British Journal of Pharmacology (2008) 154, 901–913

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Hydrogen peroxide-induced translocation of glycolipid-anchored (c)AMP-hydrolases to lipid droplets mediates inhibition of lipolysis in rat adipocytes

G Müller, S Wied, C Jung and S Over

Sanofi-Aventis Pharma Deutschland GmbH, TD Metabolism, Frankfurt am Main, Germany

Background: The insulin-independent inhibition of lipolysis by palmitate, the anti-diabetic sulphonylurea glimepiride and H_2O_2 in rat adipocytes involves stimulation of the glycosylphosphatidylinositol (GPI)-specific phospholipase-C (GPI-PLC) and subsequent translocation of the GPI-anchored membrane ectoproteins (GPI-proteins), Gce1 and cluster of differentiation antigen (CD73), from specialized plasma membrane microdomains (DIGs) to cytosolic lipid droplets (LDs). This results in cAMP degradation at the LD surface and failure to activate hormone-sensitive lipase. Reactive oxygen species (ROS) may trigger this sequence of events in response to palmitate and glimepiride.
Experimental approach: The effects of various inhibitors of ROS production on the release of H_2O_2, GPI anchor cleavage and translocation of the photoaffinity-labelled or metabolically labelled Gce1 and CD73 from DIGs to LD and inhibition of lipolysis by different fatty acids and sulphonylureas were studied with primary rat adipocytes.
Key results: Glimepiride and palmitate induced the production of H_2O_2 via the plasma membrane NADPH oxidase and mitochondrial complexes I and III, respectively. Inhibition of ROS production was accompanied by the loss of (i) GPI-PLC activation, (ii) Gce1 and CD73 translocation and (iii) lipolysis inhibition in response to palmitate and glimepiride. Non-metabolizable fatty acids and the sulphonylurea drug tolbutamide were inactive. NADPH oxidase and GPI-PLC activities colocalized at DIGs were stimulated by glimepiride but not tolbutamide.
Conclusions and implications: The data suggest that ROS mediate GPI-PLC activation at DIGs and subsequent GPI-protein translocation from DIGs to LD in adipocytes which leads to inhibition of lipolysis by palmitate and glimepiride. This insulin-independent anti-lipolytic mechanism may be engaged by future anti-diabetic drugs.
British Journal of Pharmacology (2008) **154,** 901–913; doi:10.1038/bjp.2008.146; published online 5 May 2008

Keywords: reactive oxygen species; sulphonylureas; lipid droplets; lipolysis; glycosylphosphatidylinositol-anchored proteins; adipocytes

Abbreviations: DPI, diphenylene iodonium; Gce1, glycolipid-anchored cAMP-binding ectoprotein-1; GO, glucose oxidase; (G)PI, (glycosyl)phosphatidylinositol; (G)PI-PLC, (G)PI-specific phospholipase-C; GPI-protein, GPI-anchored membrane ectoprotein; hcDIGs and lcDIGs, high- and low-cholesterol-containing detergent-insoluble glycolipid-containing membrane rafts; LD, lipid droplets; L-NAME, N^{ω}-nitro-L-arginine methyl ester; PKC, protein kinase-C; ROS, reactive oxygen species; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TTFA, thenoyltrifluoroacetone

Introduction

Hydrogen peroxide (H_2O_2), long-chain fatty acids and certain anti-diabetic drugs of the sulphonylurea class, such as the third-generation sulphonylurea, glimepiride, have been known for a long time to reduce hormone-stimulated lipolysis in primary and cultured rodent adipocytes (Maragno *et al.*, 1971; Little and de Haen, 1980; Mukherjee, 1980; Muchmore *et al.*, 1982; Vallano *et al.*, 1983; Jepson and Yeaman, 1992; Müller *et al.*, 1994c; Lei *et al.*, 2004; Müller, 2005; Guo *et al.*, 2006). Different molecular mechanisms have been proposed for each of these apparently anti-lipolytic agents. However, very recently, it has been suggested that the inhibition of the isoprenaline-, adenosine deaminase- or forskolin-induced lipolysis in isolated rat adipocytes by H_2O_2 and glimepiride and, in part, by

Correspondence: Dr G Müller, Sanofi-Aventis Pharma Deutschland GmbH, TD Metabolism, Industrial Park Höchst, Building H821, Frankfurt am Main 65926, Germany.
E-mail: Guenter.Mueller@sanofi-aventis.com
Received 4 December 2007; revised 7 February 2008; accepted 4 March 2008; published online 5 May 2008

palmitate may rely on the same mechanism, based on the finding that the anti-lipolytic activity of each agent critically depends on the upregulation of a (c)AMP-degrading activity associated with cytosolic lipid droplets (LDs) (Müller *et al*., 2008a, b). The missing protein kinase-A-dependent phosphorylation may ultimately lead to impaired access of triacylglycerol lipases, such as hormone-sensitive lipase Yeaman (2004), to the LD. Upregulation of the LD-associated cAMP degradation is based on the translocation of the two glycosylphosphatidylinositol (GPI)-anchored membrane ectoproteins (GPI-proteins), the cAMP-specific phosphodiesterase, originally called glycolipid-anchored cAMP-binding ectoprotein-1 (Gce1), and the 5′-nucleotidase, cluster of differentiation antigen (CD73), from high-cholesterol-containing detergent-insoluble glycolipid-containing membrane rafts (hcDIGs) to the LD (Müller *et al*., 2008c). The activation of a GPI-specific phospholipase-C (GPI-PLC), which cleaves the GPI anchors of Gce1 and CD73, represents the most proximally located step shared by palmitate, H_2O_2 and glimepiride so far identified. However, the (common) molecular mechanism(s) underlying the upregulation of the GPI-PLC and, in consequence, GPI-protein translocation and inhibition of lipolysis in response to these stimuli remain unknown.

A candidate mechanism may represent the production of reactive oxygen species (ROS), in general, and H_2O_2, in particular, for the following reasons: (i) the anti-diabetic second-generation sulphonylurea drug, glibenclamide, has been shown to stimulate ROS production in cultured β-cells through protein kinase-C (PKC)-dependent activation of the NAD(P)H oxidase (Tsubouchi *et al*., 2005). (ii) Pretreatment of 3T3-L1 adipocytes with 1 mM octanoate or linoleate has been reported to increase the level of intracellular H_2O_2 (Guo *et al*., 2006). This was dependent on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and (CPT-1-independent) β-oxidation, and accompanied by inhibition of isoprenaline-stimulated lipolysis (Furukawa *et al*., 2004). (iii) Exposure of cultured aortic smooth muscle and endothelial cells to 0.2 mM palmitate has been found to trigger ROS production. This was paralleled by elevated levels of diacylglycerol, PKC activity and PKC-dependent NAD(P)H oxidase activity (Inoguchi *et al*., 2000). (iv) Challenge of cultured human umbilical vein endothelial cells with 0.25 mM palmitate has been demonstrated to elicit ROS production (Bellin *et al*., 2006). (v) In adipocytes during various anti-lipolytic (submaximal) challenges, inverse correlations between H_2O_2 production and the release of fatty acids have been consistently observed (Little and de Haen, 1980; Mukherjee, 1980; Muchmore *et al*., 1982; Guo *et al*., 2006). Taken together, these findings suggest that both sulphonylureas of the second- and third-generation and exogenous fatty acids inhibit lipolysis by the induction of ROS and H_2O_2 production.

Here, the putative requirement for ROS production at distinct subcellular sites during lipolysis inhibition by palmitate, glimepiride and insulin was studied in isolated rat adipocytes. The results argue for the need of localized action of ROS at plasma membrane hcDIGs for the activation of the GPI-PLC and induction of the translocation of the GPI-proteins, Gce1 and CD73, from hcDIGs to cytosolic LD.

Materials and methods

Determination of intracellular ROS levels

Intracellular ROS generation was determined using a derivative of 6-carboxy-2′,7′-dichlorodihydrofluorescein (6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; H_2-DCFDA), which enters the cells and is hydrolysed by intracellular esterases to H_2-DCF. ROS, such as H_2O_2 and other peroxides, cause oxidation of the non-fluorescent H_2-DCF to the fluorescent product, 2′,7′-dichlorofluorescein (DCF). The method used has been described previously (Wang and Joseph, 1999; Talior *et al*., 2003) with some modifications. Briefly, H_2-DCFDA was dissolved in DMSO as 10-mM stock solution and kept frozen at −20 °C. Prior to loading of the adipocytes with H_2-DCFDA, the stock solution was mixed with KRH buffer (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 20 mM HEPES/KOH, pH 7.4), containing 1% (w/v) BSA, 100 μg mL^{-1} gentamycin and 10 mM sodium pyruvate to yield an H_2-DCFDA concentration of 1 mM. The adipocytes (5×10^6 cells) in 5 mL of KRH buffer containing 1% BSA and glucose as indicated were incubated (1 h, 37 °C) with the various stimuli under continuous bubbling with 5% CO_2, 95% O_2 and mild shaking (50 cycles per min). After addition of the 1 mM solution of H_2-DCFDA to a final concentration of 100 μM, the incubation was continued for an additional 45 min in the dark. Incubation with H_2O_2 served as a positive control, and known concentrations of H_2-DCF incubated with 20 mM NaOH were used as standards. Adipocytes were separated from the medium by flotation (200 *g*, 2 min, 25 °C), washed twice with 20 mL of KRH buffer each by flotation and then suspended in 0.5 mL of 10 mM HEPES/KOH (pH 7.4) and 1 mM EDTA to disrupt the plasma membranes. Subsequently, the fluorescence of DCF was measured in 100 μL samples in triplicate with a multiplate fluorescence reader (CytoFluor Series 4000 multiwell; PerSeptive Biosystems Inc., Framingham, MA, USA) at 37 °C and excitation/emission set at 485 ± 10 nm:530 ± 12.5 nm. The measured fluorescence was digitized using CytoFluor software (Version 4.0; PerSeptive Biosystems).

