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http://dx.doi.org/10.1016/j.bbagen.2016.08.013

A novel role for small molecule glycomimetics in the protection against lipid-induced endothelial dysfunction: involvement of Akt/eNOS and Nrf2/ARE signaling

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Short title: Mahmoud; Glycomimetics protect endothelial cells.

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ABSTRACT

Background: Glycomimetics are a diverse array of saccharide-inspired compounds, designed to mimic the bioactive functions of glycosaminoglycans. Therefore, glycomimetics represent a unique source of novel therapies to target aberrant signaling and protein interactions in a wide range of diseases. We investigated the protective effects of four newly synthesized small molecule glycomimetics against lipid-induced endothelial dysfunction, with an emphasis on nitric oxide (NO) and oxidative stress. **Methods:** Four aromatic sugar mimetics were synthesized by the stepwise transformation of 2,5-dihydroxybenzoic acid to derivatives (C1-C4) incorporating sulfate groups to mimic the structure of heparan sulfate. **Results:** Glycomimetic-treated human umbilical vein endothelial cells (HUVECs) were exposed to palmitic acid to model lipid-induced oxidative stress. Palmitate-induced impairment of NO production was restored by the glycomimetics, through activation of Akt/eNOS signaling. Furthermore, C1-C4 significantly inhibited palmitate-induced reactive oxygen species (ROS) production, lipid peroxidation, and activity and expression of NADPH oxidase. These effects were attributed to activation of the Nrf2/ARE pathway and downstream activation of cellular antioxidant and cytoprotective proteins. In *ex vivo* vascular reactivity studies, the glycomimetics (C1-C4) also demonstrated a significant improvement in endothelium-dependent relaxation and decreased ROS production and NADPH oxidase activity in isolated mouse thoracic aortic rings exposed to palmitate. **Conclusions:** The small molecule glycomimetics, C1-C4, protect against lipid-induced endothelial dysfunction through up-regulation of Akt/eNOS and Nrf2/ARE signaling pathways. Thus, carbohydrate-derived therapeutics are a new class of glycomimetic drugs targeting endothelial dysfunction, regarded as the first line of defense against vascular complications in cardiovascular disease.

Key words:

Small molecule drug discovery, glycomimetics, heparan sulfate, endothelial dysfunction, oxidative stress.

INTRODUCTION

The vascular endothelium plays a pivotal role in regulating vascular tone, controlling tissue blood flow, vessel integrity, permeability, inflammatory responses, vascular remodeling and angiogenesis [1, 2]. Pathological conditions can be detrimental to these processes and initiate endothelial dysfunction resulting in the development of vascular complications [3, 4], characterized by the reduced bioavailability of nitric oxide (NO), which is critical in maintaining the physiological function of vasculature [5]. In addition, an imbalance between anti-thrombotic and pro-thrombotic factors predisposes the endothelium to an atherogenic milieu, contributing to endothelial dysfunction [6]. Thus, endothelial dysfunction has emerged as a critical early target for preventing atherosclerosis and cardiovascular disease [7].

Endothelial dysfunction is linked to the pathogenesis of common diseases associated with increased risk of CVD, including type II diabetes mellitus, obesity and metabolic syndrome [8]. It has been reported that chronic elevations in plasma free fatty acids (FFAs) contribute to the development of endothelial damage, diminished endothelium-dependent vasodilation, and hypertension [9, 10] by triggering overproduction of reactive oxygen species (ROS), leading to uncoupling of eNOS [11]. Palmitic acid, the most prominent FFA in the bloodstream, stimulates the production of ROS, at least in part, through activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and down-regulation of eNOS and associated protective pathways, including nuclear factor-erythroid 2-related factor 2 (Nrf2) [12, 13]. Decreasing lipotoxicity and its associated oxidative stress, as well as promoting up-regulation of anti-oxidant enzymes and cytoprotective proteins, may be key components to prevent and treat cardiovascular diseases.

The glycosaminoglycan, (GAG) heparan sulfate (HS), is highly sulfated and key to a variety of physiological processes, including wound healing, angiogenesis, inflammation and development, [14] often acting as a co-receptor for signaling pathways. The multiple biological functions of HS are attributed to their variable sulfation patterning, which in turn, allows for diverse but specific ionic interactions between the negatively charged sulfates and the carboxylate groups of a variety of proteins, including proteases, growth factors and cytokines [14-16]. HS oligosaccharides can mimic HS functions in biological systems; however, their use has been hindered by the complexity of their synthesis [17]. Thus, small molecule mimics; glycomimetics, can offer a potential solution to this obstacle [18].

To the best of our knowledge, there are no published reports regarding the possible protective effects of small molecule HS mimetics against lipid-induced endothelial dysfunction. Here, we demonstrate the evaluation of a class of small molecule glycomimetics, establishing for the first time their protective effects against palmitate-induced endothelial dysfunction, using *in vitro* and *ex vivo* models. This study provides an understanding of the mechanisms underlying the vascular protective effects of glycomimetics, in terms of oxidative stress and endothelial function and their potential as a new class of therapeutic drugs to target endothelial dysfunction.

