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Cellular crosstalk between TNF-α, NADPH oxidase, PKCβ2, and C2GNT in human leukocytes

Joanna M. Tarr [a,*], Ning Ding [a,1], Kirti Kaul [a], Anna Antonell [b,2], Luis A. Pérez-Jurado [b], Rakesh Chibber [a]

[a] Institute of Biomedical and Clinical Science, Peninsula College of Medicine and Dentistry, Universities of Exeter & Plymouth, UK
[b] Genetics Unit, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Barcelona, Spain

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ABSTRACT

Increasing evidence suggests that chronic, sub-clinical inflammation plays an important role in the pathogenesis of diabetic retinopathy. We have established the potential role of the inflammatory enzyme, core 2 β-1, 6-N-acetylglucosaminyltransferase (C2GNT) in diabetic retinopathy. The present study was designed to explore the NADPH oxidase signaling pathway in the tumor necrosis factor-alpha (TNF-α)-induced activity of C2GNT in leukocytes. Human leukocytes (U937 cells) and an Epstein–Barr-transformed lymphoblastoid cell line deficient in p47phox (F10007 cells) were used for the study. Cells were exposed to TNF-α for 24 h in the presence and absence of 1) NADPH oxidase inhibitors (apocynin and scrambled and unscrambled gp91ds-tat), 2) LY379196 (specific protein kinase C β1/2 (PKCβ1/2) inhibitor), and 3) the antioxidant tiron. Subsequent C2GNT and NADPH activity was measured and the adhesion of U937 and F10007 cells to endothelial cells was assessed. TNF-α-induced C2GNT activity (1813 ± 326 pmol/h/mg protein) (mean ± SEM) in human leukocytes was significantly reversed with apocynin (153 ± 82 pmol/h/mg protein), unscrambled gp91ds-tat (244 ± 122 pmol/h/mg protein) and tiron (756 ± 87 pmol/h/mg protein). We further supported this C2GNT–NADPH oxidase link using p47phox-deficient leukocytes. The deficiency in p47phox prevented TNF-α-induced NADPH oxidase and C2GNT activity and adherence to endothelial cells. The response to TNF-α was restored by transfection with an expression plasmid containing a p47phox cDNA inserted in the sense direction. Our results demonstrate for the first time a novel signaling crosstalk between TNF-α, NADPH oxidase, PKCβ1/2 and C2GNT in leukocytes.

1. Introduction

Despite marked improvements in the treatment of diabetes and its complications, diabetic retinopathy remains a leading cause of blindness and vision impairment in working-age adults [1]. The two most threatening complications of diabetic retinopathy are diabetic macular edema and proliferative diabetic retinopathy [2,3].

Growing evidence suggests that diabetic retinopathy is an inflammatory condition associated with increased stickiness and adhesion of leukocytes in the capillaries supplying the retina [4–8]. This leads to areas of limited blood flow, retinal tissue damage and the development of retinopathy. In recent years, we have focused on leukocytes and provided evidence that modification of O-linked carbohydrate side-chains on the surface of leukocytes is responsible for their increased adhesion to endothelial cells in diabetic patients [9]. These carbohydrate side-chains involved in leukocyte–endothelial cell adhesion [10], are mainly synthesized by the golgi enzyme UDP-GlcNAc: Galβ1, 3GalNAc-R (GlcNAc to GalNAc) β1, and 6-N-acetylglucosaminyltransferase (i.e., C2GNT, EC 2.4.1.102) [11]. The enzyme converts core 1 (i.e. Gal (β1-3) GalNAc (α1-O)) to core 2 structures (i.e. Galβ 1, 3[GlcNAcβ1, 6] GalNAcα-O) expressed on serine or threonine residues [12,13]. The O-linked glycans synthesized by C2GNT are associated with cellular adhesion, immune responses, and disease states, such as malignant transformation, T-cell activation, inflammation, myocardial dysfunction, capillary morphogenesis, and myeloblastic leukemia [14–21].

