependent increases in phase shift difference between the age classes I, II and III/IV (Fig. 5A). Age class I displayed the highest phase shift difference and age classes III and IV overlapping phase shift differences. Quantitative evaluation of the phase shift differences for the means of the four representative rats (Fig. 5B), the individual values of the 16 rats (Fig. 5C) and the means of the 16 rats (Fig. 5D) between the age classes demonstrated the presence of micelle-like complexes consisting of phosphatidylserine and CD55 in all serum samples. Age class I displayed significantly higher amounts compared to class II and then classes III/IV (with no significant difference between the latter). Importantly, in contrast to the micelle-like complexes released from isolated adipocyte and liver plasma membranes (Figs. 2 and 3) and primary adipocytes (Fig. 4), full-length CD73 and aP were not detected in complexes in rat serum. There are several explanations for the apparent absence of these GPI-APs in micelle-like complexes in serum, which is independent of the age of the rats. (i) CD73 and aP are more tightly associated with and resist release from adipose and liver tissue plasma membranes *in vivo* compared to the corresponding isolated plasma membranes and cells *in vitro*. At variance, CD55 exhibits similar susceptibility towards release *in vivo* and *in vitro*. (ii) The amount of CD73 and aP released from adipose and liver tissues into the circulation of rats, even of high age, is rather low relative to CD55 and under the limit of detection (by chip-based sensing). In fact, CD55, which is predominantly expressed by blood cells (lymphocytes, monocytes, erythrocytes), represents one of the most abundant GPI-APs in peripheral blood of mice and humans (Kinoshita et al., 1985).

*3.5. Expression of GPI anchor-less GPI-APs in primary culture of intact adipocytes and in serum is not affected by the age of the rats*

Next, it was confirmed that the SAW sensing method is specific for GPI-APs having retained the complete GPI anchor (including its phospholipid moiety). Furthermore, the compatibility of a putative release of GPI-APs from plasma membranes by proteolytic or lipolytic cleavage, which has been described for a variety of cell types, including rat adipocytes, in response to a number of exogenous stimuli (for reviews see Nosjean et al., 1997; Müller, 2018), with the determination of the age-related spontaneous (i.e. not protease- or lipase-mediated) release of full-length GPI-APs was demonstrated. For this, hydrophilic versions of GPI-APs, i.e. those having lost the GPI anchor and being enriched in the aqueous detergent-depleted phase upon TX-114 partitioning (Bordier, 1981), which may be contained in the incubation medium of adipocytes (Fig. 6A) or serum (Fig. 6B) from rats of the four age classes were injected into the α-toxin-coated channels of the SAW chip and then assayed for phase shift.

 The considerable increases in phase shift differences upon injection of the aqueous-phase samples from the adipocyte incubation media of each age class and subsequently of anti-CD73 and anti-aP antibodies in combination with unaltered phase shift in response to anti-CD55 antibodies demonstrated in all samples the presence of CD73 and aP, having retained the GPI core glycan, and absence of CD55 (Fig. 6A, upper panels; two representative adipocyte pools from four rats for each age class). The non-response to annexin-V injection is compatible with lack of association of these hydrophilic versions of CD73 and aP with phospholipids in micelle-like complexes.

 Moreover, the considerable increases in phase shift difference upon injection of the aqueous phase samples from the rat sera of each age class and anti-CD73 antibodies in combination with unaltered phase shift in response to annexin-V and both anti-aP and anti-CD55 antibodies demonstrated the presence of only CD73 which has retained the GPI core glycan but escaped association with phospholipids, aP and CD55 in micelle-like complexes (Fig. 6B, upper panels; two representative sera from individual rats for each age class).

 The failure of anti-CD55 antibodies or anti-CD55 and anti-aP antibodies to elicit phase shift with medium and serum samples, respectively, may be explained by failure of their lipolytic cleavage in the cultured rat adipocytes and rats under the experimental conditions or by cleavage at a rate being too low for detection of the released products by SAW sensing. However, a number of reports have previously shown that delipidation of GPI-APs by bacterial phospholipase C or serum GPI-PLD can drastically reduce the immunoreactivity (e.g. in Western blotting) of their protein moieties towards antibodies (Barboni et al., 1995; Kukulansky et al., 1999; Seong et al., 2013). This is most likely due to a conformational change in course of loss of the phospholipid moiety mediated by (i) the GPI core glycan *vs.* the complete GPI anchor, (ii) the aqueous environment *vs.* the immediate neighbourhood of plasma membranes of the protein moiety or (iii) the monomeric *vs.* the oligomeric state of the GPI-APs, since membrane anchorage by GPI enhances their oligomerization (Seong et al., 2013). In consequence, it cannot be excluded that, in addition to CD73, CD55 and aP underwent lipolytic cleavage in adipocytes and rats under the conditions chosen but failed to be recognized by the corresponding antibodies due to reduced antigenicity of their protein moieties upon separation from the GPI phospholipids.