MATERIALS AND METHODS

Synthesis of small molecule glycomimetics

Glycomimetics were synthesized by the stepwise transformation of 2,5-dihydroxybenzoic acid to a range of 2,5-substituted benzoic acid derivatives incorporating the key sulfate groups to mimic the interactions of HS (Supplementary Fig. I). Namely, commercially available 2,5-dihydroxybenzoic acid was converted to the methyl ester using Fischer esterification conditions [19]. In turn, the methyl 2,5-dihydroxybenzoate can be mono or di-allylated to afford methyl 5-(allyloxy)-2-hydroxybenzoate or methyl 2,5-bis(allyloxy)benzoate, respectively [20]. Methyl 5-(allyloxy)-2-hydroxybenzoate can be further alkylated with 4-bromobutyl acetate to afford methyl 2-(4-acetoxybutoxy)-5-(allyloxy)benzoate. Either as the methyl ester or carboxylic acid the above olefin-containing intermediates were treated with either AD-mix-α or β under Sharpless asymmetric dihydroxylation [21] conditions to afford the chiral di or tetra-hydroxylated compounds. Finally, treatment with sulfur trioxide-trimethylamine complex gave the desired HS-mimics (**C1-C4**). The chemical methods are described in detail in the Supplementary material online. In total, 4 examples of glycomimetics (**C1-C4**) have been prepared, the structures of which are shown in Figure 1. The molecular weights of **C1-C4** range from 554-636 Da, total polar surface area (tPSA) are all ≥ 235 A^2, calculated LogP (octanol/water solubility) ≤ -0.13 and hydrogen bond acceptors ≥ 16, however the number of hydrogen bond donating groups are allowable at ≤ 5, therefore, these compounds fall outside the Lipinski's rule of 5 (Ro5) [22], for orally bioavailable drug molecules (Supplementary Table I). This is due to the necessary high level of sulfation to mimic HS, but act as a useful starting point for the discovery of repurposed glycomimetics.

Human umbilical vein endothelial cell (HUVEC) culture

HUVECs (Caltag, Medsystems, Buckingham, UK) from passage 2-7, were cultured in media (M199; Lonza, Belgium) containing 20% fetal bovine serum (FBS), penicillin/streptomycin (2 mmol/L), glutamine (2 mmol/L), HEPES (10 mmol/L), endothelial cell growth supplement (30 µg/mL) and heparin (100 µg/mL) under 5% CO_2 at 37°C.

Cell viability

HUVECs were seeded in 96-well microtiter plate and incubated at 37°C for 24 hours. The medium was removed, replaced by fresh medium and then exposed to serial dilutions of **C1-C4** (1, 10, 100 and 500 µmol/L) for 24 hours. After incubation, 20 µL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in phosphate buffered saline (PBS) was added to the cells and further incubated at 37°C for 15 min. After washing, 100 µL of DMSO were added to each well and absorbance was measured at 570 nm on a microplate reader (BioTek) with subtraction of the blank value at 630 nm, and compared with control untreated cells.

Preparation of palmitate-albumin complexes and cell treatments

To generate the *in vitro* model of disease, sodium palmitate was conjugated to bovine serum albumin (BSA) as outlined by Chavez and Summers [23], with some modifications. Sodium palmitate was dissolved in ethanol at 60ºC and diluted 1:100 in M199 media supplemented with 2% fatty acid-free bovine serum albumin (BSA) and incubated for 1 hour at 37ºC. HUVECS were treated with 1 µmol/L of the synthesized glycomimetics (**C1-C4**) in the presence of palmitate (100 µmol/L) for 3 hours or pre-treated with glycomimetics for 12 hours followed by 3 hours palmitate (100 µmol/L). In some experiments, HUVECs were incubated with 1, 10 or 100 µmol/L of the test compounds (**C1-C4**). Control cells were incubated in serum free M199 containing 2% (wt/vol) fatty acid-free BSA.

NO release assay

NO release was determined using, diaminofluorescein-2 (DAF-2) [24]. Briefly, cells were washed with PBS and incubated with L-arginine (100 µmol/L /PBS) for 5 min at 37ºC before incubating with DAF-2 (0.1 µmol/L) for 2 min, followed by the calcium ionophore calimycin (A23187, 1 µmol/L) for 30 minutes. L-NAME (100 µmol/L), a nitric oxide inhibitor (NOS), was also added 20 min before the addition of L-arginine in some experiments to demonstrate the involvement of NOS. DAF-2 fluorescence (arbitrary units, AU) was quantified using a microplate reader (BioTek) with excitation and emission wavelengths of 485 and 540, respectively. The autofluorescence was subtracted from each value.

Quantification of ROS production

Cells were incubated with 10 µmol/L of the fluorescent probe 2'-7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Sigma) for 30 min at 37ºC and the level of fluorescence was determined (excitation 490 nm; emission 540 nm) using a microplate reader (BioTek).

NADPH oxidase activity

The level of NADPH-enhanced superoxide anions ($O_2^{\bullet-}$) in HUVEC homogenates or intact mouse aortic rings was determined by lucigenin-enhanced chemiluminescence, as demonstrated previously [25]. Following the various treatment conditions, cells or aortic rings were homogenized in a buffer (20 mmol/L KH_2PO_4 and 1 mmol/L EDTA supplemented with aprotinin, leuprotinin, pepstatin and PMSF) and the total protein quantified using the Bicinchoninic acid (BCA) assay (Pierce Biotechnology). To 50 μg of total protein, NADPH (100 μmol/L) was added in a total volume of 500 μl, with lucigenin (5 μmol/L) injected automatically. Activity was determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany). Basal values of NADPH oxidase activity were subtracted from the experimental values and expressed as RLU/min/μg protein for cells or RLU/min/mg tissue for aortic rings.

Lipid peroxidation assay

The OxiSelect TBARS assay kit (Cell Biolabs, San Diego, USA) was used to determine the level of lipid peroxidation by measuring malondialdehyde (MDA). After treatment, HUVECs were washed with PBS and homogenized in RIPA buffer. SDS lysis solution was added to the samples and MDA standards followed by incubation with thiobarbituric acid for 45 minutes at 95°C. Samples were cooled to room temperature and centrifuged at 1000 g for 15 min. The supernatant was removed and the absorbance was measured at 532 nm using a microplate reader (BioTek). Lipid peroxidation was expressed as nmol MDA/mg protein.

