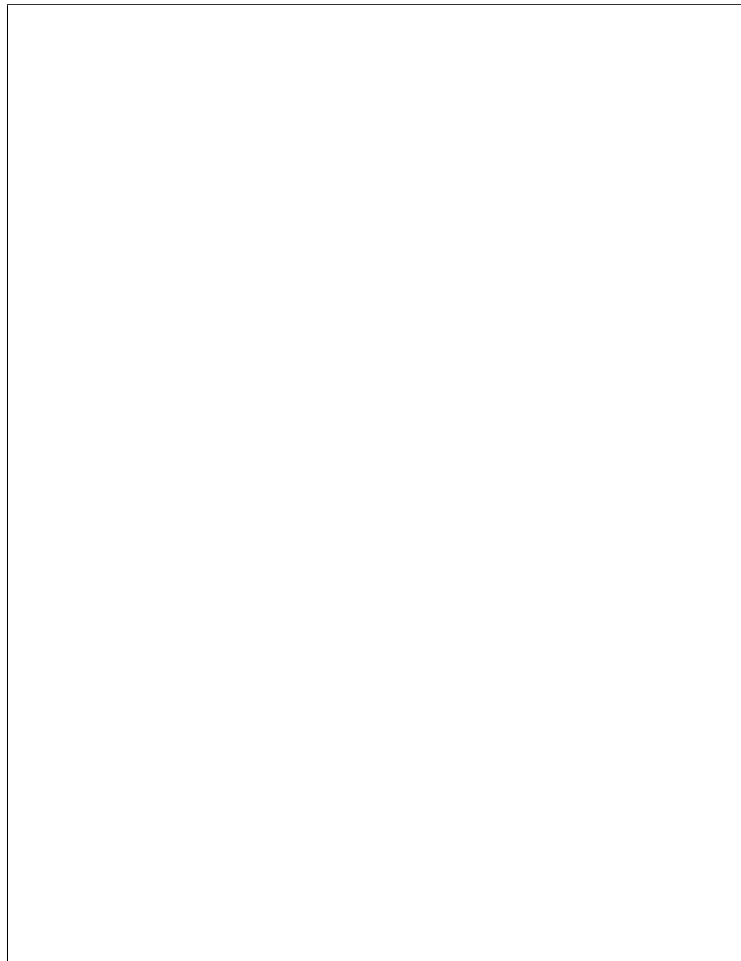


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Research Paper

Hyperglycaemia promotes human brain microvascular endothelial cell apoptosis via induction of protein kinase C- β_1 and prooxidant enzyme NADPH oxidase

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ABSTRACT

Blood–brain barrier disruption represents a key feature in hyperglycaemia-aggravated cerebral damage after an ischaemic stroke. Although the underlying mechanisms remain largely unknown, activation of protein kinase C (PKC) is thought to play a critical role. This study examined whether apoptosis of human brain microvascular endothelial cells (HBMEC) might contribute to hyperglycaemia-evoked barrier damage and assessed the specific role of PKC in this phenomenon. Treatments with hyperglycaemia (25 mM) or phorbol myristate acetate (PMA, a protein kinase C activator, 100 nM) significantly increased NADPH oxidase activity, $O_2^{\bullet-}$ generation, proapoptotic protein Bax expression, TUNEL-positive staining and caspase-3/7 activities. Pharmacological inhibition of NADPH oxidase, PKC- α , PKC- β or PKC- β_1 via their specific inhibitors and neutralisation of $O_2^{\bullet-}$ by a cell-permeable superoxide dismutase mimetic, MnTBAP normalised all the aforementioned increases induced by hyperglycaemia. Suppression of these PKC isoforms also negated the stimulatory effects of hyperglycaemia on the protein expression of NADPH oxidase membrane-bound components, Nox2 and p22-phox which determine the overall enzymatic activity. Silencing of PKC- β_1 gene through use of specific siRNAs abolished the effects of both hyperglycaemia and PMA on endothelial cell NADPH oxidase activity, $O_2^{\bullet-}$ production and apoptosis and consequently improved the integrity and function of an in vitro model of human cerebral barrier comprising HBMEC, astrocytes and pericytes. Hyperglycaemia-mediated apoptosis of HBMEC contributes to cerebral barrier dysfunction and is modulated by sequential activations of PKC- β_1 and NADPH oxidase.

