Eicosanoids participate in the regulation of cardiac glucose transport by contribution to a rearrangement of actin cytoskeletal elements

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Intact actin microfilaments are required for insulin-regulated glucose transporter isoform 4 (GLUT4) translocation to the plasma membrane. Lipoxygenase (LO) metabolites have recently been shown to contribute to the regulation of actin cytoskeleton rearrangement. In the present investigation, ventricular cardiomyocytes were used to study the effects of two structurally different LO inhibitors (esculetin and nordihydroguaiaretic acid) on insulin signalling events, glucose uptake, GLUT4 translocation and the actin network organization. Insulin stimulation increased glucose uptake 3-fold in control cells, whereas LO inhibition completely blocked this effect. This was paralleled by a slight reduction in the insulin-induced tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2. However, inhibition of 12-LO did not affect the association of phosphatidylinositol 3-kinase with IRS-1 and the phosphorylation of Akt/protein kinase B in response to insulin. Addition of 12(S)-hydroxyeicosatetraenoic acid almost completely restored the insulin action in cells exposed to nordihydroguaiaretic acid. Insulin stimulation increased cell surface GLUT4 2-fold in control cells, whereas LO inhibition abrogated the insulin-stimulated GLUT4 translocation. LO inhibition induced a prominent disassembly of actin fibres compared with control cells. In conclusion, we show here that 12(S)-hydroxyeicosatetraenoic acid plays a role in the organization of the actin network in cardiomyocytes. LO inhibition blocks GLUT4 translocation without affecting downstream insulin signalling. These data suggest that LO metabolites participate in the regulation of glucose transport by contributing to a rearrangement of actin cytoskeletal elements.

Key words: cytoskeleton, GLUT4 translocation, heart, 12-lipoxygenase, 12(S)-hydroxyeicosatetraenoic acid.

INTRODUCTION

Insulin rapidly stimulates glucose transport primarily by inducing the translocation of vesicles containing the glucose transporter isoform 4 (GLUT4) from intracellular pools to the plasma membrane (for reviews, see [1,2]). Major aspects of this part of insulin action that still remain to be elucidated relate to downstream signalling events from the insulin receptor to GLUT4-containing vesicles, but also to the mechanisms leading to the recruitment of GLUT4 vesicles to the plasma membrane in response to the hormone. Recent investigations have focused on the involvement of actin cytoskeletal elements in mediating the insulin-induced GLUT4 translocation. It has been demonstrated in both adipocytes and L6 myotubes that an intact actin network is essential for insulin-induced glucose uptake and exocytosis of GLUT4 vesicles to the plasma membrane [3–6]. However, the mechanisms of actin network rearrangement, leading to insulin-induced GLUT4 translocation, still remain undefined.

Recent studies have focused on the involvement of hydroxyeicosatetraenoic acid (HETE) in the regulation of actin network organization. These eicosanoid metabolites of the lipoxygenase (LO) pathway have been shown to bind selectively to actin fibres [7,8]. Other groups have demonstrated that 12(S)-HETE, the product of 12-lipoxygenation of arachidonic acid, induces the phosphorylation of actin fibres, leading to an increased actin filament content and enhancement of actin polymerization [9–11]. LOs are known to form a widely distributed family of lipid peroxidizing enzymes that insert molecular oxygen stereospecifically into polyunsaturated fatty acids, such as arachidonic acid or linoleic acid [12]. Increasing numbers of certain mammalian LO isoforms (5-, 12- or 15-LO) have been identified in various tissues [13,14], but, for most of them, the biological function and significance remain unclear [12,14,15]. Sigal and co-workers [13] have cloned and characterized a 12-LO isoform from cardiac muscle, and 12(S)-HETE was found to be the main LO metabolite in ventricular cardiomyocytes [16]. This isoform has also been described in a variety of rat, mouse and human tissues [17].

The potential involvement of this eicosanoid in the organization of the actin network in the cardiomyocyte has not been investigated to date, although 12(S)-HETE has been shown to play an important role in the cardioprotective effect of ischaemic preconditioning [18–20]. In the present study we have used our well characterized adult cardiomyocyte system [21–24] to (1) assess the possible contribution of the 12-LO pathway in the rearrangement of actin cytoskeletal elements and (2) determine its potential implications for insulin-regulated trafficking of GLUT4. The results suggest that the 12-LO pathway participates

Abbreviations used: Cy3, indocarbocyanine; 2-DOG, 2-deoxy-D-glucose; ECL® (Amersham), enhanced chemiluminescence; FITC, fluorescein isothiocyanate; GLUT4, glucose transporter isofom 4; HETE, hydroxyeicosatetraenoic acid; IRS, insulin receptor substrate; NHS-LC, *N*-hydroxysuccinimido-long chain; LO, lipoxygenase; NDGA, nordihydroguaiaretic acid; PI 3-kinase, phosphatidylinositol 3-kinase.

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in the organization of the actin cytoskeletal network, and thus, may operate as an important determinant in the insulin-sensitive cardiac glucose uptake system.