 Quantitative evaluation of the specific phase shift differences did not reveal significant differences between the four age classes in the expression of CD73, aP and CD55 in both adipocyte mediumand serum samples (Fig. 6A and B, lower panels).Furthermore, it is concluded that hydrophilic GPI-APs equipped with proteolytically cleaved GPI anchor were either not present in the adipocyte incubation medium and serum samples or present but not detected (i.e. not captured by the chip channels in specific or unspecific fashion). Together the findings strongly argue that the enzymic release of a subset of GPI-APs from the surface of isolated adipocytes as well as the presence of enzymically cleaved GPI-APs in the circulation does not considerably contribute to the age-dependence of the phase shifts as measured by SAW sensing of GPI-APs under the selected experimental conditions.

*3.6. GPI-PLD activity in serum becomes elevated with the age of the rats*

 Recent findings have demonstrated that full-length GPI-APs complexed to (lyso)phospholipids and cholesterol in micelles are preferred substrates for degradation by serum GPI-PLD and that their degradation becomes upregulated in course of metabolic derangement in rats and humans (Müller et al., 2020). The present findings of the age-dependent release from adipocyte plasma membranes and appearance in serum of full-length GPI-APs prompted the question as to whether serum GPI-PLD activity is increased in relation to the four age classes of the donor rats. For determination of GPI-PLD activity, dimeric AChE from bovine erythrocyte membranes, which represents a typical GPI-AP (Rosenberry et al., 1986; Stieger et al., 1986 and 1991), complexed to lipid micelles was used as substrate (Taguchi and Ikezawa, 1987). The conversion of amphiphilic to hydrophilic AChE as a consequence of GPI anchor cleavage was assessed by TX-114 partitioning. This procedure leads to enrichment of amphiphilic proteins in the detergent-enriched phase and of hydrophilic ones in the aqueous phase (Bordier, 1981). Subsequently the amount of AChE having retained the GPI core glycan in the aqueous phase by capturing by α-toxin-coated channels was measured which is directly correlated to GPI-PLD activity.

 The increases in phase shift upon injection of aqueous phase samples, which became diminished by about 70 % by subsequent injection of PIG41, indicated the generation of lipolytically cleaved GPI-APs and thus demonstrated operation of GPI-PLD in rat serum (Fig. 7A; shown are four representative serum samples for each of the four age classes). Activity was highest with rats of age class IV, followed by III and then II/I. Quantitative evaluation of the phase shift difference for the means of the four representative rats (Fig. 7B), the individual values of the 16 rats (Fig. 7C) and the means of the 16 rats (Fig. 7D) confirmed the significantly higher GPI-PLD activity in serum from rats of age class IV compared to class III and then classes II/I (with no significant difference between the latter).

**4. Discussion**

*4.1. Release from adipocyte and liver plasma membranes and from adipocytes of full-length GPI-APs, their presence in serum, serum GPI-PLD activity and the age of the donor rats are correlated*

 In the present study a microfluidic chip-based sensor system relying on SAW was used for the detection of and discrimination between full-length GPI-APs harbouring the complete GPI anchor including the phospholipid moiety, lipolytically cleaved GPI-APs still equipped with the GPI core glycan but lacking the phospholipid moiety and proteolytically cleaved GPI-APs having lost the complete GPI anchor encompassing both the GPI core glycan and the phospholipid moiety with high sensitivity and specificity. In contrast to an alternative technique for the analysis of biomolecular interactions on the basis of surface plasmon resonance (Johnsson et al., 1991; Lee et al., 2013), SAW sensing enables the measurement of analytes in complex matrices, including lipids and serum, without the need for prior extraction, enrichment or purification of the entities of interest (Gronewold et al., 2005b; Andrä et al., 2008). Moreover, the consequent adaptation of the SAW sensing method for the analysis of GPI-APs turned out to be of critical advantage: It enabled the implementation of a single platform for the versatile determination of the non-enzymic spontaneous, lipolytic or proteolytic release of GPI-APs from isolated plasma membranes or surfaces of cultured cells and of the degradation of GPI-APs in serum by GPI-PLD.