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Introduction

Diabetes and acute stress hyperglycaemia increase the rates of mortality and morbidity in patients with ischaemic stroke by exacerbating the breakdown of blood–brain barrier (BBB) [1]. Endothelial cells cover the entire inner surface of all blood vessels and help form the BBB in the central nervous system to prevent the entry of circulating molecules into the brain parenchyma. Anomalies in programmed death (apoptosis) of cells which make up the BBB, especially that of brain microvascular endothelial cells (BMEC), may represent a key phenomenon in hyperglycaemia-evoked BBB disruption. Indeed, the apoptotic effect of hyperglycaemia on endothelial cells derived from large arteries and venous system e.g. aorta and umbilical vein is well-documented [2,3]. Although the mechanisms involved remain unclear, hyperglycaemia is known to elicit cellular injury by increasing the quantities of pro-apoptotic proteins (Bad, Bax and Bid) and

possibly by decreasing those of anti-apoptotic bcl-2 proteins (Bcl-2 and bcl-XL) [4]. Sufficient elevations in Bax to Bcl-2 ratio in turn trigger the conversion of inactive procaspase-3 to caspase-3, the major executor of apoptotic process [3].

Hyperglycaemia can also induce apoptosis through stimulation of oxidative stress characterised by the exaggerated synthesis or release of free radicals, in particular superoxide anion ($O_2^{\bullet-}$) [5,6]. Higher concentrations of $O_2^{\bullet-}$ may mediate the rates of endothelial cell apoptosis through activation of a variety of redox-sensitive mechanisms including mitochondrial dysfunction, Bax protein overexpression and p38 mitogen-activated protein kinase (p38MAPK) [3,4,7]. As the main source of $O_2^{\bullet-}$ in vasculature, NADPH oxidase is anticipated to be intimately involved in BMEC survival/death [8,9].

Hyperglycaemia-mediated activation of protein kinase C (PKC) is regarded as a key step in peripheral and cerebral vasculopathies where activation of different PKC isoforms, particularly PKC- β is coupled to diabetes-induced endothelial dysfunction and neurovascular complications [10,11]. Besides PKC- β , activations of other PKC isoforms have also been shown to induce apoptosis through utilisation of different downstream effectors. For instance, stimulation of

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p38 α MAPK by PKC- δ and NADPH oxidase-mediated O₂^{•-} production by PKC- β_{II} have been shown to account for hyperglycaemia- and TNF- α -induced apoptosis of bovine retinal pericytes and human umbilical vein endothelial cells, respectively [6,12].

In light of the above, the aims of the current study were three-fold. Firstly, it was to investigate whether and to what extent hyperglycaemia could compromise BMEC apoptosis. Secondly, it was to determine if activation of PKC compromised BMEC viability in a comparable manner. Finally, it was to assess whether inhibition of PKC- β_I might prevent cells from undergoing apoptosis and thus protect BBB integrity.

Materials and methods

Cell culture

Human BMEC (HBMEC) (TCS Cellworks Ltd., Buckingham, UK), between passages 4 and 7, were cultured to almost 70% confluence in its specialised media before exposure to normoglycaemia (NG; 5.5 mM D-glucose), hyperglycaemia (HG; 25 mM D-glucose) or D-mannitol (5.5 mM D-glucose + 19.5 mM D-mannitol) for 72 h. The relevance of individual PKC isoforms to hyperglycaemia-mediated particular outcomes was investigated through simultaneous application of specific inhibitors for PKC- α (Ro-32-0432, 1 μ M), PKC- β (LY333531, 0.05 μ M) or PKC- β_{II} (CGP53353, 1 μ M) with hyperglycaemia for 72 h. Potential contribution of O₂^{•-} (MnTBAP, 2.5 μ M), NADPH oxidase (apocynin, 1 mM), nitric oxide synthase (L-NAME, 100 μ M), xanthine oxidase (allopurinol, 100 μ M), mitochondrial complex I (rotenone, 2 μ M) and cyclooxygenase (indomethacin, 50 μ M) to hyperglycaemia-related changes was also studied in similar conditions using specific inhibitors indicated in parentheses.