Abbreviations: BREC, bovine retinal endothelial cells; C2GNT, core 2 β-1,6-*N*-acetylglucosaminyltransferase; CD62E, E-selectin; CD62L, L-selectin; CD62P, P-selectin; GlcNAc, D-N-acetylglucosamine; ICAM-1, intracellular adhesion molecule-1; NADPH, nicotinamide adenine dinucleotide phosphate; PKC, protein kinase C; PMN, polymorphonuclear; sLEx, sialy-Lewis x; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species.

* Corresponding author at: Institute of Biomedical & Clinical Science, Peninsula College of Medicine and Dentistry, University of Exeter, St Luke's Campus, Magdalen Road, Exeter, EX1 2LU, UK. Tel.: +44 1392 726336; fax: +44 1392 722926.
E-mail addresses: joanna.tarr@pms.ac.uk (J.M. Tarr), sallydn1238@hotmail.com (N. Ding), kirti.kaul@pcmd.ac.uk (K. Kaul), anna.antonell@gmail.com (A. Antonell), luis.perez@upf.edu (L.A. Pérez-Jurado), rakesh.chibber@pms.ac.uk (R. Chibber).
[1] Present address: Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Ophthalmology & Visual Sciences Key Laboratory, Beijing 100730, China.
[2] Present address: Neurology Service, Hospital Clínic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), Barcelona, Spain.

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The activity of C2GNT is significantly higher in leukocytes of type 1 and type 2 diabetic patients with retinopathy and is associated with their increased adhesion to cultured retinal capillary endothelial cells [22]. To date we have shown that the increased levels of tumor necrosis factor-alpha (TNF-α) in diabetic patients induce C2GNT activity via protein phosphorylation mediated by serine/threonine protein kinase Cβ2 (PKCβ2) [23–25]. In this study we aim to further resolve the pathway by which TNF-α induces the activity of C2GNT.

TNF-α is known to cause increased production of reactive oxygen species (ROS) via signaling with nuclear factor kappa B (NFκB) and NADPH oxidase [26]. It is thought that TNF-α causes the serine phosphorylation and translocation of p47phox to combine with the other subunits of NADPH oxidase and enhance the subsequent production of ROS [27,28]. The serine phosphorylation of p47phox can occur due to the activation of PKCβ2 [29]. It has been shown that ROS are able to cause the translocation and the activation of PKCβ2 [30–32]. It is proposed that ROS causes activation of PKCβ2 by causing tyrosine phosphorylation of the catalytic site [30]. In addition it has been shown that the oxidative modification of the zinc finger structures of PKCβ2 can lead to decreased autoinhibition by the regulatory domain [32].

We explored the possibility that TNF-α-induced activity of C2GNT is regulated via the NADPH oxidase signaling pathway. Increasing evidence suggests that NADPH oxidase, which was originally identified and characterized in phagocytes, is the most important source of cellular ROS in blood vessels [33]. The NADPH oxidase complex consists of two membrane-bound components, gp91phox (also known as Nox2) and p22phox, and several cytosolic regulatory subunits, including p40phox, p47phox, p67phox, and the small GTPase Rac (Rac1 or Rac2) [34,35]. Other important NOX members identified in the vasculature: include Nox4 and Nox5 [36].

Our observations demonstrate for the first time a novel intracellular crosstalk between TNF-α, C2GNT, PKCβ1/2 and NADPH oxidase in human leukocytes which results in leukocyte adhesion to endothelial cells. This study also unveils the absence of this crosstalk in p47phox-deficient lymphoblastoid cell-line from a patient with chronic granulomatous disease (CGD), a rare inherited disorder of the NADPH oxidase complex in which phagocytes are defective in generating ROS [37–39]. The affected individuals are highly susceptible to certain life-threatening bacterial and fungal infections and require lifelong antibiotic and antifungal prophylaxis [37–39].

2. Materials and methods

2.1. Culture of bovine retinal capillary endothelial cells

Bovine retinal capillary endothelial cells (BREC) were isolated and established from bovine retinas dissected from eyes of freshly slaughtered cattle as described previously [40].