 The data presented unequivocally demonstrated that the spontaneous release from rat adipocyte and liver plasma membranes *in vitro* (Figs. 2 and 3) and from the surface of rat adipocytes in primary culture (Fig. 4) of full-length GPI-APs as well as their degradation by GPI-PLD from rat serum (Fig. 7) become upregulated with the age of the donor rats (Fig. 8). In contrast, the amount of full-length GPI-APs in serum was found to decline with age (Figs. 5 and 8). Taking into consideration that the degradation of serum GPI-APs seems to be initiated by serum GPI-PLD, exclusively (Figs. 1 and 6) (Davitz et al., 1987; Low and Prasad, 1988), it is tempting to speculate that the release of full-length GPI-APs from tissue cells into body fluids and the circulation, as reflected here as release from adipocyte and liver plasma membranes and intact adipocytes, is counterbalanced by a robust upregulation of serum GPI-PLD leading to a reduction of the serum steady-state concentration of full-length GPI-APs (Figs. 8 and 9). Each of the processes becomes considerably stimulated with the age of the donor rats as has been shown previously in course of metabolic derangement, such as diabetes and obesity (Müller et al., 2020). Thus these two exogenous cues presumably provoke subtle biophysical alterations, such as in stiffness, fluidity, viscoelasticity, membrane ordering, and mild destabilization of the plasma membrane phospholipid bilayer which apparently cause loosening of the membrane anchorage of GPI-APs in course of so far hardly understood processes.

 It is well-known that GPI-APs become enriched in certain membrane microdomains, the so-called lipid rafts, which are characterized by high concentrations of (glyco)sphingolipids and cholesterol and the atypical membrane protein caveolin-1 in a liquid-ordered manner (Fig. 1) (Schroeder et al., 1994). Partitioning of GPI-APs into lipid rafts, owing to this lipid and protein composition and caused by lipid-lipid/protein interactions, is considered to be the most important function of GPI, as it affects many biological characteristics of GPI-APs, such as signal transduction and intracellular trafficking, as well as of lipid rafts themselves, such as their stability and size (Brown and Rose, 1992; Paladino et al., 2004). Changes in the lipid (and protein) composition and thereby structure of the lipid rafts relative to non-raft membrane areas with age could play a critical role for the molecular mechanism underlying the stability of cell surface anchorage of GPI-APs.

 Interestingly, in young adult human and rodent neurons, approximately 85 % of the plasma membrane cholesterol was found associated with the cytoplasmic leaflet while the majority of glycosphingolipids, such as GM1 gangliosides, were recovered with the outer leaflet (for a review see Egawa et al., 2016). With age, a reduction in plasma membrane gangliosides and cholesterol was reported (see Fig. 9) or a redistribution of cholesterol from the cytoplasmic to the outer leaflet. Furthermore, recent studies have shown that lipid rafts change their composition in correlation with age-dependent neuronal impairment as prevalent during Alzheimer’s and Parkinson’s disease (for a review see Mesa-Herrera et al., 2019). In particular, using an agent-based model to assess the effect of lipid changes in lipid rafts on ageing and Alzheimer’s disease, lipid raft size and lipid mobility were identified as the two main factors that increase with age and are accelerated in the transgenic Alzheimer’s disease mouse model (Santos et al., 2016). Importantly, structural and functional changes in lipid rafts in hypothalamic membranes of genetically as well as high-fat induced obese mice have been demonstrated (Delint-Ramirez et al., 2015). In high-fat induced obese rats, upregulation of the release from adipocyte plasma membranes and isolated adipocytes as well as of the serum levels of full-length GPI-APs compared to lean animals has been reported (Müller et al., 2019). Together the findings of age- and obesity-induced biophysical and biochemical effects on plasma membrane lipid rafts and in parallel on plasma membrane anchorage of raft resident GPI-APs suggest that alterations in composition and structure of (the outer plasma membrane leaflet of) lipid rafts in course of ageing and obesity are responsible for weakening of the interaction of GPI-APs and lipid rafts, which is typically high for the young and healthy state.