MATERIALS AND METHODS

Materials

2-Deoxy-D[1-^{14}C]glucose (2-DOG), 3-*O*-[^{14}C]methyl-D-glucose and L-[^{14}C]glucose were obtained from Amersham Pharmacia Biotech (Braunschweig, Germany). Anti-rabbit horseradish peroxidase-conjugated IgG, used as the secondary antibody for enhanced chemiluminescence (ECL®; Amersham), was purchased from Promega (Mannheim, Germany). Indocarbocyanine (Cy3)-conjugated AffiniPure® goat anti-rabbit IgG (H + L) was from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). The polyclonal antiserum against GLUT4 was obtained from a rabbit injected with a peptide corresponding to the C-terminal 12 amino acids of GLUT4 coupled to keyhole limpet haemocyanin (Eurogentec, Serain, Belgium). Polyclonal anti-[insulin receptor substrate (IRS)-1] and anti-(IRS-2) antibodies were kindly provided by Dr. Antonie Maassen (Leiden, The Netherlands). Anti-phosphothreonine-308 Akt and anti-Akt antibodies were supplied by New England Biolabs (Beverly, MA, U.S.A.). Anti-(12-LO) antibody and 12(S)-HETE were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Phosphotyrosine antibody was obtained from Transduction Labs (Lexington, KY, U.S.A.). Sulpho-*N*-hydroxysuccinimido-long chain (NHS-LC)-biotin and streptavidin-agarose beads were supplied by Pierce (Rockford, IL, U.S.A.). Fluorescein isothiocyanate (FITC)–phalloidin, the LO inhibitors esculetin and nordihydroguaiaretic acid (NDGA) and all other chemicals were of the highest analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.).

Isolation and culture of ventricular cardiomyocytes

Male Wistar rats weighing 260–310 g were used throughout the experiments. Ca^{2+}-tolerant myocytes were isolated by perfusion of the heart with collagenase and either used immediately, or kept in primary culture, as previously described [25]. The final cell suspension was washed three times with Hepes buffer (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25 mM Hepes, 5 mM glucose, 20 g/l BSA, pH 7.4, equilibrated with O_2) and incubated in silicone-treated Erlenmeyer flasks in a rotating shaking water bath at 37 °C. After 20 min, $CaCl_2$ and $MgSO_4$ (1 mM final concentration) were added, and incubation was continued for at least 60 min until further use. Cell viability was checked by determination of the percentage of rod-shaped cells and averaged 90–95 % under all incubation conditions. For overnight primary culture of cardiomyocytes, culture flasks were precoated with laminin (approx. 2 μg/cm^2) for at least 20 min before seeding.

Immunostaining

Cells grown on coverslips were briefly rinsed twice with PBS. After fixation in 4 % paraformaldehyde in PBS for 10 min at 20 °C, the cells were covered with 0.1 % Triton X-100 in PBS for 10 min, washed with PBS and covered with 1 % BSA in PBS for 1 h. For double staining of 12-LO and actin fibres, cells were then incubated for 16 h at 6 °C in a wet chamber with the primary anti-12-LO antibody (1:200 dilution) and FITC–phalloidin (1:500 dilution). PBS alone was applied for control staining. After rinsing and washing, the slides were incubated for 2 h with a Cy3-conjugated goat anti-rabbit IgG (1:500 dilution). For single staining of actin fibres, cells were incubated for 16 h at 6 °C in a wet chamber with FITC–phalloidin (1:500) alone. After two final washing steps, the cells were covered with fluorescence mounting medium (Dako, Hamburg, Germany) and a coverslip.

Confocal laser microscopy

Immunostained cardiomyocytes on coverslips were analysed using the Leica TCS-NT confocal laser scanning system with an argon–krypton laser on a Leica DM IRB inverted microscope. Images were acquired from two channels at 488 nm and 568 nm excitation wavelength. Emission was measured at 530 ± 10 nm (green) and > 590 nm (red).

Only cells prepared in parallel were compared using the same adjustments for all parameters (i.e. laser power, filter settings, setting of the acousto-optical tunable filter, pinhole, lens, voltages at the photo multiplier tubes, number of accumulated scans, format size and zoom, scan speed and z-step-size). Images were coded to avoid bias during image selection. Pictures of whole cells (containing green and/or red signals) were then calculated using the 'projection' function of the Leica software, whilst cell cuts were calculated by use of the 'section' function to give a single picture which was stored as TIFF file.

Assay of 3-*O*-methylglucose transport

Transport experiments were performed at 37 °C in Hepes buffer containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The reaction was started by adding a 50 μl aliquot of the cell suspension to 50 μl of Hepes buffer containing 3-*O*-[^{14}C]methyl-D-glucose (100 μM final concentration) for 10 s. The uptake reaction was terminated by the addition of 900 μl of ice-cold stop solution (38 μM cytochalasin B, 0.1 % ethanol and 150 mM NaCl). Two 300 μl aliquots of the resulting suspension were immediately transferred to precooled Microfuge tubes containing 100 μl of silicone oil (density 1.04) and centrifuged at 10000 ***g*** for 40 s in a Beckman Microfuge B. The tip of the tube was cut off and, after solubilization of the pellet, the radioactivity was determined by liquid scintillation counting. Carrier-mediated glucose transport was then determined using a 10 s assay period and L-[^{14}C]glucose in order to correct for simple diffusion, as previously described [21].