To ascertain the specific role of PKC activation in the outlined parameters, in some experiments HBMEC were treated with a PKC activator, phorbol myristate acetate (PMA, 0.1 μ M) for 4 h.

TUNEL staining

This is performed for detection and quantification of apoptosis at a single cell level, based on in situ labelling of fragmented DNA by the DeadEnd colourimetric TUNEL system (Promega, WI, USA). Apoptotic cells, characterised by dark brown staining, were then counted via light microscopy.

Caspase-3/7 assay

In view of the roles of the caspase family members in mammalian cell apoptosis, the activities of caspase-3/7 were also measured in HBMEC exposed to different experimental conditions using the Apo-ONE homogeneous caspase-3/7 assay kit (Promega). Briefly, onto HBMEC grown in 96-well opaque black plates, 100 μ l of diluted caspase-3/7 profluorescent substrate were added. The mixture was incubated at room temperature for 2 h and the enzymatic activities were assessed using a fluorescent plate reader.

Establishment of an in vitro model of human BBB

A triple-culture model of human BBB was set up. For this, human astrocytes were seeded onto the outer surface of Transwell inserts (12 mm diameter, 0.4 μ m pore size; Corning Costar, High Wycombe, UK) directed upside down. On the following day, the inserts were inverted the correct way and placed into 12-well culture plates containing human pericytes. HBMEC were then seeded onto the inner side of the inserts before culturing all cell layers to confluence.

Analyses of the BBB characteristics

The integrity and function of the BBB were evaluated as previously described by transendothelial electrical resistance and the flux of high (Evan's Blue-labelled albumin, 67 kDa) and low (sodium fluorescein, 376 Da) molecular weight permeability markers, respectively [13].

Western blotting

Equal amounts of total cellular proteins (~40 μ g) were run on 10–15% sodium dodecyl sulphate-polyacrylamide gels before transferring onto polyvinylidene fluoride membranes and successively incubating with a combination of β -actin (reference protein, Sigma Aldrich, Poole, UK) and anti-Bax (Cell Signalling Technology, Danvers, MA, USA), Nox2, PKC- β_I (Santa Cruz, Dallas, Texas, USA) or p22-phox (Abcam, Cambridge, UK) primary antibodies and appropriate infrared dye-conjugated secondary antibodies (Li-Cor Biosci, Cambridge, UK). Blots were analysed by densitometry using the Li-Cor Odyssey infrared imaging system.

Measurements of O₂^{•-} production and NADPH oxidase activity

The level of O₂^{•-} production was measured by cytochrome C reduction as previously described [8,14]. In brief, cell pellets were sonicated in cold lysis buffer containing 20 mM HEPES buffer (pH 7.2), 1 mM EGTA, 210 mM mannitol and 70 mM sucrose. Equal amounts of homogenate (50 μ l) were then incubated with 50 μ M cytochrome C for 60 min at 37 °C. O₂^{•-} generation was measured as the reduction of cytochrome C and monitored as the change in absorbance at 550 nm using a FLUOstar Omega plate reader (BMG, Aylesbury, UK).

NAD(P)H oxidase activity was measured by the lucigenin chemiluminescence assay. HBMEC homogenates (50 μ l) were incubated at 37 °C with assay buffer (50 mM potassium phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose, and 5 μ M lucigenin) containing the specific inhibitors of enzymes that are known to generate reactive oxygen species (ROS), namely nitric oxide synthase (L-NAME, 100 μ M), xanthine oxidase (allopurinol, 100 μ M), mitochondrial complex I (rotenone, 50 μ M) and cyclooxygenase (indomethacin, 50 μ M). After 15 min NADPH (100 μ M; Sigma Aldrich, Poole, UK) was added to initiate the reaction. The reaction was monitored every minute for 4 h and the rate of reaction calculated. Buffer blanks were also run for both assays and subtracted from the data.