 In this sense, the release of full-length GPI-APs from tissue cells into the circulation can be regarded as spontaneous process secondary to more direct age- and obesity-induced mechanisms, such as redistribution of cholesterol or saturated (glyco)phospholipids between lipid raft and non-raft membrane domains. However, at present the operation of an age-dependent signalling cascade controlling the protein-mediated release of full-length GPI-APs cannot be completely excluded. Several mechanisms are conceivable, among them (i) expression at lipid rafts of an ‘extraction’ or ‘scavenging’ protein for the GPI-APs fatty acid moieties and (ii) exchange of the long saturated fatty acid moieties for shorter unsaturated ones. The latter process could be mediated by transacylation or a different two-step enzymic process which resembles the fatty acid remodeling of GPI-APs (Maeda et al., 2007). Remodeling occurs during GPI-AP transport through the Golgi and involves the replacement of an unsaturated fatty acid, such as arachidonate, at the *sn-2* position in the phosphatidylinositol moiety by a saturated chain, commonly stearic acid, with the aid of at least two proteins, PGAP2 (Tashima et al., 2006) and PGAP3 (Maeda et al., 2007). An ‘inverse’ remodeling could weaken the association of the GPI-APs with lipid rafts and, in fact, fatty acid remodeling of GPI-APs has been demonstrated to be prerequite for their raft association (Maeda et al., 2007). It is tempting to speculate about an ‘inversion’ of the initial biogenetic fatty acid remodeling of GPI-APs in course of their residence at the cell surface and recycling to the Golgi in response to ageing. In any case, whether being consequence or cause of ageing, the release of full-length GPI-APs may accelerate and foster its progression and adverse outcome (late complications) as well as of age-related diseases, such as obesity and diabetes, on basis of alterations in the protein composition and function of lipid raft membrane areas.

 The observed deviations in the absolute extent of the release of CD73 and aP from plasma membranes between adipocytes and liver under maintenance of the positive correlation with age (Figs. 2 and 3) hint to tissue-specific differences of those (biophysical and biochemical) characteristics of certain plasma membrane areas which are critical for the retention of GPI-APs, but are compatible with general susceptibility of plasma membranes of tissue cells for the loss of full-length GPI-APs towards extrinsic and intrinsic cues, such as age and glucose / insulin levels. It remains to be determined whether the tissue-specific differences in the release of a subset of GPI-APs as monitored by chip-based sensing are correlated to certain pathogenetic and ageing processes which affect tissues and individuals in differential fashion.

 It may be argued that full-length GPI-APs in body fluids, such as serum, will exert detrimental effects on tissue cells upon contact. This transfer may be enabled or facilitated by the pronounced amphiphilic character of GPI-APs, in particular if they are presented within micelle-like complexes together with (lyso)phospholipids and cholesterol. Problems which could be caused by transfer encompass (i) lysis of cells, (ii) perturbance of membrane function and (iii) acquirement of the transferred full-length GPI-APs by ‘secondary’ cell types resulting in unphysiological GPI-AP expression and function and ultimately pathophysiological consequences. In fact, there is accumulating evidence for the spontaneous insertion of full-length GPI-APs in solution (upon detergent solubilization) into phospholipid bilayers and for the direct transfer of GPI-APs between phospholipid bilayers (e.g. from erythrocytes to liposomes or endothelial cells, from extracellular vesicles to adipocytes) (for a review see Kooyman et al., 1998; Müller, 2020). Moreover, it is conceivable that the apparently less tight residence of a GPI-AP at plasma membranes (compared to typical transmembrane proteins) and the putative promiscuous transfer between tissue cells becomes further promoted by their expression in the recently described micelle-like complexes together with (lyso)phospholipids and cholesterol (Müller et al., 2020).

*4.2. Age and body weight stimulate release of full-length GPI-APs in independent fashion*