Determination of NADPH oxidase activity

Collected plasma membranes or hcDIGs or low-cholesterol-DIGs (lcDIGs) were washed in 30 mM MES (2-(*N*-morpholino) ethanesulphonic acid) buffer (KOH, pH 5.8) 120 mM NaCl, 4 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1 mM NaN_3 and 10 μM FAD, suspended in the same buffer at 0.1–0.3 mg protein per mL and then incubated (30 min, 37 °C) with NADPH (final concentration 0.25 mM) in 50 μL of the same buffer. After addition of HCl (final concentration 0.1 M), the samples were diluted by 10- to 100-fold with assay buffer (0.1 M Na_2HPO_4; 0.1 M KH_2PO_4, pH 8.2, 1 mM EDTA, 12.5 μM luminol and 0.1 U mL^{-1} horseradish peroxidase) and then assayed for the peroxidase-catalysed oxidation of luminol by H_2O_2 (Kather *et al*., 1987; Krieger-Brauer and Kather, 1992). The reactions were carried out in the absence or presence of 110 U mL^{-1} catalase to account for nonspecific reactions. External standards (10–100 nM) contained all components except that protein was omitted. Reaction kinetics was followed

using a luminescence analyzer (LB 950T; Berthold, Wildbad, Germany). Integrated counts from 5 to 10 s (light output stable for 20 s) were taken as a measure for H_2O_2 concentration. The detection limit for H_2O_2 was 5–10 nM, and light output was linearly related to H_2O_2 concentrations to up to 100 nM. Linear increases in NADPH-dependent H_2O_2 production were limited to 5–15 μg protein mL^{-1} and measured using 3–4 different concentrations.

Determination of GPI-PLC activity

Adipocyte GPI-PLC was assayed according to previously published protocols (Müller *et al*., 1994a, 2005) with modifications. Briefly, collected plasma membranes and non-DIGs (10–40 μg protein) or hcDIGs and lcDIGs (5–25 μg protein) were suspended in 90 μL of buffer (20 mM HEPES/KOH, pH 7.8, 144 mM NaCl, 0.1% TX-100, 0.2 mM $MgCl_2$) and then incubated (1 h, 25 °C) with 10 μL of (bovine erythrocyte) acetylcholinesterase solution (0.12 U). After addition of 5 μL of acetic acid and then 0.4 mL of 10 mM Tris (HCl, pH 7.4) and 144 mM NaCl, the mixture was subjected to partitioning into TX-114 (non-ionic detergent; Merck, Darmstadt, Germany; Müller *et al*., 2008a).

Cleavage of Gce1 and CD73 by GPI-PLC was determined by separating their uncleaved amphiphilic versions (harbouring the intact GPI anchor), which partition into the TX-114 detergent phase, from their cleaved hydrophilic versions (lacking the GPI anchor), which partition into the aqueous phase, upon phase separation induced by warming up of the mixtures up to the clouding point of this non-ionic detergent.

For this, protein samples (25–100 μg protein in 0.5 mL) were partitioned between TX-114-enriched and TX-114-depleted (aqueous) phases by mixing with 0.5 mL of ice-cold 25 mM Tris/HCl (pH 7.4), 140 mM NaCl containing 2% TX-114. After incubation (1 h, on ice), the mixture was layered onto a cushion of 0.4 mL of 0.25 M sucrose and 25 mM Tris/HCl (pH 7.4) on ice. Phase separation was induced by warming up to 37 °C and subsequent centrifugation (10 000 *g*, 1 min). The re-extracted lower TX-114-enriched phase and the pooled upper TX-114-depleted phase were precipitated by sequential addition of BSA (fraction V; Sigma, Deisenhofen, Germany) to a final concentration of 0.1 mg mL^{-1} and polyethylene glycol 6000 (25% in Mops/KOH, pH 7.4, 0.5 mM DTT and 0.5 mM $MgCl_2$) to a final concentration of 12%. After incubation (1 h, on ice), the precipitated proteins were collected by centrifugation (12 000 *g*, 10 min, 4 °C), then washed with 0.5% polyethylene glycol 6000 and finally dissolved in 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 mM DTT, 20 mM octylglucoside and 0.1% β-amidotaurocholate (Sanofi-Aventis Pharma, Frankfurt am Main, Germany) containing protease and phosphatase inhibitors at 0.5–1 mg protein per mL.

The GPI-PLC activity was calculated as the amphiphilic-to hydrophilic conversion of the acetylcholinesterase from the ratio of its activity measured in the TX-114-depleted phase (and corrected for the 10–20% hydrophilic acetylcholinesterase activity as revealed by incubations lacking protein) and the total activity measured before partitioning.

Other experimental procedures

Published procedures were used for the preparation and incubation of rat adipocytes with insulin, sulphonylureas and fatty acids (Müller *et al*., 1994a, 2003, 2008a), metabolic labelling of rat adipocytes with *myo*-[^{14}C]inositol (Müller *et al*., 1993, 1994a, b), extraction and precipitation of LD-associated proteins under native and denaturing conditions, affinity purification of Gce1 and CD73 on (c)AMP-sepharose (Müller *et al*., 2008a), determination of lipolysis as glycerol and fatty acid release (Müller *et al*., 2003), SDS-polyacrylamide gel electrophoresis (SDS-PAGE), phosphor-imaging and immunoblotting (Müller *et al*., 2001, 2002), determination of the protein concentration using the BCA method (Pierce, Perbio Science, Bonn, Germany) with BSA as standard and the preparation of LD, plasma membranes, hcDIGs, lcDIGs and non-DIGs (Müller *et al*., 2002, 2008b, c). The identity and purity of the hcDIGs, lcDIGs and non-DIG plasma membranes were confirmed by the distribution of known marker enzymes with relative enrichment of Gce1, CD73, p59^{Lyn}, caveolin, insulin receptor β-chain and GLUT4 at hcDIGs vs lcDIGs vs non-DIG plasma membranes as described previously (Müller *et al*., 2002, 2008a). Figures of phosphor-images and lumi-images were constructed by using Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA, USA).

Data analysis

Concentration–response curves were fitted by using GraphPad Prism 4.03 software. Results are shown as means ± s.d. Differences between experimental groups were determined by ANOVA with statistical significance set at *P*-values of $\leqslant 0.05$.

Materials

Glimepiride, glibenclamide, tolbutamide, glimepiride M1 and GPI-2350 were made available by the Pharma synthesis department of Sanofi-Aventis Pharma; *N*-acetyl-cysteine, apocynin, oxpurinol, TTFA, N^{ω}-nitro-L-arginine methyl ester (L-NAME), antimycin A, rotenone, dinitrophenol and DPI were obtained from Calbiochem (Bad Soden, Germany). Catalase, peroxidase, xanthine oxidase, creatine kinase, acetylcholinesterase, creatine phosphate, myristate, stearate, oleate, linoleate, dodecanoate and 8-bromopalmitate were purchased from Sigma. NBD-dodecanoate was synthesized as described previously (Müller *et al*., 2003). H_2-DCFDA was delivered by Molecular Probes (Eugene, OR, USA). H_2O_2 was bought from Merck. Pre-mixed protease (complete EASY-pack) and phosphatase (PhosSTOP) inhibitor cocktails (one tablet each for 10 mL) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). All other materials were obtained as described earlier (Müller *et al*., 1994a, 2001, 2002, 2005, 2008a, 2008b).

Results

Insulin, palmitate and glimepiride induce ROS production in rat adipocytes via different mechanisms

Palmitate, glimepiride and H_2O_2 are known to inhibit lipolysis in rodent adipocytes (see Introduction). Recent

findings indicate that the anti-lipolytic activity of each of these stimuli depends on the stimulation of the GPI-PLC that is located at hcDIGs of the adipocyte plasma membrane (Müller *et al*., 2008c). ROS, such as H_2O_2, may act as the common signalling molecules triggering activation of the GPI-PLC and in consequence lipolysis inhibition via the translocation of Gce1 and CD73 to LD in response to these stimuli.

To study this putative molecular mechanism, the generation of H_2O_2 and other radical or non-radical ROS was studied in isolated rat adipocytes exposed to various types of fatty acids and sulphonylureas by determination of the oxidation of H_2-DCFDA to fluorescent DCF (see Materials and methods). The production of cell-associated DCF by basal adipocytes during 1 h increased with increasing glucose concentrations to up to twofold at 16 vs 0.5 mM glucose (Figure 1a). Treatment of the adipocytes with insulin, palmitate or glimepiride led to significant upregulation of the ROS production in glucose-dependent (Figure 1a) and concentration-dependent (Figure 1b) fashion. Glimepiride was considerably more potent than palmitate and insulin. It stimulated ROS production by up to 5.5-fold compared to 3.4- and 1.9-fold, respectively, above the basal state with EC_{50} values of 6.2 μM compared with 0.7 mM and 0.07 nM, respectively, at 5.5 mM glucose (Figure 1b). The specificity of DCF as indicator for ROS was demonstrated by the addition of catalase to the incubation mixtures, when the increase in the fluorescence signal was completely prevented irrespective of the type of stimulus and glucose concentration used (Figure 1, filled bars). Furthermore, the extracellular production of H_2O_2 during treatment of rat adipocytes with glucose oxidase (GO) at concentrations (10–100 mU mL^{-1}), which are known to elicit anti-lipolytic activity (Mukherjee, 1980; Muchmore *et al*., 1982; Müller *et al*., 2008a), was monitored as concentration-dependent rises in the DCF signal (Figure 2), which were not observed in the presence of catalase. This argues for the specificity and sensitivity of the method used for the detection of ROS.