Assay of 2-DOG uptake

Determination of 2-DOG uptake was performed at 37 °C on monolayers of cells (2×10^5 cells per well) in serum-free culture medium containing 7.8 mM glucose as described previously [26,27]. 2-DOG (0.4 μCi per well, 7.3 μM final concentration) was added and hexose uptake assayed for 30 min. Uptake was stopped by aspirating the medium and washing the wells twice with ice-cold PBS containing 25 μM cytochalasin B. Cells were then dissolved in 1 M NaOH, neutralized with acetic acid and aliquots were taken for scintillation counting and protein determination. Carrier-independent uptake was determined in parallel incubations using L-[^{14}C]glucose (2–5 % of total uptake) and was subtracted from all determinations. Protein was determined using the Bio-Rad protein assay (Bio-Rad, Munich, Germany) with BSA as a standard.

Extraction of lipids

The extraction procedure follows that described by Powell [28] with some modifications. Cardiomyocytes maintained in primary culture were briefly rinsed three times with PBS at 20 °C and

collected after rapid trypsinization with 1 ml of trypsin/EDTA solution. Following cell detachment, 0.8 ml of extraction buffer (methanol containing 0.2 mM NaOH and 0.25 mM propyl gallate) was added to the culture plate. NaOH hydrolyses HETE esters and propyl gallate prevents non-specific oxidation. The cell suspension was then collected in an Eppendorf tube followed by three freeze/thaw cycles in liquid N_2. Freshly isolated cardiomyocytes were pelleted and briefly rinsed three times with PBS at 20 °C followed by three freeze/thaw cycles in liquid N_2. Then 0.8 ml of extraction buffer was added to the cells. Lysates from cultured and freshly isolated cardiomyocytes were then incubated in the dark under N_2 atmosphere for 40 min at 4 °C, and acidified to pH 3.0 with 1M HCl before loading on to an octadecyl-functionalized silica gel (ODC) column (2.2 g of ODC/column) that had been prewashed with 7 ml of methanol and 7 ml of water. Tracheas suction sets (UnoPlast, Hundested, Denmark) were used for columns. The columns were eluted successively and rapidly under N_2 pressure (10 lb/in^2) with 7 ml 15% ethanol, 7 ml H_2O, 2 ml of petroleum ether and finally 10 ml of ethyl acetate. The ethyl acetate fraction was collected, evaporated to dryness under N_2 stream and the dry material dissolved in 100 μl ethyl acetate.

ELISA for the determination of 12(S)-HETE

The quantity of the 12-LO metabolite 12(S)-HETE was analysed using a TiterZyme® 12(S)-HETE ELISA kit (Perseptive Biosytems, Framingham, MA, U.S.A.). The samples were prepared as described above and the ELISA was performed according the manufacturer's instructions. Briefly, samples containing variable amounts of 12(S)-HETE or 12(S)-HETE standards (0.25, 1, 2.5, 10, 50 ng/ml) competed with a constant amount of alkaline phosphatase-labelled 12(S)-HETE for binding to a rabbit anti-12(S)-HETE antibody. The unbound material was removed by washing and the reaction terminated. The absorbance was measured at 405 nm and the 12(S)-HETE concentration in the samples calculated from the standard curve.

IRS-1 and IRS-2 immunoprecipitation

Cells were preincubated with esculetin, NDGA or DMSO for 1 h, treated in the absence or presence of insulin (100 nM) for 5 min and finally lysed in modified RIPA buffer [50 mM Tris, 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM EDTA, 1% (w/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 1μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstatin, pH 7.4]. After incubation for 2 h, cell suspensions were centrifuged at 16000 ***g*** for 10 min and the supernatant was subjected to immunoprecipitation.

For immunoprecipitation of IRS-1 and IRS-2, 5 μl of each antiserum was added to 1 mg/ml of lysate and incubated for 16 h at 6 °C with gentle rotation, mixed with 50 μl of IgG–acrylamide beads and incubated again for 2 h at 6 °C. After centrifugation at 16000 ***g*** for 10 min, the immunopellet was washed three times with RIPA buffer, twice with PBS, and the pellets were subjected to Western-blot analysis.

Cell surface biotinylation

Cell surface biotinylation was adapted from previously published procedures [29,30]. Cells (1x10^6) in 100 mm culture plates were washed with ice-cold PBS and incubated with 0.5 mg/ml sulpho-NHS-LC biotin in PBS for 30 min at 4 °C. The reaction was stopped by rinsing the plates three times with 15 mM glycine in ice-cold PBS. The cells were then collected and solubilized for 30 min at 4 °C in 1 ml of solubilization buffer [165 mM NaCl, 20 mM Hepes, pH 7.4, 1% (v/v) Triton X-100, 2 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml protease-inhibitor cocktail]. The supernatant was separated by centrifugation at 16000 ***g*** for 10 min at 4 °C and mixed with 50 μl of streptavidin–agarose beads (1–2 mg of streptavidin/ml gel). The suspension was gently mixed overnight at 4 °C and the beads were sedimented by centrifugation. The pellet was washed three times in ice-cold washing buffer (150 mM NaCl, 10 mM Tris, pH 7.0, 2 μg/ml aprotinin, 2 μg/ml protease-inhibitor cocktail). The final pellet was resuspended in 50 μl of urea buffer [8.0 M urea, 2% (v/v) SDS, 100 mM Tris, pH 6.8] and incubated for 30 min at 37 °C. The supernatant was mixed with Laemmli buffer [31] and frozen at −70 °C until use. Protein content in the lysates was determined by the bicinchoninic acid protein assay (Pierce Laboratories, Rockford, IL, U.S.A.) using BSA, dissolved in RIPA buffer, as the standard.