 The present study was designed on basis of the classification of rats into four age classes ranging from 3 to 16 weeks (Table 1). By nature, this categorization leads to four classes of body weight encompassing 87 to 477 g, albeit the wildtype rats were fed normal chow diet and were not obese according to lean-to-fat mass index. This raises the question as to whether the increase in age or body weight has to be regarded as the primary cause of the enhanced release into and degradation in the rat serum of full-length GPI-APs. In rats, steers and humans, growth and development are strongly coupled to massive upregulation in number and size of adipocytes (Hubbard and Matthew, 1971; Cianzio et al., 1985; Hanamoto et al., 2013) at the various depots, including the epididymal fat pad (Johnson et al., 1978; Edwards et al., 1993; Perez de Heredia et al., 2008). Moreover, previous investigations revealed a positive correlation between cell size and release of full-length GPI-APs for epididymal adipocytes of wildtype rats (Müller et al., 2019). This could be attributed to enhanced stretching and tension of their plasma membranes in course of extensive lipid synthesis and lipid droplet biogenesis. Thus, in rats growth-induced hyperplasia and hypertrophy of lipid-loading adipocytes as the underlying mechanism(s) for the age-dependence of release from plasma membranes of full-length GPI-APs could be directly responsible for their appearance in serum and indirectly for the overcompensatory stimulation of serum GPI-PLD activity and the consequent reduction in serum steady state concentration of full-length GPI-APs.

 However, albeit total (as well as epididymal) adipose tissue considerably contributes to the massive increase in total body mass during rat development, it seems to be less likely that a major portion of the full-length GPI-APs released into and subsequently degraded in rat serum originates from adipocytes. Rather it is reasonable to assume that blood cells, such as macrophages, lymphocytes and platelets, the serum titer and size of which do not significantly change with rat growth, act as the major source for serum GPI-APs, on the basis of their total number and their level of GPI-AP expression (Resta et al., 1998; Boccuni et al., 2000; Loertscher and Lavery, 2002; Elishmereni et al., 2011). Taking these experimental findings and theoretical considerations together, it seems likely that ageing-induced alterations in the biophysics/biochemistry of blood cell plasma membranes are more relevant for the increase in the level of full-length GPI-APs in micelle-like complexes in serum with ageing than hyperplasia and hypertrophy of tissue cells, in general, and of adipocytes, in particular. To substantiate this conclusion, comparative measurements have to be performed with weight-matched rats of differing age.

*4.3. Future perspectives*

 It will be interesting to test the potential of the presented GPI-AP SAW sensing platform for the stratification of age-related diseases, for which pronounced changes in certain biophysical and biochemical properties, such as in membrane outer leaflet and transbilayer fluidity, viscoelasticity, membrane ordering, membrane rigidity, lateral lipid diffusion and lipid peroxidation, of neuronal, liver, lung and blood cell membranes derived from corresponding animal models have been amply reported, such as for insulin resistance (Martin-Segura et al., 2019), amyotrophic lateral sclerosis (Miana-Mena et al., 2011), Alzheimer’s (Zhu et al., 2015), Parkinson’s and Down syndrome (Scott et al., 1994). Age-dependent differences in the phenotype of those diseases could rely on the well-described structural and functional changes of plasma membranes along ageing (Freund et al., 1986; Horie et al., 1986; Sawada et al., 1992; Fraeyman et al., 1993; Igbavboa et al., 1996; Marinho et al., 1997) which are reflected in the differential release of full-length GPI-APs.

 Moreover, the platform may be used as screening system for “membrane-active” substances which manage to counteract unfavourable plasma membrane characteristics and function, as has already been demonstrated for S-adenosylmethionine (Cimino et al., 1984), centrophenoxine (Wood et al., 1986), Ginkgo Biloba extract (Stoll et al., 1996), docosahexaenoic acid (Hashimoto et al., 2017), aniracetam (Li et al., 2007) and piracetam (Müller et al., 1997b), and represent potential drug candidates for age-related impairment of health, in general, and neuronal diseases, in particular.

**AUTHOR CONTRIBUTIONS**

 GAM, SU, MHT, and TDM conceived and designed the research. GAM performed the experiments and analyzed the data. GAM, SU, MHT, and TDM interpreted the experimental results. GAM prepared the figures, wrote, edited and revised the manuscript. GAM, SU, MHT, and TDM reviewed the manuscript and approved its final version.

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**Declaration of Competing Interest**

 No conflicts of interest, financial or otherwise, are declared by any author. This declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research recommended by funding agencies, publishers, and other organizations engaged with supporting research.