Different types of fatty acids and sulphonylureas exhibited distinct potencies in stimulating ROS production (Figure 2). Stearate, oleate and linoleate were approximately as effective as palmitate, whereas myristate, dodecanoate and the poorly or non-metabolized fatty acid analogues, NBD-dodecanoate and 8-bromopalmitate, had almost no effect (hatched bars). The second-generation sulphonylurea, glibenclamide, as well as the major metabolite of glimepiride, M1, which both exhibit considerably lower anti-lipolytic activity than the third-generation sulphonylurea, glimepiride (Müller *et al*., 1994c; data not shown), were significantly less active in inducing ROS production compared with glimepiride (filled bars). This was also reflected in the 2.5- to 4.5-fold lower EC_{50} value of glimepiride vs glibenclamide (data not shown). The first-generation sulphonylurea, tolbutamide, which does not exert anti-lipolytic activity (Müller *et al*., 1994c, 2005), did not affect intracellular ROS levels (Figure 2). These observations suggest that in rat adipocytes ROS production is stimulated by those metabolizable fatty acids and sulphonylureas, which are capable of inhibiting lipolysis *in vitro*.

Next, the subcellular sites of the insulin-, fatty acid- and sulphonylurea-induced ROS production in rat adipocytes were studied. For this, the ROS-producing enzymes, plasma

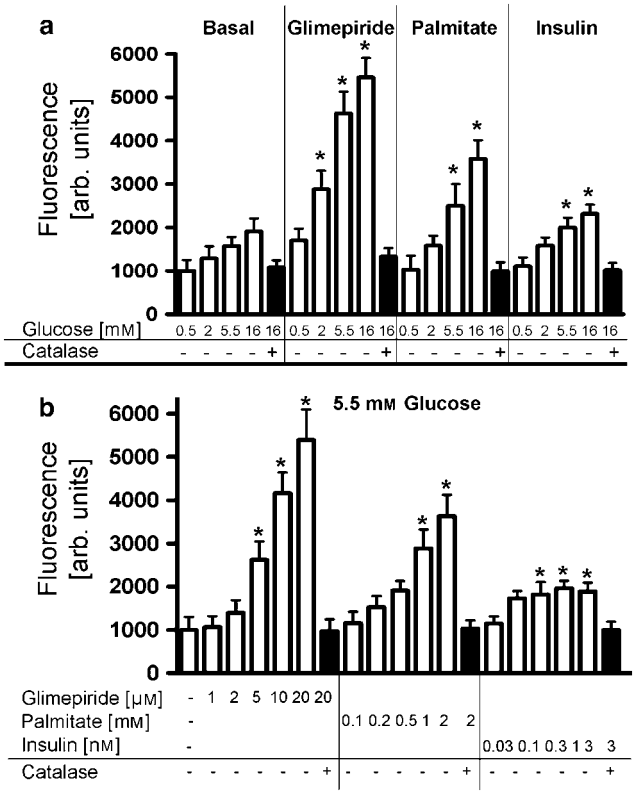

Figure 1 Effect of insulin, palmitate and glimepiride on reactive oxygen species (ROS) production. Isolated rat adipocytes were incubated (1 h, 37 °C) with 0.5, 2, 5.5 or 16 mM glucose (**a**) or 5.5 mM glucose (**b**) in the absence or presence of insulin (5 nM), glimepiride (10 μM) and palmitate (1 mM) (**a**) or with increasing concentrations of insulin, palmitate and glimepiride as indicated (**b**) in the absence or presence of catalase (0.5 U ml^{-1}). After addition of 6-carboxy-2′,7′-dichlorodihydrofluorescein (H_2-DCF) and further incubation, the adipocytes were separated from the medium, washed and then analysed for fluorescence as described in Materials and methods. Means ± s.d. of three adipocyte preparations with incubations in duplicate; *indicates significant difference compared with basal treatment at the same glucose concentration (**a**) or at 5.5 mM glucose (**b**, left bar).

membrane NADPH oxidase, mitochondrial xanthine oxidase, mitochondrial respiratory complexes and NOS were blocked with inhibitors at concentrations exceeding the corresponding IC_{50} values by 10- to 20-fold (Figure 3). The presence of the general ROS scavenging agent, *N*-acetylcysteine, dramatically reduced the fluorescent DCF signals observed with glimepiride, palmitate and insulin. This confirmed the upregulation of ROS production by these agents in rat adipocytes and the specificity of the ROS detection method. Diphenylene iodonium (DPI), apocynin and vanillylnonanamide, three widely used inhibitors of the plasma membrane NADPH oxidase, significantly diminished the glimepiride-, palmitate- and insulin-induced ROS production by 80–90, 60–70 and 25–40%, respectively. Inhibition of the mitochondrial complex I by rotenone or TTFA and complex III by antimycin A significantly reduced palmitate-induced ROS production by 30–40% and insulin-induced ROS levels by 45–55%, but had no effect on the induction by glimepiride. Inhibition of the NOS by L-NAME and of xanthine oxidase by oxpurinol did not significantly

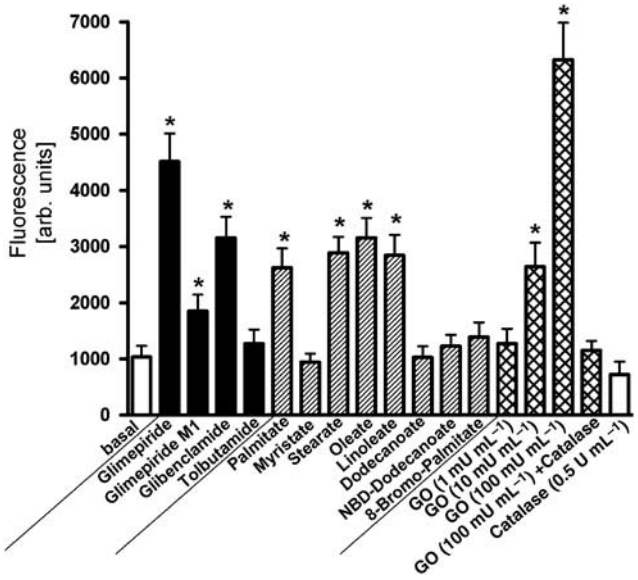

Figure 2 Effect of various fatty acids, sulphonylureas and glucose oxidase (GO) on reactive oxygen species (ROS) production. Isolated rat adipocytes were incubated (1 h, 37 °C) with 2 mM glucose in the absence or presence of various sulphonylureas (10 μM) and fatty acids (1 mM) or various concentrations of GO as indicated. After addition of 6-carboxy-2′,7′-dichlorodihydrofluorescein (H_2-DCF) and further incubation, the adipocytes were separated from the medium, washed and then analysed for fluorescence. Means ± s.d. of two adipocyte preparations with incubations in duplicate; *indicates significant difference compared with basal treatment (left bar).

impair ROS production in response to glimepiride and palmitate, but moderately lowered insulin-induced ROS levels. These data suggest the involvement of distinct cellular sites of ROS production in rat adipocytes in response to insulin (NADPH oxidase, complex I and III, NOS and xanthine oxidase, to varying degrees), palmitate (NADPH oxidase and complex I and III, with comparable degree) and glimepiride (NADPH oxidase). In consequence, simultaneous inhibition of the NADPH oxidase and complex I by DPI and rotenone almost completely prevented the rise in ROS levels induced by glimepiride or palmitate, but was less efficient with regard to insulin. Taken together, NADPH oxidase, mitochondrial complex I and III, xanthine oxidase and NOS contribute to the total ROS production in rat adipocytes and are differentially engaged by insulin, palmitate and glimepiride.

ROS generation is required for the induction of the translocation of Gce1 and CD73 from hcDIGs to LD and stimulation of the GPI-PLC by palmitate and glimepiride

On basis of the observed upregulation of ROS production by palmitate and glimepiride in combination with the previously reported activation of the GPI-PLC and translocation of Gce1 and CD73 from hcDIGs to LD in response to H_2O_2 (Müller *et al*., 2008c), it was tempting to speculate that ROS may also mediate the corresponding effects of palmitate and glimepiride.

To study the role of ROS in the activation of the GPI-PLC, rat adipocytes were metabolically labelled with *myo*-[^{14}C]inositol and then incubated with various inhibitors of ROS production. After exposure to glimepiride or palmitate,

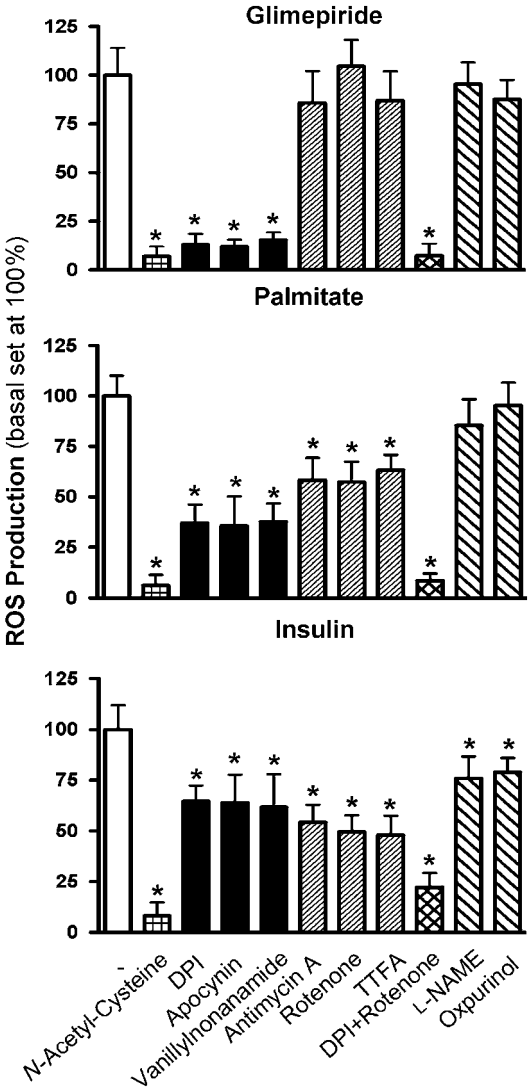

Figure 3 Effect of inhibitors of oxidases, mitochondrial respiratory chain or NOS on the induction of reactive oxygen species (ROS) production by insulin, palmitate and glimepiride. Isolated rat adipocytes were incubated (5 min, 37 °C) with 5.5 mM glucose in the absence or presence of *N*-acetyl-cysteine (0.5 mM), diphenylene iodonium (DPI; 20 μM), apocynin (100 μM), vanillylnonanamide (100 μM), antimycin A (10 μM), rotenone (1 μM), thenoyltrifluoroacetone (TTFA; 10 μM), N^{ω}-nitro-L-arginine methyl ester (L-NAME; 25 μM) and oxpurinol (100 μM) prior to addition of insulin (5 nM final concentration), palmitate (1 mM) or glimepiride (10 μM) as indicated. After addition of 6-carboxy-2′,7′-dichlorodihydrofluorescein (H_2-DCF) and further incubation, the adipocytes were separated from the medium, washed and then analysed for fluorescence. The stimulus-induced production of ROS in the absence of inhibitor was set at 100% in each case (left bars). Means ± s.d. of three adipocyte preparations with incubations in duplicate; *indicates significant difference compared with the corresponding induction in the absence of inhibitor (left bars).