Superoxide dismutase (SOD) and catalase (CAT) activity assays

The activity of the enzymes, SOD and CAT, was determined in cell homogenates using assay kits (Cayman; Cat No 706002 and 707002 respectively), according to the manufacturer's instructions. A unit of SOD was defined as the amount of enzyme required to demonstrate 50% dismutation of superoxide radicals. Similarly, a unit of CAT was defined as the amount required to the form 1.0 nmol of formaldehyde per min at 25ºC.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Trizol (Invitrogen) was used to isolate total RNA from HUVECs and was quantified at 260 nm using a Nanodrop. RNA samples with A260/A280 ratios < 1.7 were discarded. cDNAs were synthesized from total

RNA (2 µg), using an oligo deoxythymidine primer and SuperScript II reverse transcriptase (Sigma) and were amplified using the SYBR Green master mix (Bioline, UK) in a total volume of 20 µl (Surecycler 8800 thermocycler; Agilent Technologies) with the primer sets outlined in Supplementary Table II. Real-time PCR was performed using the following conditions; 10 min at 95°C, followed by 40 cycles of 30 sec at 95ºC, 60 sec at annealing temperature of respective primer set, and extension for 30 sec at 72ºC. PCR products were assessed for quality using standard melting curve analysis and an agarose gel electrophoresis. The cycle threshold (CT) values were analyzed using the $2^{-\Delta\Delta Ct}$ method and data normalized to GAPDH.

Western blot analysis

HUVECs were lysed in RIPA buffer, containing proteinase inhibitors and total protein quantified using BCA protein assay kit (Pierce Biotechnology). Proteins (30 µg/well) were denatured and separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Blots were blocked for 1 hour in 5% non-fat milk/Tris-Buffered Saline Tween-20 (TBS-T), probed with primary rabbit anti-phospho-protein kinase B (Akt), rabbit anti-Akt (Santa Cruz Biotechnology), rabbit polyclonal anti- phospho-eNOS (Cell Signaling), rabbit anti-eNOS, rabbit anti-HO-1 (Stressgen), rabbit anti-Nrf2, rabbit anti-NQO-1 (Santa Cruz Biotechnology) or mouse anti-β-actin (Sigma), overnight at 4ºC followed by washing and incubation with the corresponding horse radish peroxidase-labelled secondary antibody for 1 hour at room temperature. After washing with TBS-T, membranes were incubated with ECL reagent and visualized (Amersham Pharmacia Biotech). Densitometry analysis using ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/) was used to quantify protein signal.

***Ex vivo* vascular reactivity**

Thoracic aortas from the experimental male BALB/c mice (Janvier, St Berthevin Cedex, France) were harvested, cleared of periadventitial tissue and cut transversely into 2.0-mm rings. All the procedures conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health (NIH) publication no. 85-23, revised 2011) and approved by the Institutional Committee for the ethical care of animals (University of Granada, Spain). Mouse thoracic aortic (MTA) rings were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, and antibiotics (100 U/mL penicillin, and 100 µg/mL streptomycin). MTA rings were then incubated in serum-free DMEM containing 2% fatty acid-free BSA (control) or palmitate-conjugated BSA (100 µmol/L) in the presence of glycomimetics (1 or 10 µmol/L) for a 24 hour duration. Care was taken with the vascular rings to avoid damage of the endothelial layer and transferred to a chamber filled with fresh Kreb's solution and mounted in a myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) for isometric tension measurement, as described previously [26]. Cumulative dose–response relaxation curves to acetylcholine (ACh; 1 nmol/L – 10 µmol/L) were performed in intact rings pre-contracted by U46619 (10 nmol/L) in control or L-NAME (100 µmol/L)-treated aortic rings. The mouse aorta were incubated with mitochondrial antioxidant mitoQ (0.1 µmol/L) or the NADPH oxidase inhibitor apocynin (10 µmol/L) for 60 min before the addition of U46619 to establish the source of ROS following palmitate treatment and relaxant responses to ACh were subsequently measured and expressed as a percentage of pre-contraction.

In situ detection of vascular ROS content

MTA rings were incubated in serum-free DMEM containing 2% fatty acid-free BSA (control) or palmitate conjugated BSA (100 µmol/L) in the presence of glycomimetics (1 or 10 µmol/L) for 24 hours. Unfixed MTA rings were cryopreserved in 0.1 mol/L PBS plus 30% sucrose for 1 hour, included in OCT compound medium (Tissue-Tek; Sakura Finetechnical), and kept frozen (−80ºC). By using a cryostat (Microm InternationalModelHM500 OM), 10 µm cross sections were obtained and incubated for 30 min in Hepes-buffered solution containing 10 µmol/L dihydroethidium (DHE), which incalates with DNA in the presence of ROS and is detected by fluorescence. Cells were counterstained with the nuclear stain DAPI at 0.3

μmol/L. In the following 24 hours, sections were examined using a fluorescence microscope (Leica DMIRB,). Sections were photographed and fluorescence was quantified using ImageJ. All parameters (pinhole, contrast, gain and offset) were held constant for all sections from the same experiment. ROS production was estimated from the ratio of ethidium/DAPI fluorescence [27].

Statistical analysis

Data were analysed using GraphPad Prism 5 (GraphPad Software, San Diego, USA) and comparisons were made using one-way analysis of variance (ANOVA) test and Tukey's post-hoc analysis. Two-way ANOVA tested for group interactions and results were expressed as mean ± standard error of the mean and $P = \leq 0.05$ was considered significant.

RESULTS

Glycomimetics do not affect cell viability

We tested the effects of glycomimetics **C1-C4** (Fig.1) on HUVEC viability using concentrations ranging from 1 to 500 μmol/L. After incubation for 24 hours, no detrimental effects on HUVEC viability was detected with any glycomimetic compound at any concentration (Supplementary Fig. II).