Immunoblotting

Protein samples were separated by SDS/PAGE using 8–18% gradient gels or 10% horizontal gels and transferred to PVDF membranes (Millipore, Düsseldorf, Germany). Membranes were blocked for 90 min in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.5; TBS) containing 0.1% (v/v) Tween (TBST) and 10% (w/v) milk powder. Subsequently, membranes were incubated for 16 h at 4 °C with antibodies against IRS-1, IRS-2, phospho-tyrosine, p85, Akt-1, phospho-Akt, GLUT4 or 12-LO. After incubation, membranes were washed ten times for 5 min with TBST buffer and incubated for 90 min with anti-rabbit horseradish peroxidase-conjugated IgG diluted in TBST containing 5% (w/v) milk powder. After washing ten times for 5 min with TBST, the membranes were incubated with ECL solution. Proteins were revealed and signals were quantified on a Lumi-Imager system using image analysis software (Boehringer, Mannheim, Germany). Significance of reported differences was evaluated using the null hypothesis and *t*-statistics for paired data.

RESULTS

Characterization of 12-LO localization and activity in cardiomyocytes

Freshly isolated adult rat cardiomyocytes markedly express 12-LO, as shown in Figure 1. Cells were double stained for actin fibres by FITC–phalloidin and for 12-LO by a primary anti-12-LO antibody, followed by Cy3-conjugated goat anti-rabbit IgG. We show here that immunofluorescence specific for actin and 12-LO could be readily detected in the different sections of ventricular cardiomyocytes. The actin network was found to be organized in strictly parallel fibres (in green) in both perinuclear and plasma-membrane-near sections. For 12-LO (in red), a more punctuate staining appeared in a section near the plasma membrane, whereas a more homogeneous distribution of 12-LO-bound immunofluorescence was found in perinuclear sections. 12-LO could not be detected co-localized to the actin network.

Cardiomyocytes were exposed to the structurally different LO inhibitors NDGA for 1 h (freshly isolated cells), esculetin for 16 h (cultured cardiomyocytes) or to 0.1% DMSO as a control. Extracts of cells were prepared and the amount of 12(S)-HETE was determined by ELISA. In freshly isolated cardiomyocytes 42.0 ± 3.7 ng of 12(S)-HETE per mg of protein ($n = 3$) could be detected under control conditions, with a comparable level in cultured cells. Upon treatment with NDGA or esculetin, we observed a marked reduction in the cellular content of 12(S)-HETE to 7.1 ± 3.9 and 13.8 ± 5.8 ng/mg of protein ($n = 3$) re-

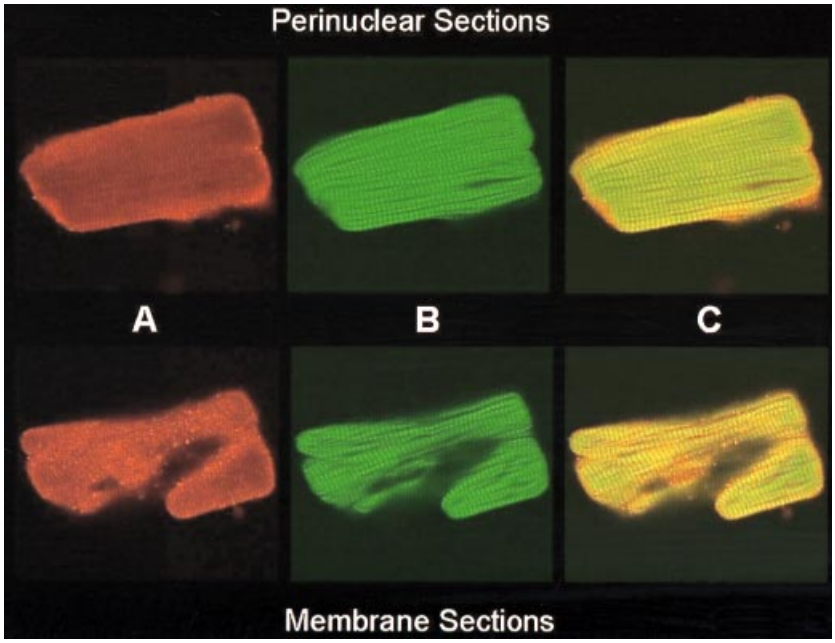

Figure 1 Immunofluorescence detection of 12-LO in primary cultured cardiomyocytes

Ventricular cardiomyocytes were cultured for 16 h in a medium containing 8 mM glucose and 17 pM insulin, followed by an incubation for 3 h in medium with 5.5 mM glucose. Cells were fixed, permeabilized and incubated for 16 h at 6 °C in a wet chamber with anti-12-LO antibody (diluted 1 : 200) and with FITC–phalloidin (diluted 1 : 500) for actin staining. Slides were then incubated for 2 h with a combination of Cy3-conjugated goat anti-rabbit IgG (diluted 1 : 500). Cell samples were prepared for confocal laser microscopy, viewed and analysed using the Leica TCS-NT confocal laser scanning system. (**A**) 12-LO bound immunofluorescence (red), (**B**) actin network (green) and (**C**) combined 12-LO and actin staining. Whole cells are marked as perinuclear sections (top) and cell cuts are membrane sections (bottom). Representative pictures from three independent experiments are shown.