plasma membranes were prepared and then subjected to TX-114 partitioning. Gce1 and CD73, which harbours the lipolytically cleaved GPI anchor including the terminal inositol moiety, were recovered from the aqueous phase by affinity purification. As revealed by the phosphor-images from SDS-PAGE, the amounts of hydrophilic [^{14}C]inositol-

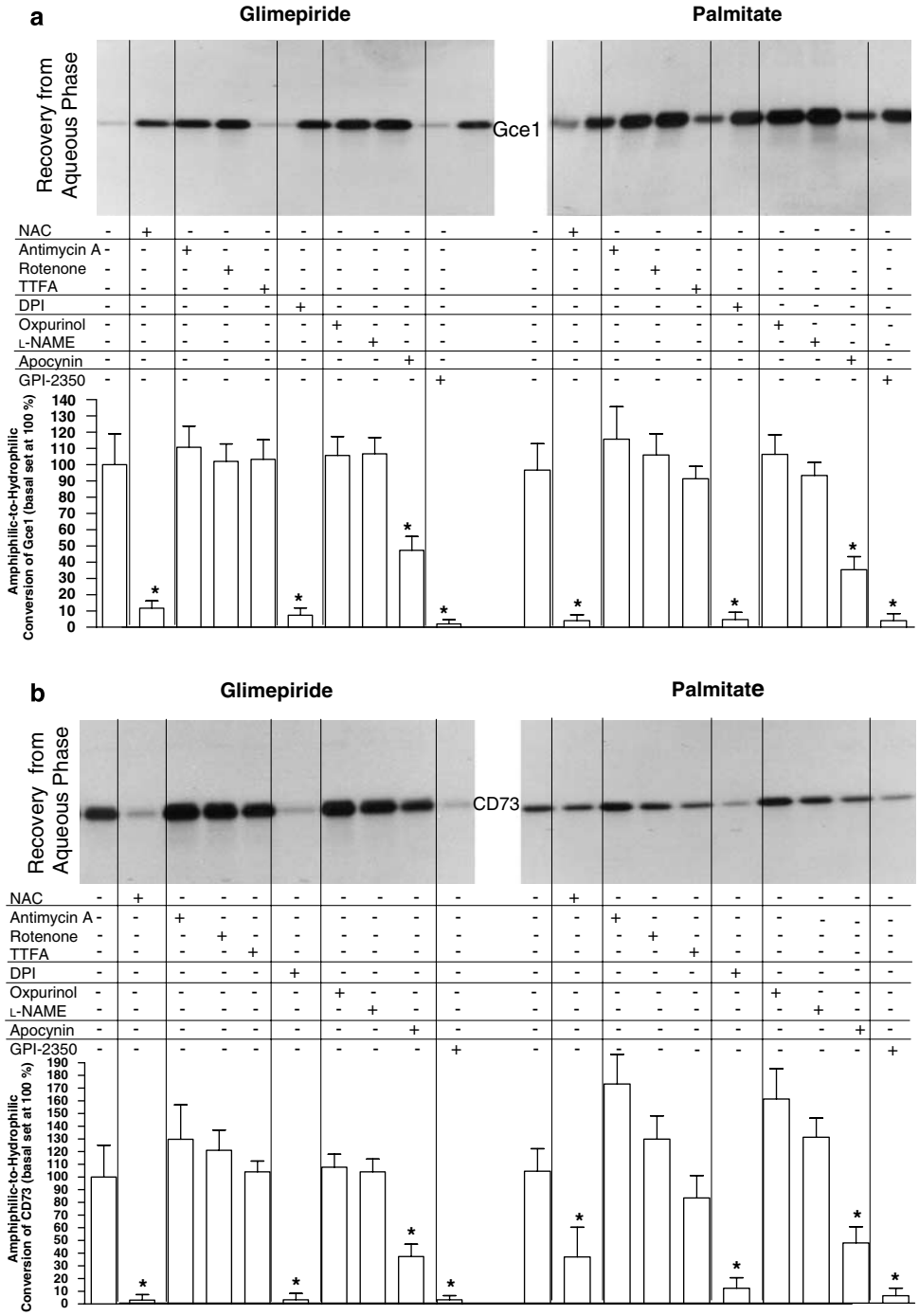

Figure 4 Effect of the inhibition of reactive oxygen species (ROS) production on the insulin-, palmitate- and glimepiride-induced lipolytic cleavage of glycolipid-anchored cAMP-binding ectoprotein-1 (Gce1) and CD73. Isolated rat adipocytes were metabolically labelled with *myo*-[^{14}C]inositol and then incubated (5 min, 37 °C) with 2 mM glucose in the absence or presence of *N*-acetyl-cysteine (NAC; 0.5 mM), antimycin A (10 μM), rotenone (1 μM), thenoyltrifluoroacetone (TTFA; 10 μM), diphenylene iodonium (DPI; 20 μM), oxpurinol (100 μM), N^{ω}-nitro-L-arginine methyl ester (L-NAME; 25 μM), apocynin (100 μM) and GPI-2350 (50 μM) prior to the addition of palmitate (1 mM final concentration) or glimepiride (10 μM) as indicated. After further incubation (20 min), the adipocytes were separated from the medium. Plasma membranes were prepared and then subjected to TX-114 partitioning. Proteins of the aqueous phase were precipitated under native conditions. Solubilized Gce1 (**a**) and CD73 (**b**) were affinity purified, then precipitated under denaturing conditions and after solubilization analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and phosphorimaging. Quantitative evaluation is given with the amphiphilic-to-hydrophilic conversion calculated by subtraction of the basal value in each case and for each stimulus in the absence of inhibitor set at 100% (left bars). Means ± s.d. of three adipocyte preparations with incubations in triplicate; *indicates significant difference compared with the corresponding induction in the absence of inhibitor (left bars). GPI, glycosylphosphatidylinositol.

labelled Gce1 (Figure 4a) and CD73 (Figure 4b) generated in glimepiride- and palmitate-treated adipocytes were significantly reduced by *N*-acetyl-cysteine, DPI and apocynin, but remained almost unaffected by antimycin A, rotenone, thenoyltrifluoroacetone (TTFA), oxpurinol and L-NAME. The amphiphilic-to-hydrophilic conversion of Gce1 and CD73 as monitored by the TX-114 partitioning was completely abolished in the presence of the GPI-PLC inhibitor, GPI-2350. This demonstrates the specific lipolytic cleavage of the GPI anchors of Gce1 and CD73 by the GPI-PLC, which can be assayed by TX-114 partitioning of the [^{14}C]inositol-labelled GPI-proteins. As expected, the induction of the GPI-protein translocation and activation of the GPI-PLC in course of H_2O_2 production by GO was compromised by the presence of *N*-acetyl-cysteine and catalase rather than by DPI and apocynin (data not shown). This confirms that the observed inhibition of the palmitate- and glimepiride-induced GPI-PLC activation and GPI-protein translocation (see below) by DPI and apocynin was due to (site-specific) interference with the ROS production.

To study the role of ROS in the induction of GPI-protein translocation, rat adipocytes were metabolically labelled with *myo*-[^{14}C]inositol (for the detection of the GPI anchors of Gce1 and CD73) and then incubated with various inhibitors of ROS production. After exposure to glimepiride or palmitate, LD were prepared and then used for the affinity purification of Gce1 and CD73. As revealed by the phosphorimages from SDS-PAGE, the amounts of LD-associated [^{14}C]inositol-labelled Gce1 and CD73 were considerably higher in glimepiride- and palmitate-treated, compared with basal adipocytes (Figure 5). These increases were significantly reduced in the presence of inhibitors of NADPH oxidase, *N*-acetyl-cysteine, DPI and apocynin, but remained almost unaffected by rotenone, TTFA, antimycin A, L-NAME and oxpurinol.