Glycomimetics rescue palmitate-induced impairment of NO production

First, to study the concentration-dependent effect of the synthesized glycomimetics on NO production, HUVECs were incubated with 1, 10 or 100 μmol/L of the test compounds (**C1-C4**) for 12 hours, followed by 3 hours palmitate (100 μmol/L). **C1** significantly increased A23187-stimulated NO production from HUVECs at the 1 μmol/L ($P<0.05$), 10 μmol/L ($P<0.001$) and 100 μmol/L ($P<0.01$) concentrations (Supplementary Fig. IIIA). Pre-treatment with different doses of **C2** significantly ($P<0.001$) protected HUVECS against palmitate-induced reduction in NO (Supplementary Fig. IIIB). The lower dose (1 μmol/L) of **C3** produced a significant ($P<0.05$) increase in A23187-stimulated NO production from HUVECs and the higher doses (10 and 100 μmol/L) produced a more potent effect (P<0.001, Supplementary Fig. IIIC). Similar to **C1**, **C4** significantly increased NO production from HUVECs at the 1 μmol/L (P<0.05), 10 μmol/L (P<0.001) and 100 μmol/L (P<0.01) concentrations (Supplementary Fig. IIID). In HUVECs treated with 3 different doses of **C1**, **C2**, **C3** or **C4**, the eNOS inhibitor L-NAME significantly (P<0.001) abolished A23187-stimulated NO production from HUVECS when compared with the corresponding glycomimetic dose. However, in light of the consistent response with all four compounds, we selected to use the low dose of 1 μmol/L for all future glycomimetic treatments.

Next, to determine the restorative effect of the glycomimetics on palmitate-induced reduction in NO production, HUVECs were incubated either with 1 μmol/L of the synthesized glycomimetics (**C1-C4**) in the presence of palmitate (100 μmol/L) for 3 hours, or pre-treated with glycomimetics for 12 hours followed by 3 hours palmitate (100 μmol/L) respectively. HUVECs incubated with palmitate, exhibited a significant (P<0.001) decrease in A23187-stimulated NO production. In HUVECs pre-treated with palmitate, followed by incubation with **C1**, **C2**, **C3** or **C4** for 3 hours, the dramatic decrease in NO production was significantly (P<0.001) attenuated compared to palmitate controls (Fig. 2A). Pre-incubation with 1 μmol/L **C1-C4,** significantly protected HUVECs against the significant decline in A23187-stimulated NO production in the presence of palmitate (Fig. 2B).

Glycomimetics modulate mRNA and phosphorylation levels of eNOS and Akt under palmitate-induced oxidative stress

To determine whether the enhanced NO release observed after treatment with glycomimetics under palmitate-induced stress conditions was due to activation of the Akt/eNOS pathway, both the level of mRNA abundance and protein phosphorylation of Akt and eNOS were investigated. HUVECs were pre-incubated with 1 μmol/L of the glycomimetics (**C1-C4**), followed by palmitate for 3 hours. Palmitate treatment alone produced a marked decline in both Akt mRNA (P<0.01, Fig. 3A) and protein phosphorylation (pAkt; P<0.05, Fig. 3B). Pretreatment of HUVECs with **C1**, **C2, C3** or **C4** significantly

(P<0.05) increased Akt mRNA abundance, and Akt protein phosphorylation, with pAkt restored to untreated control levels. A more marked effect in terms of Akt phosphorylation (P<0.01) was observed with **C4**.

Similarly, palmitate significantly reduced eNOS mRNA abundance (P<0.01, Fig. 3C) and protein phosphorylation (peNOS; P<0.05, Fig. 3D) in HUVECs treated for 3 hours. eNOS mRNA abundance exhibited a marked increase in HUVECS pretreated with **C1** (P<0.05), **C2** (P<0.01), **C3** (P<0.05) or **C4** (P<0.05). There was a significant increase in eNOS protein phosphorylation in HUVECs pretreated with **C1** (P<0.05), **C2** (P<0.05), **C3** (P<0.05) or **C4** (P<0.001) followed by palmitate treatment, compared to palmitate-treated controls, with the greatest effect observed with **C4**. Again, peNOS was restored to untreated control levels when pre-treated with all four glycomimetics.

Glycomimetics attenuate palmitate-induced ROS production and oxidative stress

Next, we investigated the effect of the glycomimetics on palmitate-induced oxidative stress by assessing ROS production, lipid peroxidation and activity of the antioxidant enzymes, SOD and CAT. HUVECs treated with palmitate for 3 hours exhibited a significant (P<0.001) increase in ROS production as expected. This ROS induction was significantly reduced (P<0.001) after co-treatment with 1 µmol/L of **C1**, **C2**, **C3** or **C4** to untreated control levels (Fig. 4A). The same protective effect was apparent when pre-treated with C1-C4 for 12 hours (P<0.001; Fig. 4B. The concentration-dependent effect of the glycomimetic compounds **C1-C4** on palmitate-induced ROS production was also studied in the pre-treatment model. All compounds at the different doses (1, 10 or 100 µmol/L) significantly abolished the palmitate-induced ROS production to the same extent and to untreated control levels (Supplementary Fig. IV).

Supporting the ROS data, lipid peroxidation (assayed as MDA) showed a significant (P<0.001) increase in HUVECs treated with palmitate for 3 hours which was significantly reduced (P<0.05) after pretreatment with 1 µmol/L **C1-C4**, (Fig. 4C).