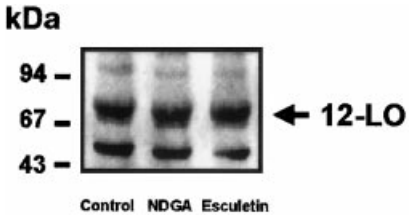

Figure 2 Effect of 12-LO inhibition on 12-LO expression in cardiomyocytes

Ventricular cardiomyocytes (1×10^6 cells per incubation) were treated with NDGA (50 μM) for 1 h or with esculetin (100 μM) for 16 h. Cell lysates from pretreated adult cardiomyocytes were analysed by SDS/PAGE, transferred to PVDF membranes and immunoblotted with a polyclonal anti-12-LO antibody. A representative blot from three experiments is shown. Molecular-mass makers (kDa) are indicated at the left.

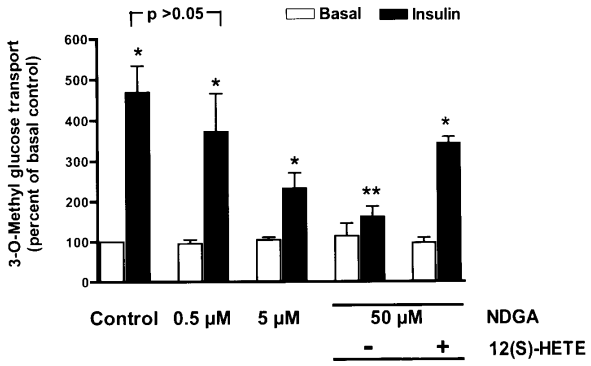

Figure 3 Effect of NDGA on basal- and insulin-stimulated 3-*O*-methylglucose transport

Freshly isolated adult rat ventricular cardiomyocytes were incubated for 1 h with increasing concentrations of NDGA (0.5 μM–50 μM) or 0.1% DMSO as a control in Hepes buffer containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, followed by an incubation for 5 min with or without 100 nM insulin. Cells exposed to 50 μM NDGA were additionally treated with or without 12(S)-HETE (3 μM). 3-*O*-Methylglucose transport was then determined by incubating cells with 3-*O*-[^{14}C]methyl-D-glucose (100 μM final concentration) for 10 s. Results are means $\pm$ S.E.M. of four independent experiments. $^*P < 0.05$ compared with basal control, **not significantly different from basal control.

spectively. Cell lysates were analysed by Western blotting with a polyclonal anti-12-LO antibody. As shown in Figure 2, 12-LO was detected at a molecular mass of approx. 70–75 kDa. Compared with control cells, 12-LO expression was not altered in cells exposed to esculetin or NDGA.

Involvement of 12-LO in insulin signalling, glucose uptake and GLUT4 translocation in cardiomyocytes

In order to investigate the involvement of 12-LO in cardiac glucose uptake, cells were incubated with increasing concentrations of the LO inhibitor NDGA or 0.1% DMSO as control, followed by an incubation for 5 min in the absence or presence of insulin (100 nM). 3-*O*-Methylglucose transport was then determined following a 10 s exposure of the cells to 3-*O*-[^{14}C]methyl-D-glucose. A 4–5-fold increase in glucose transport could be detected in control cells in response to insulin (Figure 3). In the NDGA-treated cells, basal glucose transport remained unchanged compared with control cells. Insulin-induced glucose transport was not altered in cells exposed to 0.5 μM NDGA. However, at 5 μM NDGA the insulin effect was significantly

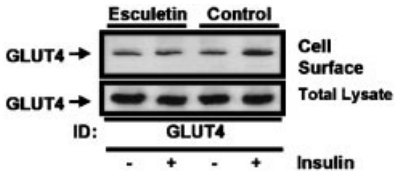

Figure 4 Immunodetection of GLUT4 in total lysates and cell surface-biotinylated protein fractions of esculetin-treated cardiomyocytes

Cardiomyocytes were cultured for 16 h with esculetin (100 μM) or 0.1% DMSO (control) and incubated further in the absence or presence of 1 μM insulin for 45 min. The cells were then treated with sulpho-NHS-LC-biotin and solubilized, as described in the Materials and methods section. The biotinylated lysates were processed for affinity-purification or directly used for Western blotting. Total lysates (15 μg) or cell surface biotinylated fractions (3 μg) were analysed by SDS/PAGE, transferred to PVDF membranes and immunoblotted (ID) with an anti-(GLUT4) antibody. Representative blots from three independent experiments are shown.