The sulphonylurea-dependent GPI-PLC and NADPH oxidase are colocalized at hcDIGs

Our findings that the induction of the translocation of Gce1 and CD73 to LD by glimepiride depends on the NADPH oxidase-dependent activation of the GPI-PLC raised the possibility of a causal relationship between the two plasma membrane enzymes in rat adipocytes. As the first evidence for the mechanistic coupling of NADPH oxidase and GPI-PLC, their putative colocalization at plasma membrane DIGs of basal adipocytes was studied (Figure 6). Both the GPI-2350-sensitive GPI-PLC and the DPI-sensitive NADPH oxidase activity were found to be significantly enriched compared with plasma membranes at hcDIGs and lcDIGs but not non-DIG plasma membranes. The specific enrichments were higher for hcDIGs than for lcDIGs and comparable for

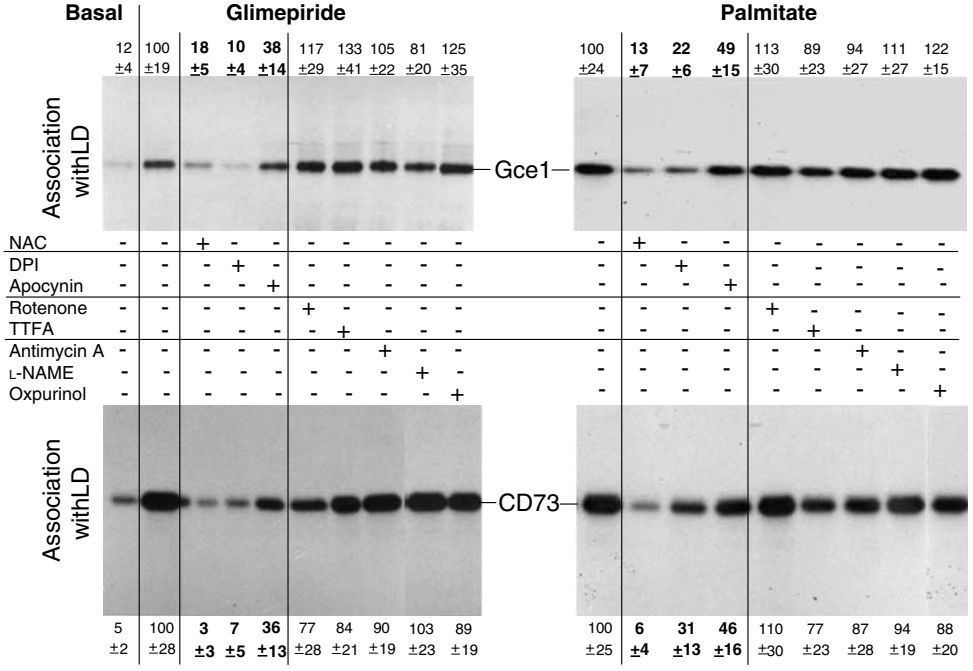

Figure 5 Effect of the inhibition of reactive oxygen species (ROS) production on the insulin-, palmitate- and glimepiride-induced translocation of glycolipid-anchored cAMP-binding ectoprotein-1 (Gce1) and CD73 to lipid droplet (LD). Isolated rat adipocytes were metabolically labelled with *myo*-[^{14}C]inositol and then incubated (5 min, 37 °C) with 2 mM glucose in the absence or presence of *N*-acetyl-cysteine (NAC; 0.5 mM), diphenylene iodonium (DPI; 20 μM), apocynin (100 μM), rotenone (1 μM), thenoyltrifluoroacetone (TTFA; 10 μM), antimycin A (10 μM), N^{ω}-nitro-L-arginine methyl ester (L-NAME; 25 μM) and oxpurinol (100 μM) prior to the addition of insulin (5 nM final concentration), palmitate (1 mM) or glimepiride (10 μM) as indicated. Thereafter, the adipocytes were separated from the medium. Proteins were extracted from the prepared LD and precipitated under native conditions. Solubilized glycolipid-anchored cAMP-binding ectoprotein-1 (Gce1) and CD73 were affinity purified, then precipitated under denaturing conditions and after solubilization analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and phosphorimaging. Quantitative evaluation is given with the amount of LD-associated Gce1 and CD73 for each stimulus in the absence of inhibitors set at 100. Means ± s.d. of two adipocyte preparations with incubations in triplicate; bold numbers indicate significant difference compared with the corresponding induction in the absence of inhibitor (set at 100).

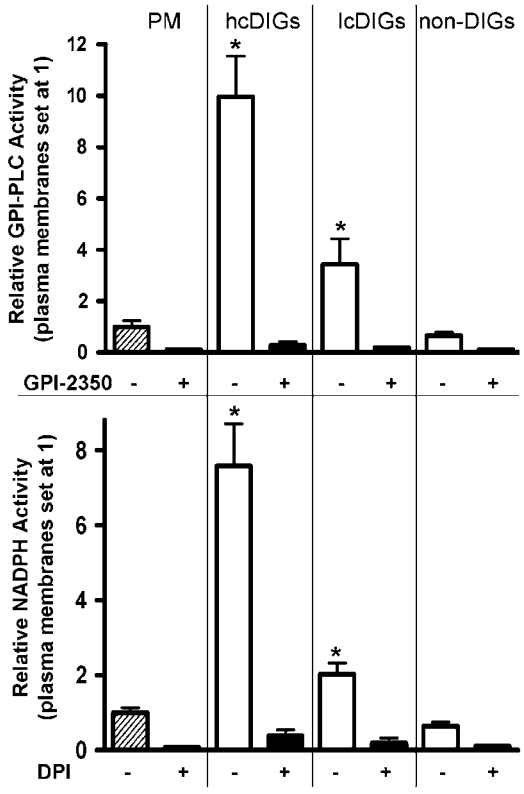

Figure 6 Distribution of the GPI-specific phospholipase-C (GPI-PLC) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activities between high-cholesterol-containing detergent-insoluble glycolipid-containing membrane rafts (hcDIGs), low-cholesterol-containing detergent-insoluble glycolipid-containing membrane rafts (lcDIGs) and non-DIG plasma membranes in rat adipocytes. Plasma membranes (PM), hcDIGs, lcDIGs and non-DIG plasma membranes were prepared from isolated rat adipocytes and then assayed for GPI-PLC and NADPH activities in the absence or presence of GPI-2350 (50 μM) and diphenylene iodonium (DPI; 20 μM) as indicated. Relative specific activities of the GPI-PLC and NADPH oxidase measured with the PM in the absence of inhibitors were set at 1 each. Means ± s.d. of three adipocyte preparations with activity determinations in quadruplicate; *indicates significant difference compared with the PM in the absence of inhibitors (hatched bars). GPI, glycosylphosphatidylinositol.

both activities. This argues for colocalization of the NADPH oxidase and the GPI-PLC at hcDIGs of the adipocyte plasma membrane.

Upon challenge of the adipocytes with glimepiride, both the DPI-sensitive NADPH oxidase (Figure 7a) and the GPI-2350-sensitive GPI-PLC (Figure 7b) activity at hcDIGs increased in concentration-dependent fashion to up to 11.5- and 5.9-fold, respectively, above basal with similar EC_{50} values (11.5 and 8.1 μM, respectively). Glibenclamide and the glimepiride metabolite, M1, were considerably less potent as revealed by their higher EC_{50} values and lower maximal responses (Figure 7). Tolbutamide was almost ineffective. The identical ranking of the various sulphonylureas in stimulating the NADPH oxidase and GPI-PLC at hcDIGs of the adipocyte plasma membrane are in agreement with their mechanistic coupling, which may rely on their colocalization at hcDIGs. Insulin stimulation of the NADPH oxidase and GPI-PLC was only moderate (Figure 7) making a major role of these enzymes in metabolic insulin signalling unlikely.

ROS production is required for the inhibition of lipolysis by glimepiride and palmitate

The apparent involvement of ROS in the glimepiride- and palmitate-induced GPI-PLC stimulation and GPI-protein translocation predicts a role for ROS in the inhibition of lipolysis by glimepiride and palmitate in adipocytes too. To study this, isolated rat adipocytes were incubated with various inhibitors of ROS production prior to challenge with glimepiride or palmitate and then assayed for isoprenaline-stimulated glycerol and fatty acid release (Figure 8). The about half-maximal inhibition of lipolysis by glimepiride and palmitate was almost completely abolished by DPI, apocynin and vanillylnonanamide. Their specificity for inhibition of ROS production rather than interference with the regulation of lipolysis *per se* was confirmed by their failure to counteract the potent lipolysis inhibition exerted by GO (data not shown). Rotenone, antimycin A, TTFA, oxpurinol and L-NAME did not impair lipolysis inhibition by glimepiride and palmitate. In contrast, scavenging or degradation of ROS by *N*-acetyl-cysteine or catalase, respectively, completely blocked the anti-lipolytic activity of glimepiride and palmitate as well as of GO (data not shown). Remarkably, each of these inhibitors of ROS production had no significant effect on the almost complete inhibition of the isoprenaline-induced lipolysis by insulin (100 ± 8% in the absence of inhibitors vs 93 ± 5% after DPI, 87 ± 6% after apocynin, 95 ± 8 after vanillylnonamide, 79 ± 9 after *N*-acetyl-cysteine and 82 ± 6% after catalase). These data suggest that lipolysis inhibition by glimepiride and palmitate is mediated by ROS, which is produced predominantly by the plasma membrane NADPH oxidase. In contrast, lipolysis inhibition by insulin does not depend on ROS production.

Discussion and conclusions

Stimulation of localized ROS production by insulin, sulphonylureas and fatty acids

Previous studies indicated that ROS, such as H_2O_2, are generated transiently in mature and differentiated 3T3-L1 adipocytes in response to insulin (Krieger-Brauer and Kather, 1992; Mahadev *et al*., 2004; Goldstein *et al*., 2005). The enzyme responsible is assumed to be one of the plasma membrane NADPH oxidase (Nox) homologues (Mahadev *et al*., 2004; Park *et al*., 2005; Quinn *et al*., 2006). However, the mechanism of its coupling to the insulin receptor remain ill defined. Recently, the sulphonylurea drug, glibenclamide, has been found to stimulate ROS production in cultured MIN6 β-cells, most likely via the PKC-triggered activation of Nox (Tsubouchi *et al*., 2005). For other cell types, inhibition of carnitine palmitoyltransferase-1 and mitochondrial fatty acid oxidation by glibenclamide has been reported (Lehtihet *et al*., 2003), which may lead to enhanced formation of diacylglycerol and in turn activation of PKC. Palmitate (0.1 mM) has previously been shown to increase the