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**Tables**

**Table 1**

Age, weight, blood glucose and plasma insulin of the classified rats

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Age****[class]** | **Age [weeks]** | **Weight [g]****mean + SEM** | **Fasting blood glucose [mM]** | **Fasting plasma insulin [µg/l]** |
| **I** | **3** | 80.585.381.389.591.382.487.588.491.689.083.582.887.686.488.782.3**87.0 + 3.5** | 5.455.676.125.986.346.867.015.916.346.896.466.196.885.735.897.08**6.30 + 0.50** | 0.610.530.650.740.660.810.720.520.700.870.961.030.790.600.510.55**0.70 + 0.15** |
| **II** | **6** | 159.4165.3162.5177.8181.8175.4166.3169.7162.8175.4171.2178.5180.3172.5163.6171.5**171.4 + 6.8** | 5.435.685.926.315.996.726.426.836.956.316.405.625.595.825.906.08**6.12 + 0.45** | 0.720.550.540.620.780.811.050.920.890.730.570.600.840.970.580.98**0.76 + 0.17** |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Age [class]** | **Age [weeks]** | **Weight [g]****mean + SEM** | **Fasting blood glucose [mM]** | **Fasting plasma insulin [µg/l]** |
| **III** | **9** | 305.2329.6312.4337.9342.1309.7318.3329.6336.1309.0298.5318.4326.8331.6339.5323.4**325.1 + 12.9** | 5.875.635.976.246.817.007.197.036.366.025.885.715.906.727.066.96**6.40 + 0.54** | 0.640.780.730.500.680.890.921.110.730.930.650.800.971.210.970.63**0.82 + 0.18** |
| **IV** | **16** | 478.2451.2476.3489.1502.5493.1462.5469.9448.7456.0485.1494.2506.4473.2456.9494.0**477.3 + 18.0** | 5.855.926.226.847.177.036.566.026.376.827.056.726.916.566.136.40**6.54 + 0.41** | 0.790.921.151.250.941.220.840.760.951.131.310.920.850.751.031.31**1.01 + 0.19** |

**Legends to figures**

Fig. 1. Schematic representation of the structure, membrane anchorage and lipolytic cleavage of a full-length GPI-AP. GPI-APs consist of a large polypeptide domain (red), typically harbouring N-/O-glycosidically linked glycan chains (blue) and intrachain disulfide bonds (brown), and a GPI anchor which is constituted by a phosphatidylinositol moiety (P-Ino) with long saturated fatty acids (dark green) and a core glycan (light green; detailed structure for AChE see inset). The carboxyl terminus of the polypeptide domain and the terminal mannose residue of the core glycan are coupled *via* an ethanolamine moiety (EtNH2) through amide and phosphodiester linkages, respectively. The fatty acids of the GPI anchor are embedded in the outer leaflet of plasma membranes, typically within lipid rafts (characterized by high cholesterol content and the expression of caveolin-1), with the polypeptide domain protruding into the extracellular space. The cleavage specificity of GPI-PLD is indicated (pink) separating the phosphatidic acid moiety from the inositol (Ino)-glycan protein moiety.

Fig. 2. Chip-based sensing of the release of full-length GPI-APs from adipocyte plasma membranes of rats of the four age classes using the “lab-on-the-chip” configuration. Plasma membranes prepared from adipocytes (four preparations from adipocyte pools derived from four rats each for each age class) were immobilized on the chip surface by injection of three 100-μl portions into α-toxin-coated channels (age class I-IV) or uncoated “blank” (B) channels (only for age class IV) in the presence of Ca2+ with washing cycles using running buffer after each injection during an initial 3000-sec period. Following injection of running buffer and incubation of the channels (6600 sec, no buffer flow) for triggering the putative release of full-length GPI-APs from the immobilized plasma membranes, their capture by the α-toxin-coated chips was measured after detachment of the plasma membranes from chip surface upon injection of glycine (pH 10) and EGTA for the next 1200 sec by consecutive injection of annexin-V in the presence of Ca2+, anti-CD73 and then anti-aP antibodies with washing cycles using running buffer after each injection. The specificity of the capturing of full-length GPI-APs was demonstrated by their release upon injection of PIG41 and then mannose (Man). A) The phase shifts corrected for unspecific interactions with uncoated chips and normalized for differences in capturing efficacy and signal strength between the channels of the same chip (and starting with the injection of annexin-IV *plus* Ca2+) are given for the four plasma membrane preparations for each age class (adipocyte pools from four rats each) using distinct chips. The runs were repeated three times for each plasma membrane preparation using the same chip each after its full regeneration (with PIG41 and mannose), which provided very similar results. Specific phase shifts are indicated as difference (∆) between total signal (after washing with running buffer before injection of PIG41) and unspecific signal (left after detachment with PIG41 before injection of mannose). B) The differences in specific phase shift are given as means + S.D. from four independent adipocyte and plasma membrane preparations (four rats each) for each age class (AGEI-IV) and measurements in quadruplicate each (using the same chip). \* p < 0.01, # p < 0.02, § p < 0.05.