2.2. Measurement of NADPH oxidase activity

Measurement of superoxide ion (O_2^-) was based on the capacity to reduce ferricytochrome c to ferrocytochrome at pH 7.8 [41]. Briefly, total cell lysate (50 μg protein/experiment), cytochrome c (250 μg/l final concentration), and NADPH (100 μM) were incubated at 37 °C for 120 min, either in the presence or absence of diphenyleneiodonium (DPI, 100 μM). The reduction of cytochrome c was measured by reading absorbance at 550 nm. O_2^- production in nmol/mg protein was calculated from the difference between absorbance of samples at 0 and 120 min and the extinction coefficient 21 mmol/l/cm.

2.3. Culture of human myelocytic cell line (U937) and p47phox-deficient cell-line

U937 cells and CGD cell-lines (European Collection of Cell Culture, ECACC) were cultured in glucose-free RPMI medium (Sigma, Poole, UK) supplemented with 10% FCS, antibiotics and 5 mM glucose.

Cells were treated in 3 cm dishes with 8 pg/ml human recombinant TNF-α (Sigma, Dorset, UK) in the presence and absence of 1) NADPH oxidase inhibitors (30 μM apocynin (Sigma, Dorset, UK) and 1 μM scrambled and unscrambled gp91ds-tat (Cambridge Bioscience, Cambridge, UK), 2) LY379196 (specific PKCβ1/2 inhibitor, 50 nM, (Eli Lilly, Indianapolis, USA)), and 3) the antioxidant tiron (5 mM, 5-dihydroxy-1,3-benzene disulfonic acid (Sigma, Dorset, UK)). After 24 h incubation at 37 °C, the cells were washed with ice-cold PBS and stored at −80 °C until used for the measurement of C2GNT activity.

2.4. Transfection

Full-length p47phox gene [42] was used for the transfection experiments. The cell density was adjusted to 10×10^5/ml using complete media and 2 ml of the cell suspension was added to a 3 cm dish. To 1.5 ml microfuge tube, 250 μl of serum free media was added followed by 7.5 μl of Transit LT1 transfection reagent (Mirus, Geneflow, Staffordshire, UK), the contents were mixed for 5 s. To the microfuge tube 2.5 μg of p47 phox plasmid DNA was added and thoroughly mixed for 5 s and then incubated at room temperature for 30 min. The prepared transfection reagent with p47phox gene was added drop wise to the cells. The dish was gently shaken to ensure thorough mixing and incubated for 48 h.

2.5. Leukocyte adhesion assay

In order to monitor leukocyte adhesion to endothelial cells, a flow system was established, whereby a leukocyte suspension was flowed over a monolayer of BREC. The use of a commercially available flow slide (ibidi, Thistle Scientific, Glasgow, Scotland, UK) (Fig. 5A) which was characterized to allow calculation of flow-rate-dependent shear stress, allowed this to be done at a physiological shear stress. Medium was pumped (Fig. 5A) at a flow rate of 0.8 ml/min, which generates a shear stress of 1 dyn/cm^2 in the flow slide. U937 and p47phox deficient cells were exposed to 8 pg/ml TNF-α. After 24 h incubation at 37 °C, cells were centrifuged, re-suspended and flowed over the BREC monolayer. After washing out non-adherent leukocytes, attached cells were counted with phase-contrast microscopy (Fig. 5B).

2.6. Measurement of C2GNT activity

Frozen cells were removed from −80 °C, lysed in 0.45% NaCl and 0.9% Triton X-100 at 0 °C, and the activity of C2GNT measured as described previously [43].

2.7. Western blot analysis

An antibody directed against the NADPH oxidase p47phox subunit was used for Western blot analysis (Millipore, Watford, UK). A tubulin antibody (Sigma, Dorset UK) was used as a loading control.

2.8. Protein measurement

Total protein was measured using the BCA protein assay kit (Sigma, Dorset, UK).