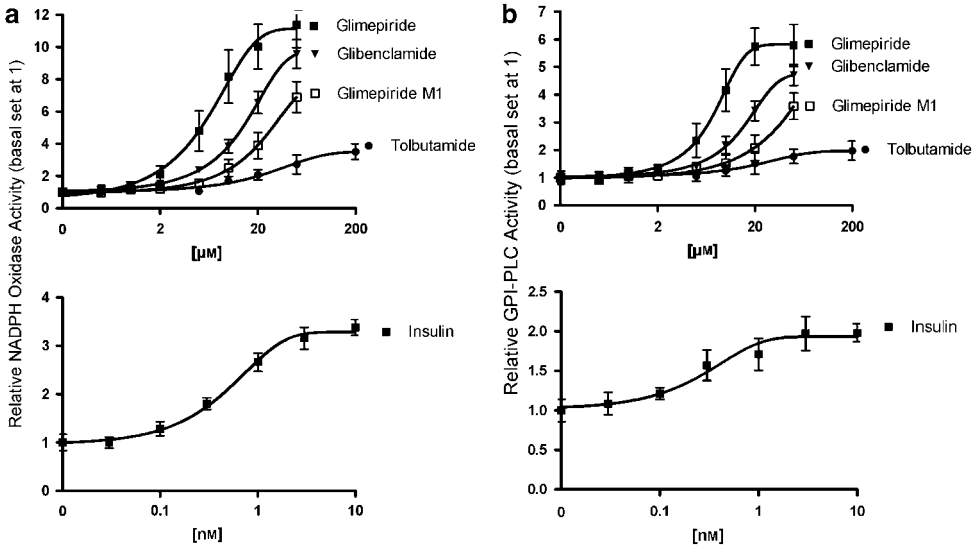

Figure 7 Effect of insulin and various sulphonylureas on the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and GPI-specific phospholipase-C (GPI-PLC) activities in high-cholesterol-containing detergent-insoluble glycolipid-containing membrane rafts (hcDIGs) of rat adipocytes. Isolated rat adipocytes were incubated (20 min, 37 °C) in the absence or presence of increasing concentrations of various sulphonylureas as indicated with 2 mM glucose as described in Materials and methods. hcDIGs were prepared and assayed for the NADPH oxidase (**a**) and GPI-PLC (**b**) activities in the absence or presence of diphenylene iodonium (DPI; 20 μM) and GPI-2350 (50 μM), respectively. Relative NADPH oxidase and GPI-PLC activities were calculated as the difference between the absence and presence of DPI or GPI-2350, respectively. Relative activities in the absence of stimulus were set at 1. Means ± s.d. of two adipocyte preparations with three incubations each and activity determinations in quadruplicate. GPI, glycosylphosphatidylinositol.

expression and activity of a DPI-sensitive Nox in rat pancreatic islets and a clonal β-cell line apparently during palmitate-driven diacylglycerol *de novo* synthesis and PKC activation (Morgan *et al*., 2007). PKC-dependent phosphorylation and activation of a Nox has previously been demonstrated (Kadri-Hassani *et al*., 1995; Bedard and Krause, 2007). Alternatively, coupling of the insulin-stimulated plasma membrane Nox to the *Pertussis* toxin-sensitive $G_{\alpha i2}$ has been described (Krieger-Brauer and Kather, 1992, 1995). It remains to be elucidated whether one of these mechanisms and which Nox homologue is involved in the upregulation of ROS production by insulin and sulphonylurea drugs and, in part, by fatty acids in rat adipocytes as revealed in the present study (Figures 1–3).

Nicotinamide adenine dinucleotide phosphate oxidases are targeted to specific subcellular sites, which is required for localized ROS production and temporally and spatially controlled activation of specific redox-signalling pathways. Some Nox subunits have been shown to be included in DIGs in various cell types (Li and Shah, 2002; Hilenski *et al*., 2004; Vilhardt and van Deurs, 2004). Furthermore, ROS production was severely compromised in intact cells or in a cell-free reconstituted system following cholesterol depletion (Vilhardt and van Deurs, 2004). This is compatible with the present findings that the glimepiride-dependent Nox (and GPI-PLC) activity is located at DIGs (Figure 6) and gets destroyed upon disruption of the DIGs by cholesterol depletion (G Müller and S Wied, unpublished data). The apparent association of Nox proteins with DIGs may explain why H_2O_2, which is assumed to be produced at the extracellular face of the plasma membrane, can affect intracellular targets. Plasma membrane DIGs may continue to produce H_2O_2, after internalization as caveolar vesicles, which then leaves the lumen of the caveolae into the cytoplasm by diffusion.

The Nox-dependent ROS production may be supported by impaired ROS degradation. Interestingly, phosphoinositolglycans prepared from plants and bacteria have been shown to inhibit the peroxidase and haeme-containing catalase in non-competitive fashion (Thomasz *et al*., 2007). Strikingly, similar phosphoinositolglycan structures may be released from the GPI anchors of GPI-proteins, such as Gce1 and CD73, upon lipolytic cleavage by the GPI-PLC (Stralfors, 1997; Jones and Varela-Nieto, 1998), which is apparently activated by palmitate, glimepiride and H_2O_2 (Müller *et al*., 1993, 1994a, 2008c; Movahedi and Hooper, 1997; see Figure 4). This would open the possibility for a feed-forward cycle in which ROS would dampen their own degradation via activation of the GPI-PLC and inhibition of peroxidases or catalases through the generated phosphoinositolglycans, thereby further amplifying the concentration of ROS.

Activation of the GPI-PLC and GPI-protein translocation by ROS, sulphonylureas and fatty acids

The present study suggests that in rat adipocytes, ROS, in particular H_2O_2, mediates the activation of the GPI-PLC and, in consequence, of the GPI-protein translocation. First, the reduction of ROS levels by inhibition of Nox as well as scavenging or degradation of ROS (Figure 3) is accompanied by impaired cleavage of the GPI anchors of Gce1 and CD73 by the GPI-PLC (Figure 4) and translocation of the GPI-proteins, Gce1 and CD73, from hcDIGs to LD (Figure 5) in response to palmitate and glimepiride. Second, Nox and GPI-PLC are colocalized at hcDIGs of the adipocyte plasma membrane (Figure 6), and, third, stimulated by different

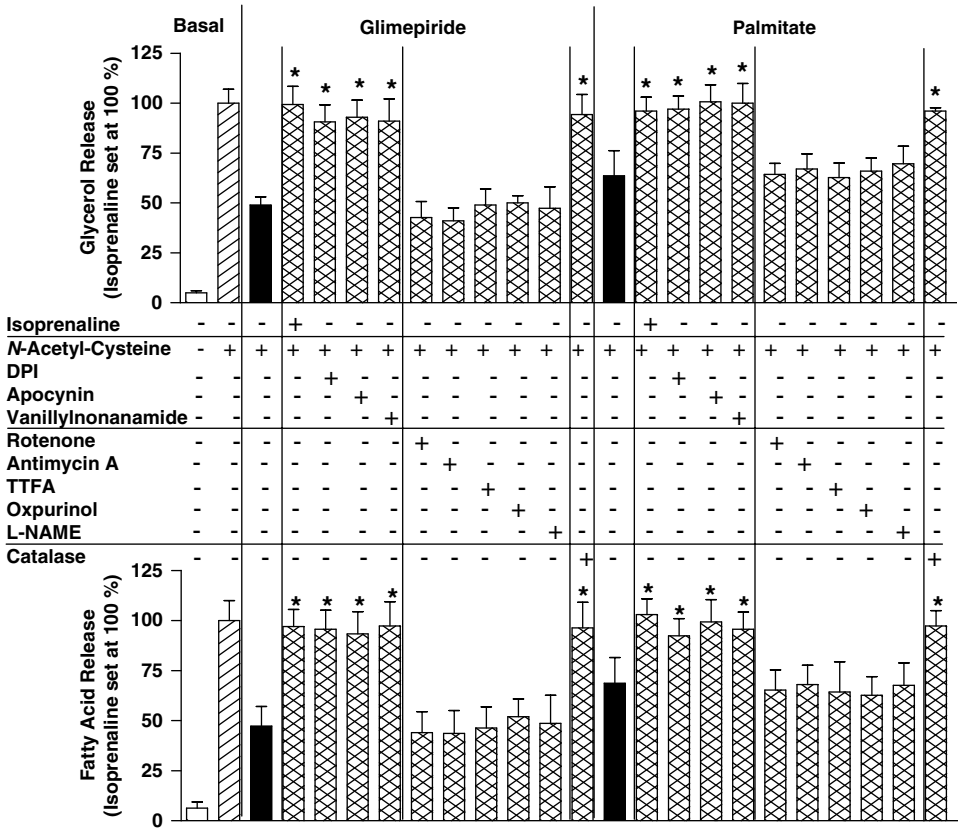

Figure 8 Effect of the inhibition of reactive oxygen species (ROS) production on the inhibition of lipolysis by palmitate and glimepiride. Isolated rat adipocytes were incubated (5 min, 37 °C) with 2 mM glucose in the absence or presence of *N*-acetyl-cysteine (0.5 mM), diphenylene iodonium (DPI; 20 μM), apocynin (100 μM), vanillylnonanamide (100 μM), rotenone (1 μM), antimycin A (10 μM), thenoyltrifluoroacetone (TTFA; 10 μM), oxpurinol (100 μM), N^{ω}-nitro-L-arginine methyl ester (L-NAME; 25 μM) and catalase (0.5 U ml^{-1}) prior to the addition of palmitate (final concentration 1 mM) or glimepiride (10 μM) as indicated. After further incubation (5 min), isoprenaline (final concentration 1 μM) was added and the incubation continued (2 h). Thereafter, the medium was separated from the adipocytes and analysed for the glycerol and fatty acids released. Isoprenaline-induced glycerol and fatty acid release in the absence of stimulus or inhibitor was set at 100% (hatched bars). Means ± s.d. of three adipocyte preparations with incubations in duplicate; *indicates significant difference compared with the corresponding stimulus in the absence of inhibitor (filled bars).

sulphonylureas with similar sensitivity and responsiveness (Figure 7). Thus, upregulation of the GPI-PLC and GPI-protein translocation upon challenge of isolated rat adipocytes with palmitate and glimepiride depends on H_2O_2 production by Nox located at the plasma membrane. ROS produced by mitochondrial complexes I and III in palmitate- and insulin-treated adipocytes (Figure 3) do not contribute significantly to stimulation of the GPI-PLC and GPI-protein translocation. Apparently, mitochondrial and plasma membrane ROS do not exchange to equilibrium but remain compartmentalized in rat adipocytes. Remarkably, even though DPI and apocynin reduced the glimepiride- and palmitate-induced ROS production to similar extent (Figure 3), DPI was more potent than apocynin in inhibiting the corresponding activation of the GPI-PLC (Figure 4) as well as the translocation of Gce1 and CD73 to LD (Figure 5). Control experiments demonstrated that the simultaneous generation of H_2O_2 by GO completely abolished the DPI inhibition, whereas the simultaneous presence of catalase or *N*-acetyl-cysteine enhanced the apocynin inhibition to the extent observed with DPI alone (data not shown). These findings exclude additional ROS-independent and nonspecific effects of DPI on the mechanisms of GPI-PLC activation and GPI-protein translocation. The reason for the apparently different downstream efficacies of the two non-selective Nox inhibitors remains unclear. It could be related to interference of DPI with another production site of compartmentalized ROS, which contribute to the GPI-PLC activation and GPI-protein translocation to a limited degree, but do not equilibrate with total cellular ROS and thereby escape assaying.