The activity of the anti-oxidant enzyme SOD was, as expected significantly ($P<0.01$) decreased in HUVECs treated with palmitate for 3 hours, an effect which was prevented when pre-treated with glycomimetic **C1** ($P<0.05$), **C2** ($P<0.001$), **C3** ($P<0.001$) or **C4** ($P<0.01$; Fig. 4D). CAT activity exhibited a similar pattern where it was significantly ($P<0.001$) lower in palmitate treated cells and restored to untreated control levels in HUVECs pretreated with **C1** ($P<0.01$), **C2** ($P<0.01$), **C3** ($P<0.001$) or **C4** ($P<0.05$) glycomimetics, with C3 showing the most marked effect (Fig. 4E).

Glycomimetics reduce palmitate-stimulated NADPH oxidase activity

To further validate the protective effect of the glycomimetic compounds **C1-C4** against palmitate-induced oxidative stress, NADPH oxidase activity and mRNA expression of its subunits were assayed. Palmitate induced a significant ($P<0.001$) increase in NADPH oxidase activity when compared with untreated control HUVECs. Pretreatment with 1 µmol/L **C1-C4** significantly ($P<0.001$) decreased NADPH oxidase activity in palmitate-treated HUVECs (Fig. 5A). Furthermore, HUVECs treated with palmitate for 3 hours exhibited a significant increase in mRNA abundance of the NADPH oxidase subunits $p22^{phox}$ ($P<0.001$; Fig. 5B), NOX1 ($P<0.001$; Fig. 5C), NOX4 ($P<0.01$; Fig. 5D), and $p47^{phox}$ ($P<0.001$; Fig. 5E). However, in the presence of all 4 glycomimetics, a significant protective effect was detected with a down-regulation of mRNA abundance of NOX1, NOX4, $p47^{phox}$ and $p22^{phox}$ (Figure 5, $P<0.05$) compared to palmitate alone.

Glycomimetics protect against palmitate-induced oxidative stress via the Nrf2/ARE pathway

To establish the downstream protective anti-oxidant effects of the glycomimetic compounds **C1-C4,** against palmitate-induced oxidative stress, mRNA and protein expression of Nrf2, NQO-1 and HO-1 were interrogated using qRT-PCR and western blotting, respectively (Fig. 6).

Under palmitate-induced oxidative stress conditions, a significant reduction in the mRNA abundance and protein levels of Nrf2, NQO-1 and HO-1 was observed, when compared to homeostatic controls (Fig. 6).

Palmitate-induced HUVECs exhibited a significant up-regulation and restoration of Nrf2, NQO-1 and HO-1 mRNA abundance when pretreated with **C1** ($P<0.001$), **C2** ($P<0.01$), **C3** ($P<0.01$) or **C4** ($P<0.001$) (Fig. 6). In support of the mRNA data, a correlation was found with, Nrf2, NQO-1 and HO-1 protein expression where we demonstrate a significant increase in HUVECs pretreated with **C1** ($P<0.05$), **C2** ($P<0.01$), **C3** ($P<0.001$) or **C4** ($P<0.05$), with **C2** and **C3** exhibiting the most potent effects (Fig. 6).

Glycomimetics restore endothelium-dependent vasodilatation *ex vivo* after palmitate treatment

In order to establish whether these effects could be validated in a more physiological model, an *ex vivo* contractility study was conducted to evaluate the effect of **C1-C4** treatment on the endothelium-dependent vasodilatation (Fig. 7). Mouse aortic rings were incubated with 100 µmol/L palmitate and 1 or 10 µmol/L of **C1-C4** for 24 hours and concentration-relaxation response curves to ACh were performed in intact rings pre-contracted by the prostaglandin analog, U46619.

Aortas treated with palmitate showed a significant ($P<0.001$) decrease in endothelium-dependent vasodilator responses to ACh, compared with the control aortas. **C1** and **C2**, at both doses (1 and 10 µmol/L), significantly ($P<0.001$) improved the palmitate-reduced endothelium-dependent vasodilatation (Fig. 7A and B, respectively). Both doses of **C3** produced a significant ($P<0.01$) increase in endothelium-dependent vasodilatation when compared with the palmitate-induced aortas (Fig. 7C). However, the improved endothelium-dependent vasodilatation produced by **C3** treatment was significantly ($P<0.05$) lower than the control aortas. The lower concentration of **C4** (1 µmol/L) showed a marked ($P<0.01$) alleviation in endothelium-dependent vasodilatation, but was still significantly ($P<0.01$) lower than the control aortas (Fig. 7D). The higher dose of **C4** (10 µmol/L) significantly ($P<0.001$) improved endothelium-dependent vasodilatation when compared with the palmitate-induced aortas and was similar to untreated controls.

To validate the endothelial-dependent relaxation responses to ACh, the eNOS inhibitor L-NAME was added, which abolished the relaxant response induced by ACh in all experimental groups as shown in Supplementary Figure V. To examine whether ROS are involved in endothelial dysfunction induced by palmitate in mouse aorta, the endothelium-dependent relaxant response to ACh in the presence of the mitochondrial antioxidant mitoQ (Fig. 7E) or the NADPH oxidase inhibitor apocynin were analyzed (Fig. 7F). Both mitoQ and apocynin significantly improved the impaired aortic relaxation in response to ACh induced by palmitate, suggesting involvement of ROS-induced pathways.

Glycomimetics attenuate palmitate-induced vascular ROS levels by reducing NADPH oxidase activity *ex vivo*

To characterize and localize ROS levels within the vascular wall, aortic rings were incubated with 100 µmol/L palmitate and 1 or 10 µmol/L of **C1-C4** for 24 hours and the level of red fluorescence was determined in sections of aorta incubated with DHE (Fig. 8A). Rings treated with palmitate showed significantly ($P<0.001$) increased staining in adventitial, medial and endothelial cells compared with the control group. Sections from palmitate-treated rings exhibited a marked decrease in red fluorescence ($P<0.01$) when co-incubated with any glycomimetic at either dose, suggesting attenuation of ROS production.

NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence (Fig. 8B) was significantly ($P<0.001$) increased by palmitate. Co-incubation of aortic rings with either dose of **C1**or **C2** significantly ($P<0.001$) reduced NADPH oxidase activity when compared to palmitate alone. **C3** significantly decreased NADPH oxidase activity at both the 1 ($P<0.01$) and 10 µmol/L ($P<0.001$) doses. **C4** exhibited a similar pattern as it significantly ($P<0.01$) reduced NADPH oxidase activity at both applied concentrations.

DISCUSSION

Glycosaminoglycans represent the most abundant class of molecules in nature. They are involved in disease indications; however, they lack the properties necessary for efficacious drugs. Therefore, design and synthesis of small molecule glycomimetics, exhibiting drug-like properties could represent a new class of therapeutic drugs to target molecular mechanisms of disease [18, 28]. Here, we report the discovery and evaluation of the effects of glycomimetics in a FFA-induced model of endothelial dysfunction. We demonstrate that glycomimetics exert protective effects against palmitate-induced endothelial dysfunction. Our data point to the involvement of Akt/eNOS and Nrf2/ARE signaling pathways in mediating these protective effects on the endothelium.

HUVECs were used in both a pre-treatment and co-incubation regime to reflect a potential preventative mechanism and possible treatment of disease in progress respectively. Palmitate-treated HUVECs showed diminished A23187-stimulated NO production mediated via reduced Akt-dependent phosphorylation of eNOS at Ser1177. This observation confirms previous work demonstrating palmitate induced eNOS uncoupling, decreased eNOS phosphorylation and ultimately NO production [11, 26]. We show that small molecule glycomimetics markedly prevented the palmitate-induced decline in NO production through up-regulation of the mRNA abundance and protein phosphorylation of both Akt and eNOS.

The FFA-induced decline in NO production could be explained by the overproduction of ROS. Palmitic acid stimulates the production of superoxide radicals through activating NADPH oxidases and down-regulation of eNOS [12, 13]. Superoxide reacts with NO, forming the potent and versatile oxidant peroxynitrite (ONOO•−) which may oxidize the eNOS cofactor tetrahydrobiopterin or destabilize the eNOS dimer, causing uncoupling of eNOS [29]. Uncoupled eNOS produces superoxide rather than NO, thus further augmenting oxidative stress and decreasing NO bioavailability [29]. In addition, ROS activates membrane oxidases leading to increased dimethylarginine, an arginine decoy for the active sites on eNOS and L-arginine transporters [30]. Here, palmitate induced a marked increase in ROS production through activating NADPH oxidase, as previously reported [26, 31, 32]. In addition, we demonstrated increased expression of the NADPH oxidase subunits, NOX1, NOX4, p22phox and p47phox, in palmitate-induced

HUVECS. The formation of ROS is further aggravated by reduced antioxidant defenses, leading to oxidative damage and endothelial cell dysfunction [33]. The intracellular antioxidant system includes SOD and CAT that metabolize toxic oxidative stress intermediates; SOD catalyzes the conversion of superoxide to H_2O_2 and O_2 and CAT converts H_2O_2 to water [34] and given that we demonstrate a significant restoration of SOD and CAT activity in the presence of C1-C4, we propose that reducing ROS production by these glycomimetic compounds act in the management of FFA-induced endothelial dysfunction and vascular complications.

Small molecule glycomimetics were able to reduce ROS production through decreasing NADPH oxidase activity and down-regulating the expression of its subunits. Moreover, the tested glycomimetics decreased lipid peroxidation, an important intermediary process in oxidative stress, and ameliorated intracellular redox status, as shown by the enhanced activity of SOD and CAT. The potentiation of antioxidant defenses and normalization of ROS would appear to contribute to the restoration of endothelial function.

To understand the molecular basis for the protective effects of the glycomimetics, we hypothesized a specific mechanism that could be associated to their potential, to elicit cell adaptive responses involving the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2). Nrf2 is a ubiquitous transcription factor, which plays an important role in cellular resistance to oxidative stress. The Nrf2 protein is sequestered in the cytosol, by the actin-binding protein, Kelch-like ECH-associated protein 1 (Keap1). Under physiological conditions, Keap1 serves as an adapter for cullin 3 (Cul3)/ring-box 1 (Rbx1)-mediated ubiquitination and degradation of Nrf2, which decreases Nrf2 activity [35]. The generation of ROS causes KEAP1 to undergo a conformational change, thus, dissociating from Nrf2, which in turn, translocates to the nucleus. Within the nucleus, it regulates antioxidant response element (ARE)-mediated expression of anti-oxidant enzyme and cytoprotective proteins, including the transcription of downstream target genes, such as NAD(P)H:quinone oxido-reductase 1 (NQO1), heme oxygenase-1 (HO-1), glutathione peroxidase (GPx) and the superoxide dismutase (SOD) family [36, 37] and CAT [31, 38].

We therefore tested the hypothesis that Nrf2 activation plays a crucial role in protecting the endothelium from oxidative injury when endothelial cells were treated with palmitate. Our results demonstrate a significant decline in Nrf2 and its downstream cytoprotective proteins. These findings could be explained in terms of palmitate-induced overproduction of ROS. It has been reported by Mann et al. [39] that the basal activity of NADPH oxidase produces ROS which activate Nrf2/ARE-mediated antioxidant genes in order to maintain redox homeostasis. However, in this acute model, using palmitate, we suggest that excessive ROS production enhances the feedback mechanism and leads to Nrf2 down-regulation. Our hypothesis is based on results of studies demonstrating down-regulation of Nrf2 pathway under oxidative stress conditions. In this context, oscillating high glucose [40] and disturbed shear stress on the vascular wall resulting from oscillatory blood flow [41], diminishes the Nrf2-mediated activation of ARE-linked genes. A high-fat diet also evoked a significant increase in the mRNA abundance of HO-1 in the aortas of Nrf2+/+ mice but not Nrf2-/- mice, suggesting that adaptive activation of the Nrf2/ARE pathway confers endothelial protection [42]. More recently, Fratantonio et al. [43] reported a significantly down-regulated Nrf2, NQO-1 and HO-1 expression in HUVECS exposed to palmitic acid for 3 hours, lending support for the findings of this study.