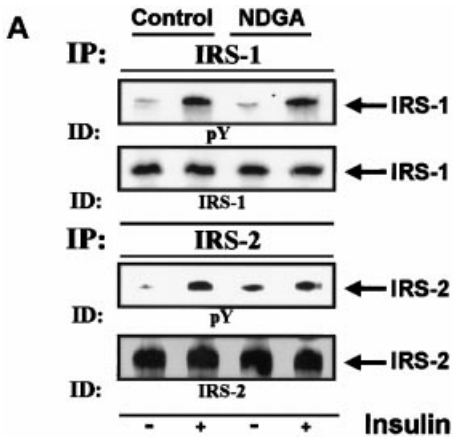

Figure 5 Effect of 12-LO inhibition on tyrosine phosphorylation of IRS proteins

Cardiomyocytes were treated with NDGA (50 μM) or with 0.1% DMSO (control) for 1 h, followed by a 5 min incubation in the absence or presence of 100 nM insulin. Cells were lysed in modified RIPA buffer and IRS-1 and IRS-2 immunoprecipitated (IP). Immunopellets were subjected to SDS/PAGE analysis and immunoblotted (ID) with antibodies against phosphotyrosine (pY), IRS-1 or IRS-2. Representative blots from four independent experiments are shown.

reduced by approx. 50%, with a complete loss of insulin-induced glucose transport at 50 μM (Figure 3).

In order to assess the specificity of this inhibitory effect, cells exposed to NDGA (50 μM) were additionally treated with exogenous 12(S)-HETE (3 μM). Under these conditions, insulin action on glucose transport could be restored to approx. 70% of that observed in control cells (Figure 3). Cardiomyocytes were then cultured overnight with or without the LO inhibitor esculetin (100 μM), followed by an incubation in the absence or presence of insulin. Under control conditions, an increase in 2-DOG uptake was observed in response to insulin from 11.9 ± 1.1 to 24.8 ± 8.9 pmol/100 μg of protein in 30 min ($n = 4$; $P < 0.05$). Esculetin completely inhibited this effect of insulin in the cultured cells.

Additional experiments were then performed to elucidate whether the effect of the 12-LO inhibitors could be attributed to an inhibition of GLUT4 translocation. Cardiomyocytes were therefore treated with esculetin and insulin, followed by biotinylation of the cell surface glucose transporters. Cells were

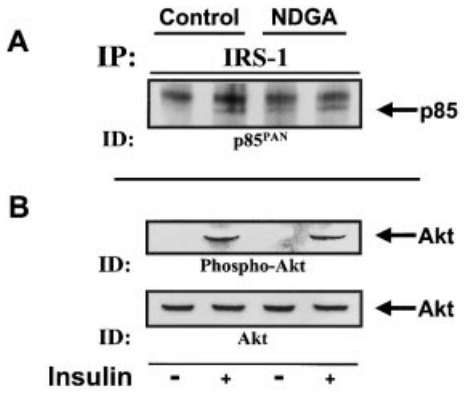

Figure 6 Effect of 12-LO inhibition on downstream insulin signalling events

Cardiomyocytes were treated with NDGA (50 μM) or 0.1% DMSO (control) for 1 h, followed by a 5 min incubation absence or presence of 100 nM insulin and lysed in modified RIPA buffer. (**A**) IRS-1 was immunoprecipitated (IP) and immunopellets were subjected to SDS/PAGE analysis and immunoblotted (ID) with anti-p85^{PAN} antibodies. (**B**) Total lysates were subjected to SDS/PAGE analysis and immunoblotted (ID) with anti-phosphothreonine-Akt or anti-Akt antibodies. Representative blots from four independent experiments are shown.

lysed and proteins separated by SDS/PAGE, transferred to PVDF membranes and immunoblotted with an anti-(GLUT4) antiserum (Figure 4). In control cells, insulin stimulation (1 μM) induced an increase in GLUT4 at the cell surface to 214 ± 39% of basal ($n = 3$; $P < 0.05$). Exposure to esculetin did not modify the total cell content of GLUT4. However, after esculetin treatment insulin was completely unable to increase the abundance of GLUT4 at the surface of the cardiomyocytes (Figure 4). We therefore conclude that the reduction of 12(S)-HETE in cardiac cells induces the complete loss of insulin-regulated GLUT4 translocation.

It may be argued that 12(S)-HETE might participate in the insulin signalling pathway upstream of the GLUT4 translocation machinery. Therefore we studied the insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2. As shown in Figure 5, no significant change of total expression of IRS-1 or IRS-2 was detectable in cells exposed to the LO inhibitor NDGA compared with control cells. Insulin (100 nM) increased the tyrosine phosphorylation of IRS-1 and IRS-2 by approx. 4.6- and 3.5-fold respectively in control cells. NDGA slightly reduced IRS-1 tyrosine phosphorylation by 15 ± 7.9% ($n = 8$; $P < 0.05$), whereas the IRS-2 tyrosine phosphorylation was reduced by 26 ± 6.9% ($n = 4$; $P < 0.05$) by this LO inhibitor. Subsequently, we investigated the association of IRS-1 with the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) and the phosphorylation of the protein kinase Akt under the same experimental conditions. No change in the insulin-induced association of the p85 subunit of PI 3-kinase with IRS-1 was detected after NDGA treatment. In addition, inhibition of LO also had no effect on the insulin-dependent phosphorylation of Akt (Figure 6).