The molecular mechanism(s) of downstream signalling of ROS remain(s) a matter of speculation at present, but may be related to the regulation of oxidation-sensitive enzymes, such as transcription factors and signalling proteins (Goldstein *et al*., 2005). An increasing number of protein tyrosine phosphatases has been shown to be regulated by oxidative modification, owing to their invariant catalytic cysteine, such as PTP1B in response to insulin (Meng *et al*., 2002, 2004). It remains to be investigated whether activation of the GPI-PLC by ROS involves an oxidative mechanism, which would require oxidized cysteine thiol side chains being essential for the catalytic activity of the GPI-PLC.

Inhibition of lipolysis by palmitate, glimepiride and localized ROS production

The present finding that blockade of the plasma membrane Nox leads to complete or partial abolition of the lipolysis inhibition by glimepiride and palmitate, respectively, in rat adipocytes (Figure 8) can be reconciled together with previously published observations (Müller *et al*., 2008a, b, c) into a unifying hypothesis about the molecular mechanism underlying the anti-lipolytic activity of agents structurally as diverse as fatty acids, sulphonylureas and H_2O_2. The common mediator seems to be ROS, in particular H_2O_2, which is produced by Nox in response to palmitate and glimepiride. ROS activate the GPI-PLC, presumably favoured by its colocalization with Nox in plasma membrane hcDIGs. Activated GPI-PLC then triggers the translocation of the GPI-proteins, Gce1 and CD73, from hcDIGs to the surface of cytosolic LD leading to cAMP degradation on the basis of their intrinsic cAMP-specific phosphodiesterase and 5′-nucleotidase activities. Lowering of the cAMP concentration in the vicinity of the LD may lead to unphosphorylated neutral lipases and LD coat proteins (Müller and Petry, 2005). Thereby, upregulation of lipolysis by cAMP-raising stimuli, such as isoprenaline, adenosine deaminase and forskolin, is blocked. Remarkably, although apocynin turned out to be less efficient than DPI in reducing the glimepiride- and palmitate-induced GPI-PLC activation (Figure 4) as well as GPI-protein translocation (Figure 5), both Nox inhibitors reversed the lipolysis inhibition by glimepiride and palmitate to 90–95% (Figure 8). This suggests that partial (that is, 40% compared with maximal) upregulation of the expression of Gce1 and CD73, and, thus, presumably of the accompanying (c)AMP degradation activity at LD is not sufficient for significant downregulation of the isoprenaline-stimulated lipolysis in rat adipocytes.

A number of different molecular mechanisms for lipolysis inhibition by fatty acids and H_2O_2 in adipocytes have been proposed. Prolonged elevation of β-oxidation of fatty acids might increase the generation of H_2O_2 that activates mitochondrial uncoupling proteins or increases the oxidation state of mitochondrial pyridine nucleotides. The resulting decrease in ATP synthesis and levels (Vallano *et al*., 1983) may interfere with ATP-dependent lipolytic mechanisms, such as the phosphorylation and activation of neutral lipases and LD coat proteins. However, blockade of the isoprenaline- and adenosine deaminase-induced lipolysis has still been observed by using the non-metabolized fatty acid analogue, bromopalmitate or palmitate in the presence of the fatty acid oxidation inhibitor, etomoxir (Spurway *et al*., 1997). This suggests that the anti-lipolytic activity of fatty acids does not depend on their β-oxidation. Alternatively, palmitate has been shown to directly inhibit the isolated hormone-sensitive lipase (Jepson and Yeaman, 1992). However, this putative feedback mechanism has been observed only at high concentrations (exceeding those used in the present study) and seems unlikely to operate in intact cells. The anti-lipolytic activity of H_2O_2 has previously been explained by the direct covalent inhibition of PTP1b, which dephosphorylates and inactivates the insulin receptor (Mahadev *et al*., 2001; Meng *et al*., 2002, 2004). This mechanism should result in the potentiation of insulin inhibition of lipolysis only and be ineffective in the absence of insulin. However, as shown in this study, the anti-lipolytic activity of palmitate and glimepiride does not depend on insulin.

It seems to be paradoxical that localized and transient production of ROS can mediate beneficial effects, whereas chronically elevated levels of total cytoplasmic superoxide and H_2O_2 have been assumed to mediate ageing processes (Frisard and Ravussin, 2006; Giorgio *et al*., 2007) and, if associated with hyperglycaemia, to have an important pathophysiological role in the development of type II diabetes including vascular-associated pathologies (Brownlee, 2005). However, numerous studies support the hypothesis that cellular signalling via ROS is mediated by discrete localized redox circuitries (Terada, 2006; Ushio-Fukai, 2006; Zhang *et al*., 2006), which are distinct from the general 'oxidative stress' effect (Li and Shah, 2002; Hilenski *et al*., 2004; Zhang *et al*., 2006; Morgan *et al*., 2007). The repeatedly reported insulin-induced transient increase in ROS production in various insulin target tissues (Krieger-Brauer and Kather, 1992, 1995), including adipocytes as shown here (Figure 1), further supports the hypothesis that cellular ROS may exert beneficial and detrimental effects depending on the cell type and the sites and time courses of their generation and destruction. Thus, localizing the ROS signal at distinct subcellular compartments, such as at the DIGs, for a limited period of time, such as during acute glimepiride administration, in adipocytes, which harbour the complete machinery for the translocation of the (c)AMP-degrading enzymes, Gce1 and CD73, to LD including the ROS-stimulated GPI-PLC, may be essential for some of the specific and ROS-mediated beneficial downstream signalling processes in these cells, such as the inhibition of lipolysis. Interestingly, preliminary results indicate that glimepiride fails to augment ROS production in cultured human vascular endothelial cells (N Schughardt and G Müller, unpublished data). In light of the assumed detrimental roles of ROS in vascular pathology, the apparent cell type-specific regulation of the Nox isoforms may be of advantage for the glimepiride therapy. Future animal studies have to address the potential involvement of up- or downregulated ROS production at DIGs and other production sites in adipocytes and other cell types in hyperlipidaemic and hyperglycaemic states as well as during their therapy, in general. It is tempting to speculate that the adipocyte-specific ROS production at DIGs with the resulting GPI-PLC activation and GPI-protein translocation forms the molecular basis for some of the demonstrated advantages of the third-generation sulphonylurea drug, glimepiride, compared with sulphonylureas of the first and second generation in the therapy of diabetes (Davis, 2004; Korytkowski, 2004; Müller, 2005; Koshiba *et al*., 2006; Matsuki *et al*., 2007).

Conflict of interest

The authors are employees of Sanofi-Aventis Pharma.

References

Bedard K, Krause KH (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* **87**: 245–313.

Bellin C, de Wiza DH, Wiernsperger NF, Rösen P (2006). Generation of reactive oxygen species by endothelial and smooth muscle cells: influence of hyperglycemia and metformin. *Horm Metab Res* **38**: 732–739.

Brownlee M (2005). The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* **54**: 1615–1625.

Davis SN (2004). The role of glimepiride in the effective management of type 2 diabetes. *J Diabetes Complications* **18**: 367–376.

Frisard M, Ravussin E (2006). Energy metabolism and oxidative stress: impact on the metabolic syndrome and the ageing process. *Endocrine J* **29**: 27–32.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y *et al*. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* **12**: 1752–1761.

Giorgio M, Trinei M, Migliaccio E, Pelicci PG (2007). Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nature Rev* **8**: 722–728.

Goldstein BJ, Mahadev K, Wu X (2005). Redox paradox: insulin action is facilitated by insulin-stimulated reactive oxygen species with multiple potential signalling targets. *Diabetes* **54**: 311–321.

Guo W, Xie W, Han J (2006). Modulation of adipocyte lipogenesis by octanoate: involvement of reactive oxygen species. *Nutr Metab (Lond)* **3**: 30.

Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK (2004). Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **24**: 677–683.

Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M *et al*. (2000). High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* **49**: 1939–1945.

Jepson CA, Yeaman SJ (1992). Inhibition of hormone-sensitive lipase by intermediary lipid metabolites. *FEBS Lett* **310**: 197–200.

Jones DR, Varela-Nieto I (1998). Diabetes and the role of inositol-containing lipids in insulin signaling. *Mol Med* **5**: 505–514.

Kadri-Hassani N, Leger CL, Descomps B (1995). The fatty acid bimodal action on superoxide anion production by human adherent monocytes under phorbol 12-myristate 13-acetate or diacylglycerol activation can be explained by the modulation of protein kinase C and p47phox translocation. *J Biol Chem* **270**: 15111–15118.

Kather H, Wieland E, Waas W (1987). Chemiluminescent determination of adenosine, inosine, and hypoxanthine xanthine. *Anal Biochem* **163**: 45–51.

Korytkowski MT (2004). Sulfonylurea treatment of type 2 diabetes mellitus: focus on glimepiride. *Pharmacotherapy* **24**: 606–620.