For the first time, we demonstrate that small molecule glycomimetics are able to induce Nrf2 under FFA conditions, thus protecting against oxidative stress. As a consequence of Nrf2 activation, NQO-1 and HO-1 gene and protein expression was up-regulated following glycomimetic pretreatment. Multiple studies support the notion that induced expression of Nrf2/ARE-regulated cytoprotective proteins may contribute to the atheroprotective phenotype in endothelial cells [44,45]. A negative feedback regulatory loop between NADPH oxidase and Nrf2 has been suggested. Under homeostatic conditions, NOX4 generates superoxide and H_2O_2 which activate Nrf2 by inactivating Keap1 [46]. The activated Nrf2 then inhibits the transcription of NOX4 to lower ROS production [47,48]. HO-1 reduces oxidative stress-induced cell damage by restoring the balance of antioxidants and pro-oxidants in the vasculature. HO-1 is also known to degrade the pro-oxidant haem to biliverdin, which is subsequently converted to the radical scavenger bilirubin [49]. By interrupting the assembly and activation of the enzyme, bilirubin may directly inhibit NADPH oxidase in endothelial cells, both *in vitro* and *in vivo* [50,51]. Hence, Nrf2/HO-1 pathway represents a key adaptive mechanism for moderating the severity of oxidative stress provoked cell damage.

Having demonstrated the protective effects of small molecule glycomimetics on FFA-induced endothelial dysfunction *in vitro*, we have evaluated their effects on endothelium-dependent vasodilatation and ROS production *ex vivo* in the presence of palmitate. Mouse aortic rings incubated with palmitate for 24 hours exhibited impaired ACh-induced relaxation. The diminished vasodilatation was associated with increased NADPH oxidase activity and ROS production, confirmed by DHE staining, in palmitate-induced mouse aortic rings, in agreement with Toral et al. [26]. They also reported diminished endothelium-dependent vasodilatation in aortas from high fat diet-fed mice. When the NADPH oxidase inhibitor apocynin and the mitochondrial antioxidant mitoQ were applied, both partially counteracted the palmitate-induced impairment of endothelium-dependent vasodilatation, strengthening the findings of Toral et al. [26], thus confirming activation of oxidative stress pathways. Of note, all tested small molecule glycomimetics were able to improve endothelium-dependent relaxation, diminish ROS production and decrease activity of NADPH oxidase in mouse aortic rings. Moreover, incubation of the aortic rings with a high concentration of the eNOS inhibitor L-NAME almost abolished the ACh-induced vasodilatation, indicating that it is completely dependent on eNOS-derived NO. These findings suggest that small molecule glycomimetics exert their effects on vascular relaxation, mainly through inhibition of ROS production and an increase in NO bioavailability.

In conclusion, we provide the first evidence that small molecule glycomimetics exert protective effects against FFA-induced endothelial dysfunction. Our mechanistic evidence suggests the involvement of Akt/eNOS and Nrf2/ARE signaling pathways in mediating the effects of glycomimetics, summarized schematically in Figure 9. These chemical probes increased NO bioavailability through their ability to positively regulate Akt and eNOS gene expression as well as protein phosphorylation. In addition, the coordinate induction of cytoprotective proteins through activation of the Nrf2/ARE pathway potentially protected the endothelium against FFA-induced oxidative stress. Therefore, small molecule glycomimetics represent a field of great interest for application in the prevention and therapy of FFA-induced endothelial dysfunction as well as for other diseases related to oxidative stress and warrant further investigation.

ACKNOWLEDGMENTS

The authors wish to thank the Science & Technology Development Fund (STDF; EGYPT) and the International Atherosclerosis Society (IAS; USA) for supporting the travel of Ayman Mahmoud. The authors thank Faye Owen and Nicola Neale for technical assistance.

DISCLOSURES

The authors and Manchester Metropolitan University have a commercial interest in glycomimetics C1-C4 and their method of use (UK Patent Application No. 1515850.4 in the name of Manchester Metropolitan University).

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Figure legends:

Figure 1. The four glycomimetics C1-C4 synthesized and used in this study.

Figure 2. Glycomimetics rescue palmitate-induced impairment of NO production. HUVECS incubated with 1 µmol/L of the glycomimetics **C1-C4** in the presence of palmitate for 3 hours (**A**) or pretreated with **C1-C4** for 12 hours followed by 3 hours palmitate (**B**). Treatment with either 1 µmol/L **C1-C4** for 3 hours along with palmitate or for 12 hours followed by the addition of palmitate for 3 hours protects against palmitate-induced reduction in NO production. Results are mean ± SEM; N = 8-12. ***P<0.01 versus control, and #P<0.05, ##P<0.01 and ###P<0.001 versus PAL. PAL, palmitate; AUC, area under curve; DAF-2, diaminofluorescein-2.

Figure 3. Glycomimetics modulate mRNA abundance and protein phosphorylation of eNOS and Akt under palmitate-induced oxidative stress. HUVECs were pretreated with **C1-C4** for 12 hours followed by 3 hours of palmitate. Treatment with palmitate decreases both Akt mRNA (**A**) and protein phosphorylation (**B**), and reduces mRNA (**C**) and phosphorylation of eNOS (**D**). All tested glycomimetics at 1 µmol/L concentration up-regulate Akt and eNOS mRNA abundance and phosphorylation in HUVECs treated with palmitate. Results are mean ± SEM; N = 6. *P<0.05 and **P<0.01 versus control, and #P<0.05, ##P<0.01 and ###P<0.001 versus PAL. PAL, palmitate; Akt, protein kinase B; eNOS, endothelial nitric oxide synthase.