Fig. 3. Chip-based sensing of the release of full-length GPI-APs from liver plasma membranes of rats of the four age classes using the “lab-on-the-chip” configuration. The experiment was performed with plasma membranes prepared from livers (four preparations derived from four rats each for each age class) and immobilized on the chip surface by injection of three 100-μl portions into α-toxin-coated channels (age class I-IV) or uncoated “blank” (B) channels (only for age class IV) in the presence of Ca2+ as described for Fig. 2. Specific phase shifts are indicated as difference (∆) between total signal (after washing with running buffer before injection of PIG41) and unspecific signal (left after detachment with PIG41 before injection of mannose). B) The differences in specific phase shift are given as means + S.D. from four plasma membrane preparations (four rats each) for each age class (AGEI-IV) and measurements in quadruplicate each (using the same chip). # p < 0.02, § p < 0.05.

Fig. 4. Chip-based sensing of the release of full-length GPI-APs from isolated adipocytes of rats of the four age classes into the incubation medium. Samples from the incubation medium of adipocytes (four pools derived from four rats each for each age class) were injected into α-toxin-coated channels (age class I-IV) or uncoated “blank” (B) channels (only for age class IV). Following injection of running buffer, the full-length GPI-APs present in the incubation medium and captured by the α-toxin-coated chips were detected by consecutive injection of annexin-V in the presence of Ca2+, anti-CD73, anti-aP and finally anti-CD55 antibodies with washing cycles using running buffer after each injection. The specificity of the capturing of full-length GPI-APs was demonstrated by their release upon injection of PIG41, followed by running buffer and finally by mannose (Man). A) The phase shifts corrected for unspecific interactions with uncoated chips and normalized for differences in capturing efficacy and signal strength between the channels of the same chip are given for four measurements of the four medium preparations for each age class (from four adipocyte pools each) using four distinct chips. The runs were repeated three times for each medium sample using the same chip after its full regeneration (with PIG41 and mannose), which provided very similar results. Specific phase shifts are indicated as difference (∆) between total signal (after washing with running buffer before injection of PIG41) and unspecific signal left (after detachment with PIG41 before injection of mannose). B) The differences in specific phase shift are given as means + S.D. from four independent adipocyte preparations and incubations for each age class (AGEI-IV) and measurements in quadruplicate each (using the same chip). \* p < 0.01, # p < 0.02, § p < 0.05.

Fig. 5. Chip-based sensing of the release of full-length GPI-APs into serum of rats of the four age classes. Samples from the serum of 16 rats for each age class were injected into α-toxin-coated channels (age class I-IV) or uncoated “blank” (B) channels (only for age class IV). Following injection of running buffer, full-length GPI-APs present in the serum and captured by the α-toxin-coated chips were detected by consecutive injection of annexin-V in the presence of Ca2+, anti-CD73, anti-aP and finally anti-CD55 antibodies with washing cycles using running buffer after each injection. The specificity of the capturing of full-length GPI-APs was demonstrated by their release upon injection of PIG41, followed by running buffer and finally by mannose (Man). A) Specific phase shifts indicated as the difference (∆) between total signal (after washing with running buffer before injection of PIG41) and unspecific signal left after detachment with PIG41 before injection of mannose) are given for four representative serum samples for each age class using four distinct chips. The measurements were repeated three times with the same medium sample injected into the same channel after full regeneration of the chip (with mannose between time point 14 and 15 min), which provided very similar results. B) Specific phase shifts differences are indicated as means + S.D. from four independent measurements of the four representative serum samples for each age class (AGEI-IV) using distinct chips and repeated four times using the same channel. \* p < 0.01, # p < 0.02, § p < 0.05. C) The differences in specific phase shift are given for the individual serum samples (16 *per* age class I-IV). D) The differences in specific phase shift are given as means + S.D. for each age class with the 16 individual serum samples each. \* p < 0.01, # p < 0.02, § p < 0.05.