Koshiba K, Nomura M, Nakaya Y, Ito S (2006). Efficacy of glimepiride on insulin resistance, adipocytokines, and atherosclerosis. *J Med Invest* **53**: 87–94.

Krieger-Brauer HI, Kather H (1992). Human fat cells possess a plasma membrane-bound H_2O_2-generating system that is activated by insulin via a mechanism bypassing the receptor kinase. *J Clin Invest* **89**: 1006–1013.

Krieger-Brauer HI, Kather H (1995). Antagonistic effects of different members of the fibroblast and platelet-derived growth factor families on adipocyte conversion and NADPH-dependent H_2O_2 generation in 3T3-L1 cells. *Biochem J* **307**: 549–556.

Lehtihet M, Welsh N, Berggren PO, Cook GA, Sjoholm A (2003). Glibenclamide inhibits islet carnitine palmitoyltransferase 1 activity, leading to PKC-dependent insulin exocytosis. *Am J Physiol Endocrinol Metab* **285**: E438–E446.

Lei T, Xie W, Han J, Corkey BE, Hamilton JA, Guo W (2004). Medium-chain fatty acids attenuate agonist-stimulated lipolysis, mimicking the effects of starvation. *Obesity Res* **12**: 599–611.

Li JM, Shah AM (2002). Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. *J Biol Chem* **277**: 19952–19960.

Little SA, de Haen C (1980). Effects of hydrogen peroxide on basal and hormone-stimulated lipolysis in perifused rat fat cells in relation to the mechanism of action of insulin. *J Biol Chem* **255**: 10888–10895.

Mahadev K, Motoshima H, Wu X, Ruddy JM, Arnold RS, Cheng G *et al*. (2004). The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H_2O_2 and plays an integral role in insulin signal transduction. *Mol Cell Biol* **24**: 1844–1854.

Mahadev K, Wu X, Zilbering A, Zhu L, Lawrence JT, Goldstein BJ (2001). Hydrogen peroxide generated during cellular insulin stimulation is integral to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes. *J Biol Chem* **276**: 48662–48669.

Maragno I, Dorigo P, Fassina G (1971). Effect of antimycin A on hormone-stimulated lipolysis *in vitro*. *Biochem Pharmacol* **20**: 2149–2154.

Matsuki M, Matsuda M, Kohara K, Shimoda M, Kanda Y, Tawaramoto K *et al*. (2007). Pharmacokinetics and pharmacodynamics of glimepiride in type 2 diabetic patients: compared effects of once- versus twice-daily dosing. *Endocrine J* **54**: 571–576.

Meng TC, Buckley DA, Galic S, Tiganis T, Tonks NK (2004). Regulation of insulin signaling through reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B. *J Biol Chem* **279**: 37716–37725.

Meng TC, Fukada T, Tonks NK (2002). Reversible oxidation and inactivation of protein tyrosine phosphatases *in vivo*. *Mol Cell* **9**: 387–399.

Morgan D, Oliveira-Emilio HR, Keane D, Hirata AE, Santos da Rocha M, Bordin S *et al*. (2007). Glucose, palmitate and pro-inflammatory cytokines modulate production and activity of a phagocyte-like NADPH oxidase in rat pancreatic islets and a clonal beta cell line. *Diabetologia* **50**: 359–369.

Movahedi S, Hooper N (1997). Insulin stimulates the release of the glycosyl phosphatidylinositol-anchored membrane dipeptidase from 3T3-L1 adipocytes through the action of a phospholipase C. *Biochem J* **326**: 531–537.

Muchmore DB, Little SA, de Haen C (1982). Counterregulatory control of intracellular hydrogen peroxide production by insulin and lipolytic hormones in isolated rat epididymal fat cells: a role of free fatty acids. *Biochemistry* **21**: 3886–3892.

Mukherjee SP (1980). Mediation of the antilipolytic and lipogenic effects of insulin in adipocytes by intracellular accumulation of hydrogen peroxide. *Biochem Pharmacol* **29**: 1239–1246.

Müller G (2005). The mode of action of glimepiride—beyond insulin secretion. *Curr Med Chem* **5**: 499–518.

Müller G, Dearey E-A, Korndörfer A, Bandlow W (1994a). Stimulation of a glycosyl phosphatidylinositol-specific phospholipase by insulin and the sulfonylurea, glimepiride, in rat adipocytes depends on increased glucose transport. *J Cell Biol* **126**: 1267–1276.

Müller G, Dearey E-A, Pünter J (1993). The sulphonylurea drug, glimepiride, stimulates release of glycosylphosphatidylinositol-anchored plasma-membrane proteins from 3T3 adipocytes. *Biochem J* **289**: 509–521.

Müller G, Hanekop N, Wied S, Frick W (2002). Cholesterol depletion blocks redistribution of lipid raft components and insulin-mimetic signaling by glimepiride and phosphoinositolglycans in rat adipocytes. *Mol Med* **8**: 120–136.

Müller G, Jordan H, Jung C, Kleine H, Petry S (2003). Analysis of lipolysis in adipocytes using a fluorescent fatty acid derivative. *Biochimie* **85**: 1245–1256.

Müller G, Jung C, Wied S, Welte S, Jordan H, Frick W (2001). Redistribution of glycolipid raft domain components induces insulin-mimetic signaling in rat adipocytes. *Mol Cell Biol* **21**: 4553–4567.

Müller G, Over S, Wied S, Frick W (2008a). Association of (c)AMP-degrading glycosylphosphatidylinositol-anchored proteins with lipid droplets is induced by palmitate, H_2O_2 and the sulfonylurea drug, glimepiride, in rat adipocytes. *Biochemistry* **47**: 1274–1287.

Müller G, Petry S (2005). Triacylglycerol, storage and mobilization of human. In: Meyers A (ed). *Encyclopedia in Biochemistry and Molecular Biology*, vol 14. Wiley-VCH: Weinheim, Germany. pp 621–704.

Müller G, Schulz A, Wied S, Frick W (2005). Regulation of lipid raft proteins by glimepiride- and insulin-induced glycosylphosphati-

dylinositol-specific phospholipase C in rat adipocytes. *Biochem Pharmacol* **69**: 761–780.

Müller G, Wetekam E-M, Jung C, Bandlow W (1994b). Membrane association of lipoprotein lipase and a cAMP-binding ectoprotein in rat adipocytes. *Biochemistry* **33**: 12149–12159.

Müller G, Wied S, Over S, Frick W (2008b). Inhibition of lipolysis by palmitate, H_2O_2 and the sulfonylurea drug, glimepiride, in rat adipocytes depends on cAMP degradation by lipid droplets. *Biochemistry* **47**: 1259–1273.

Müller G, Wied S, Walz N, Jung C (2008c). Translocation of glycosylphosphatidylinositol-anchored proteins from plasma membrane microdomains to lipid droplets in rat adipocytes is induced by palmitate, H_2O_2 and the sulfonylurea drug, glimepiride. *Mol Pharmacol* **73**: 1513–1529.

Müller G, Wied S, Wetekam E-M, Crecelius A, Unkelbach A, Pünter J (1994c). Stimulation of glucose utilization in 3T3 adipocytes and rat diaphragm *in vitro* by the sulfonylureas, glimepiride and glibenclamide, is correlated with modulations of the cAMP regulatory cascade. *Biochem Pharmacol* **48**: 985–996.

Park HS, Jin DK, Shin SM, Jang MK, Longo N, Park JW *et al*. (2005). Impaired generation of reactive oxygen species in leprechaunism through downregulation of Nox4. *Diabetes* **54**: 3175–3181.

Quinn MT, Ammons MCB, DeLeo FR (2006). The expanding role of NADPH oxidases in health and disease. No longer just agents of death and destruction. *Clin Sci* **111**: 1–20.

Spurway TD, Pogson CI, Sherratt HS, Agius L (1997). Etomoxir, sodium 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate, inhibits triacylglycerol depletion in hepatocytes and lipolysis in adipocytes. *FEBS Lett* **404**: 111–114.

Stralfors P (1997). Insulin second messengers. *BioEssays* **19**: 327–335.

Talior I, Yarkoni M, Bashan N, Eldar-Finkelman H (2003). Increased glucose uptake promotes oxidative stress and PKC-δ activation in adipocytes of obese, insulin-resistant mice. *Am J Physiol Endocrinol Metab* **285**: E295–E302.

Terada LS (2006). Specificity in reactive oxidant signalling: think globally, act locally. *J Cell Biol* **174**: 615–623.

Thomasz L, Aran M, Pizarro RA, Ibanez J, Pisarev MA, Converso D *et al*. (2007). Inhibition of peroxidase and catalase activities and modulation of hydrogen peroxide level by inositol phosphoglycan-like compounds. *Horm Metab Res* **39**: 14–19.

Tsubouchi H, Inoguchi T, Inuo M, Kakimoto M, Sonta T, Sonoda N *et al*. (2005). Sulfonylurea as well as elevated glucose levels stimulate reactive oxygen species production in the pancreatic β-cell line, MIN6—a role of NAD(P)H oxidase in β-cells. *Biochem Biophys Res Commun* **326**: 60–65.

Ushio-Fukai M (2006). Localizing NADPH oxidase-derived ROS. *Sci STKE* **2006**: re8.

Vallano ML, Lee MY, Sonenberg M (1983). Hormones modulate adipocyte membrane potential, ATP and lipolysis by free fatty acids. *Am J Physiol* **245**: E266–E272.

Vilhardt F, van Deurs B (2004). The phagocyte NADPH oxidase depends on cholesterol-enriched membrane microdomains for assembly. *EMBO J* **23**: 739–748.

Wang H, Joseph JA (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic Biol Med* **27**: 612–616.

Yeaman SJ (2004). Hormone-sensitive lipase: new roles for an old enzyme. *Biochem J* **379**: 11–22.

Zhang AY, Yi F, Zhang G, Gulbins E, Li PL (2006). Lipid raft clustering and redox signalling platform formation in coronary arterial endothelial cells. *Hypertension* **47**: 74–80.