Figure 4. Glycomimetics reduce palmitate-induced ROS production and oxidative stress. Glycomimetics exhibit protective effect against palmitate-induced ROS production in HUVECS either incubated with 1 µmol/L **C1-C4** in the presence of palmitate for 3 hours (**A**) or pretreated with **C1-C4** for 12 hours followed by 3 hours palmitate (**B**). HUVECs treated with palmitate for 3 hours show significant increase in MDA, a marker of lipid peroxidation (**C**), and concomitant decrease in the activity of SOD (**D**) and CAT (**E**). Pretreatment with 1 µmol/L **C1-C4** prevent palmitate-induced lipid peroxidation and diminished activity of antioxidant enzymes in HUVECs. Results are mean ± SEM; N = 6-10. **P<0.01 and ***P<0.001 versus control, and #P<0.05, ##P<0.01 and ###P<0.001 versus PAL. PAL, palmitate; ROS, reactive oxygen species; MDA, malondialdehyde, SOD, superoxide dismutase; CAT, catalase.

Figure 5. Glycomimetics modulate NADPH oxidase activity in HUVECs in response to palmitate. HUVECS pretreated with **C1-C4** for 12 hours followed by 3 hours palmitate. (**A**) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence. (**B-E**) Expression of NADPH oxidase subunits NOX1, NOX4, $p22^{phox}$ and $p47^{phox}$ at the level of mRNA by RT-PCR. Palmitate increases both NADPH oxidase activity and expression of the enzyme subunits. **C1-C4** reduce palmitate-induced NADPH oxidase activation and expression in HUVECs treated 12 hours with the addition of palmitate for 3 hours. Results are mean ± SEM; N = 6-8. **P<0.01 and ***P<0.001 versus control, and #P<0.05, ##P<0.01 and ###P<0.001 versus PAL. PAL, palmitate; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase.

Figure 6. Glycomimetics protect against palmitate-induced oxidative stress via the Nrf2/ARE pathway. The effect of **C1-C4** and palmitate treatment on HUVECs with regard to Nrf2 (**A** & **B**), NQO-1 (**C** & **D**) and HO-1 (**E** & **F**) levels was determined using qRT-PCR and western blotting. Treatment with palmitate produces a significant decrease in both mRNA and protein levels of Nrf2, NQO-1 and HO-1. Pretreatment with 1 µmol/L **C1-C4** increases levels of Nrf2, NQO-1 and HO-1 in HUVECs treated with palmitate. Results are mean ± SEM; N = 6. *P<0.05, **P<0.01 and ***P<0.001 versus control, and #P<0.05, ##P<0.01 and ###P<0.001 versus PAL. PAL, palmitate; Nrf2, nuclear factor-erythroid 2-related factor 2; NQO-1, NAD(P)H:quinone oxido-reductase 1; HO-1, heme oxygenase-1.

Figure 7. Glycomimetics restore endothelium-dependent vasodilatation *ex vivo*. Mice aortic rings were incubated with 100 µmol/L palmitate and 1 or 10 µmol/L of (**A**) **C1**, (**B**) **C2**, (**C**) **C3** or (**D**) **C4** for 24 hours. Concentration-relaxation response curves to ACh were performed in intact rings pre-contracted by U46619. Treatment of aortic rings with palmitate for 24 hours diminished endothelium-dependent vasodilator responses to ACh, an effect that was significantly reversed by either dose of the glycomimetics **C1-C4**. (**E** & **F**) ROS are involved in endothelial dysfunction induced by palmitate. MitoQ and apocynin improve the impaired aortic relaxation in response to ACh, induced by palmitate. Results are mean ± SEM; N = 8-12. *P<0.05, **P<0.01 and ***P<0.001 versus control, and #P<0.05, ##P<0.01 and ###P<0.001 versus PAL; CT, control; PAL, palmitate; ACh, acetylcholine.

Figure 8. Glycomimetics reduce vascular ROS levels by reducing NADPH oxidase activity *ex vivo*. (**A**) aortic rings were incubated with 100 µmol/L palmitate and 1 or 10 µmol/L of **C1-C4** for 24 hours and the level of red fluorescence was measured in sections of aorta incubated with DHE. Treatment of aortic rings with palmitate for 24 hours increased staining in adventitial, medial and endothelial cells. Co-incubation of rings with either 1 or 10 µmol/L **C1-C4** produced a significant (P<0.001) reduction in the red fluorescence. (B) NADPH oxidase activity increased in aortic rings treated palmitate for 24 hours. Both doses of **C1-C4** reduce palmitate-induced NADPH oxidase activity. Results are mean ± SEM; N = 6. ***P<0.001 versus control, and ##P<0.01 and ###P<0.001 versus PAL. PAL, palmitate; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; DHE, dihydroethidium. Scale bar = 50 µm. X400.

Figure 9. Schematic diagram demonstrating the protective effects of small molecule glycomimetics against FFA-induced endothelial dysfunction. FFA, free fatty acid; Akt, protein kinase B, eNOS, endothelial nitric oxide synthase; NO, nitric oxide; Nrf2, nuclear factor-erythroid 2-related factor 2; NQO-1, NAD(P)H:quinone oxido-reductase 1; HO-1, heme oxygenase-1; $O_2^{\bullet-}$, superoxide radical; $ONOO^{\bullet}$, peroxynitrite; GPx; glutathione peroxidase; GST, glutathione-S-transferase; SOD, superoxide dismutase; CAT, catalase; ARE, antioxidant response element.