Involvement of 12-LO in the actin network organization

Actin filaments are known to play a critical role in insulin-stimulated glucose transport and GLUT4 translocation, as demonstrated in adipocytes [3] and skeletal-muscle cells [32,33]. In order to elucidate the involvement of actin network organization in insulin-regulated glucose transport in cardiomyocytes, we used the fungal metabolite cytochalasin D, which

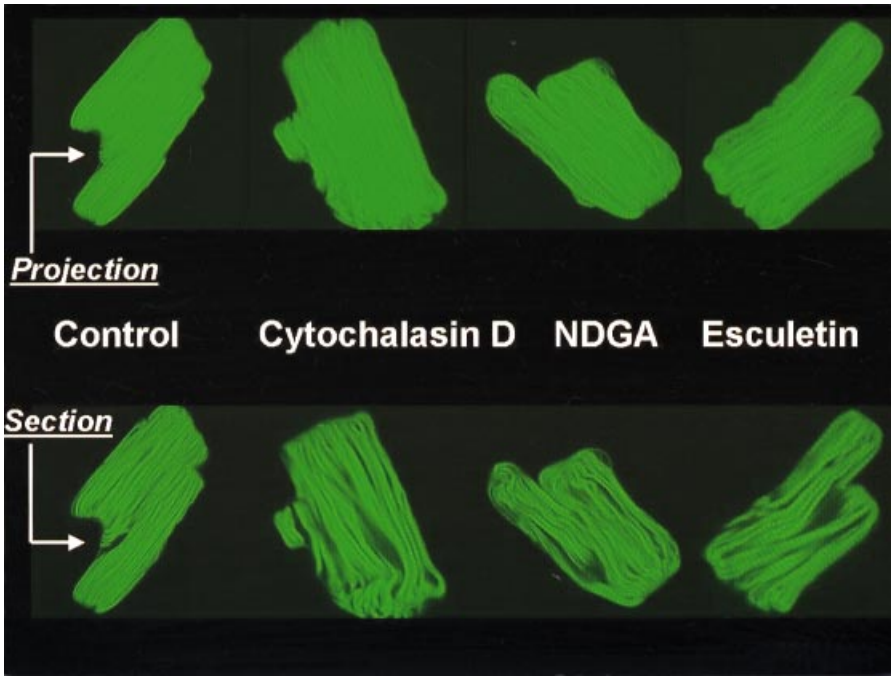

Figure 7 Effect of 12-LO inhibitors and cytochalasin D on the actin network organization

Cardiomyocytes were cultured overnight and treated with cytochalasin D (1 μM), NDGA (50 μM) or 0.1 % DMSO (control) for 1 h, or with esculetin (100 μM) for 16 h. Cells were fixed, permeabilized and then incubated for 16 h at 6 °C in a wet chamber with FITC–phalloidin (1:500 dilution) in PBS. Cell samples were prepared for confocal laser microscopy, viewed and analysed using the Leica TCS-NT confocal laser scanning system. Whole cells are marked as 'Projection' in the upper panel; cell cuts are marked as 'Section' in the lower panel. Representative pictures from three independent experiments are shown.

is known to inhibit insulin-induced glucose uptake by disruption of actin fibres. Freshly isolated cardiomyocytes were incubated for 1 h with cytochalasin D (1 μM) and stimulated with insulin. Under control conditions the rate of 3-*O*-methylglucose transport was increased to $526 \pm 15\,\%$ ($n = 4$) in response to the hormone. This stimulatory action of insulin was completely inhibited in cells treated with cytochalasin D. It is worth noting that the basal transport rate remained unaffected, most likely excluding non-specific cellular stress reactions.

In order to correlate the effects of cytochalasin D and 12-LO inhibition with actin network organization in cardiomyocytes, we cultured cardiomyocytes on coverslips with cytochalasin D or the LO inhibitors esculetin or NDGA. Cells were fixed, permeabilized and stained for actin filaments by FITC–phalloidin (Figure 7). In the whole cells (upper panel, 'projection'), the dense actin network is tightly separated into parallel fibres. Exposure to LO inhibitors or cytochalasin D was not able to change the general actin filament pattern or the typical rod shape, compared with untreated cells. Looking at a representative cut of untreated cells (lower panel, 'section'), extremely parallel fibres can clearly be seen. In contrast, cells exposed to esculetin or NDGA, revealed a prominent disassembly of actin fibres in a fashion similar to the effect induced by cytochalasin D. These data clearly indicate that LO inhibition leads to a marked disassembly of actin filaments, finally resulting in a block of GLUT4 translocation in response to insulin.

DISCUSSION

In the present investigation using ventricular cardiomyocytes, attempts have been made to correlate the activity of the 12-LO pathway with the organization of actin cytoskeletal elements, and to discover potential implications for GLUT4 trafficking and cardiac glucose uptake. This approach was based on (1) the recent demonstration that actin filaments participate in the exocytotic movement of GLUT4 [3], and (2) data showing that actin fibres might represent possible target proteins for eicosanoid metabolites of the LO pathway [7,8]. We report here a complete inhibition of insulin-induced glucose uptake by cytochalasin D in adult ventricular cardiomyocytes. Additionally, cytochalasin D caused a marked disassembly of actin fibres, indicating that, in these cells, the actin network essentially contributes to the regulation of insulin-induced translocation of GLUT4. In line with our data, recent investigations by Klip and co-workers [4–6, 32] in adipocytes and L6 myotubes revealed that insulin-induced glucose uptake and exocytosis of GLUT4 vesicles to the plasma membrane were markedly inhibited by inducing actin network disassembly using latrunculin A or cytochalasin D. Their data suggest that an intact actin network is essential for insulin-induced glucose uptake and exocytosis of GLUT4 vesicles. Furthermore, Omata and co-workers [3] used latrunculin A and peptide D^{K}-(62-85), a potent inhibitor of GLUT4 endocytosis, to measure glucose uptake in isolated adipocytes. Their findings indicate that actin filaments play a crucial role in exocytotic GLUT4 recruitment to the plasma membrane, but not in endocytosis. However, the regulatory pathways that lead to actin network rearrangement still remain poorly defined.