Fig. 6. Chip-based sensing of the release of GPI anchor-less GPI-APs into adipocyte incubation medium (A) and serum (B) of rats of the four age classes. Adipocyte incubation medium (A) and serum samples (B) were subjected to TX-114 partitioning. The aqueous TX-114-depleted phases were injected into α-toxin-coated channels (age class I-IV) or uncoated “blank” (B) channels (only for age class IV). Following injection of running buffer, GPI-APs captured by the α-toxin-coated chips were tested for the presence of phospholipids and the GPI-AP protein moiety by consecutive injection of annexin-V in the presence of Ca2+, anti-CD73, anti-aP and finally anti-CD55 antibodies with washing cycles using running buffer after each injection. The specificity of the capturing of full-length GPI-APs was demonstrated by their release upon injection of PIG41, followed by running buffer and finally by mannose (Man). Upper panels) The phase shifts corrected for unspecific interactions with uncoated chips and normalized for differences in capturing efficacy and signal strength between the channels of the same chip are given for measurement of two representative incubation medium (A) and serum (B) samples from 8 rats each for each age class using distinct chips. The runs were repeated three times for each sample using the same channel after its full regeneration (with PIG41 and mannose), which provided very similar results. Specific phase shifts are indicated as difference (∆) between total signal (after washing with running buffer before injection of PIG41) and unspecific signal (left after detachment with PIG41 before injection of mannose). Lower panels) The differences in specific phase shift are given as means + S.D. for each age class from the four independent adipocyte preparations and incubations (adipocyte pools derived from four rats for each age class) with measurements in quadruplicate each (using the same chip)(A) and from 16 individual serum samples (B).

Fig. 7. Chip-based measurement of GPI-PLD activity in serum of rats of the four age classes. AChE reconstituted into lipid micelles at the “optimized” constituent ratio was incubated with identical volumes of individual serum samples from 16 rats each of the four age classes. After TX-114 partitioning of the total incubation mixtures, the aqueous phases harbouring the hydrophilic protein moiety of AChE were injected into α-toxin-coated chips (time point 0). Specific capture of AChE having lost the phosphatidate moiety of its GPI anchor but retained the core glycan portion was tested by injection of 30 μM PIG41 at time point 203 s. The measured phase shift was corrected for unspecific interaction of components in the aqueous phase and altered viscosity of the sample fluid and normalized for varying responsiveness of distinct chips toward GPI-AP capture. The maximal serum-induced change in specific phase shift is indicated as the difference (∆) between total signal (after washing with running buffer before injection of PIG41 at time point 203 s) and unspecific signal (left after detachment with PIG41 at time point 300 s). A) The phase shifts are given for four measurements of the same serum sample as representatives (four from the 16 measured) for each age class using four distinct chips. The measurements were repeated three times with the same medium sample injected into the same channel after full regeneration of the chip (with mannose after time point 300 s), which provided very similar results. B) Specific phase shifts differences are indicated as means + S.D. from four independent measurements of the same serum samples as representatives for each age class using distinct chips and repeated four times using the same channel. \* p < 0.01, # p < 0.02, § p < 0.05. C) The differences in specific phase shift are given for the individual serum samples (16 *per* age class I-IV) measured with distinct chips. D) The differences in specific phase shift are given as means + S.D. for each age class (AGEI-IV) with 16 individual serum samples, each. \* p < 0.01, # p < 0.02, § p < 0.05.

Fig. 8. Correlation of the release of full-length GPI-APs from adipocyte plasma membranes, into adipocyte incubation medium and into serum as well as of the serum GPI-PLD activity with the age of the rats. Data for the release of full-length GPI-APs from adipocyte (■) and liver (●) plasma membranes were derived from Fig. 2B and 3B, respectively, from adipocytes into incubation medium (**x**) from Fig. 4B, into serum (▲) from Fig. 5D and for the GPI-PLD activity (♦) from Fig. 7D and are given upon normalization of each parameter for age class I (set at 100 %). The results of linear regression analysis are shown.

Fig. 9. Model for the age-dependent release and lipolytic degradation of full-length GPI-APs explaining their steady-state concentration in serum of rats. At low age, the moderate release of full-length GPI-APs, such as CD73 and aP, from the outer plasma membrane leaflet of tissue cells into the blood in micelle-like complexes together with (lyso)phospholipids, glycolipids and cholesterol is counterbalanced by moderate degradation by GPI-PLD in the blood, resulting in high steady-state concentration of the complexes (left panel). At high age, the extensive release of full-length GPI-APs is counterbalanced by pronounced degradation by GPI-PLD, resulting in low steady-state concentration of the complexes (right panel).