By labelling of F-actin fibres we show in the present study that inhibition of the 12-LO pathway, coupled with a strong reduction in the cellular content of 12(S)-HETE, leads to a prominent disassembly of the actin network in ventricular cardiomyocytes in a similar fashion to the cytochalasin D-induced actin filament

disassembly. Earlier data support the notion that 12(S)-HETE contributes to the regulation of actin network organization [9–11]. Thus it has been reported that 12(S)-HETE induces the phosphorylation of major cytoskeletal proteins, like actin, specifically leading to an increased actin filament content and an enhancement of actin polymerization. Breitbart and co-workers [16] first reported the presence of 12-LO activity in rat ventricular cardiomyocytes, with enzyme activity being detected in both the cytosol and microsomal fractions. Consistent with these findings, our confocal data presented here confirm that immunofluorescence specific for actin and 12-LO could be detected in ventricular cardiomyocytes. The actin network was organized in strictly parallel fibres, whereas for 12-LO-bound immunofluorescence, a more homogeneous distribution pattern was found in perinuclear sections, and a more punctuate staining appeared in sections near the plasma membrane. 12-LO did not co-localise to the actin network. However, the 12-LO metabolite 12(S)-HETE was recently shown by Vanderhoek and co-workers [7,8] to be a ligand for actin cytoskeletal elements. Interestingly, the 12-LO pathway is stimulated in the myocardium by hypoxia or ischaemia [19,20,34]. It may be speculated that, under these conditions, the enhanced production of 12(S)-HETE may operate to retain proper actin network organization in the cardiac cell, providing the basis for ongoing GLUT4 trafficking. This view is supported by recent data from Funk, Witztum and co-workers [35], who demonstrated that specific inhibition of the 12/15-LO isoform, or disruption of the corresponding gene, led to a strong reduction in actin polymerization. Thus it is suggested that this enzyme plays an important role in the local control of actin polymerization. However, additional work will be needed to study the regulation of cardiac 12-LO activity and to determine the relative contribution of different 12(S)-HETE levels to cytoskeletal rearrangement.

Inhibition of 12-LO activity completely abrogated insulin-induced glucose uptake and GLUT4 translocation without altering the total expression of GLUT4. In addition, rates of basal glucose transport were not affected by exposure to the LO inhibitors, most likely excluding non-specific perturbations of cardiomyocyte function. A slight reduction in insulin action by 12-LO inhibition was observed at the level of IRS-1 and IRS-2. It should be noted, however, that the complete abrogation of insulin-induced glucose uptake and GLUT4 translocation can not be explained by this observation, as inhibition of 12-LO was not able to modify elements of the insulin signalling pathway upstream of the glucose transporter, including PI 3-kinase and Akt.

In the light of the prominent disassembly of the actin network in response to LO inhibition, we propose a model in which 12(S)-HETE may function as an important element of the cardiac glucose transport machinery. Our view agrees with a recent investigation by O'Rahilly and co-workers [36]. These workers demonstrated that arachidonic acid pretreatment increased glucose uptake by adipocytes, whereas both basal and insulin-dependent glucose uptake could be substantially blocked by the LO inhibitor NDGA. Thus the importance of an intact LO pathway for insulin-induced glucose uptake was highlighted by this group, demonstrating that these eicosanoids may also act as endogenous ligands for the peroxisome proliferator activated receptor ('PPAR') γ. In contrast, Azhar and co-workers [37] demonstrated that NDGA increased glucose uptake in adipocytes in both the absence and presence of insulin. However, it was suggested that this insulin-like effect of NDGA was unrelated to its role as a LO inhibitor. Furthermore, a different pattern of LO expression in the heart and adipose tissue [14,15] may also explain these divergent findings. In the present study the specificity of 12-LO inhibition by NDGA was clearly confirmed by the addition of 12(S)-HETE to cardiomyocytes exposed to the drug, resulting in an almost complete restoration of insulin-sensitivity. This is consistent with the findings of Yamada and Proia [38], who recently showed that the inhibitory effects of esculetin on F-actin organization could be totally reversed by exogenously added LO metabolites.

In summary, the present study shows that inhibition of 12-LO activity in ventricular cardiomyocytes leads to the disassembly of the actin network. This results in an inhibition of insulin-induced GLUT4 translocation without affecting insulin signalling events downstream of the IRS level leading to GLUT4 translocation. We suggest that 12-LO metabolites stabilize actin network organization in cardiomyocytes and operate as an important element of the cardiac glucose uptake system.

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