Small interfering RNA knockdown

Semi-confluent HBMEC were transfected for 24 h with DharmaFECT small interfering RNA (siRNA) transfection reagent 4 containing 50 nM of ON-TARGET plus SMART pool human siRNA against PKC- β_I (Thermo Scientific Dharmacon, Lafayette, CO, USA). HBMEC transfected with non-targeting pool of siRNA served as controls. After exposure to different experimental conditions, HBMEC were harvested for different assays.

Statistical analysis

Data are presented as mean \pm SEM. Statistical analyses were performed using GraphPad Prism 6.0 statistical software package. Data were analysed by nonparametric Mann-Whitney *U* test or one-way ANOVA followed by Dunnett's post-hoc analyses, where appropriate. *P* < 0.05 was considered significant.

Results

Hyperglycaemia evokes oxidative stress in endothelial cells via activation of NADPH oxidase

Exposure of HBMEC to hyperglycaemia led to a significant increase in total $O_2^{\bullet-}$ generation which was normalised by specific inhibition of NADPH oxidase, PKC- α , PKC- β and PKC- β_{II} but not those of nitric oxide synthase, mitochondrial complex I, cyclooxygenase or xanthine oxidase. Treatment of HBMEC with a potent free radical scavenger, MnTBAP almost completely abolished the hyperglycaemia-evoked $O_2^{\bullet-}$ generation. Inhibition of the aforementioned PKC isoforms negated hyperglycaemia-mediated increases observed in protein expressions of NADPH oxidase membrane-bound components, namely p22-phox and Nox2 which determine enzymatic activity and stability as a whole (Fig. 1A–C).

Hyperglycaemia promotes endothelial cell apoptosis through NADPH oxidase and PKC activations

Hyperglycaemia significantly increased TUNEL-positive staining (DNA fragmentation), caspase-3/7 activities and Bax protein expression in HBMEC which appeared to be independent of hyperglycaemia-mediated increases in osmolality. Inhibition of NADPH oxidase, PKC- α , PKC- β and PKC- β_{II} and neutralisation of $O_2^{\bullet-}$ effectively suppressed all the increases observed in the apoptotic parameters. However, reductions in DNA fragmentation rates stayed significantly higher in MnTBAP-treated cells compared to controls (Fig. 2A–E).

Induction of PKC promotes endothelial cell apoptosis

To determine whether elevations in PKC activity during a hyperglycaemic insult may constitute the main cause of apoptosis, in some experiments the PKC activity was increased by exposing HBMEC to increasing concentrations of PMA (0.1–1 μ M) for 2–4 h. As these revealed time-dependent increases in total PKC activity, in subsequent studies the cells were treated with 0.1 μ M of PMA for 4 h which produced significant increases in PKC- β_1 activity and all apoptotic parameters i.e. DNA fragmentation rates, caspase-3/7 activities and Bax protein expression (Fig. 3A–E). Silencing of PKC- β_1 gene dramatically diminished its protein expression and attenuated both hyperglycaemia- and PMA-evoked increases observed in NADPH oxidase activity and $O_2^{\bullet-}$ production (Fig. 4A–E).

Suppression of PKC- β_1 prevents apoptosis and protects cerebral barrier

Knockdown of PKC- β_1 also effectively suppressed both hyperglycaemia-induced apoptosis as evidenced by radical decreases observed in all apoptotic parameters (Fig. 5A–D). Although silencing of PKC- β_1 gene neutralised PMA-mediated increases in apoptosis rate and Bax protein expression, it failed to normalise caspase-3/7 activities (Fig. 6A–D). PKC- β_1 knockdown also negated the deleterious effects of both hyperglycaemia and PMA on cerebral barrier integrity and function as proven by marked increases in transendothelial electrical resistance and concurrent decreases in paracellular flux of Evan's Blue-labelled albumin and sodium fluorescein, respectively (Fig. 7A–C).

Discussion

Increased prevalence of BBB failure in stroke patients with diabetes bestows a pivotal role on hyperglycaemia in the pathogenesis of this defect. However, the mechanisms involved remain ambiguous and require in depth investigation in order to devise efficacious novel therapies to prevent barrier leakage. Using an in vitro model

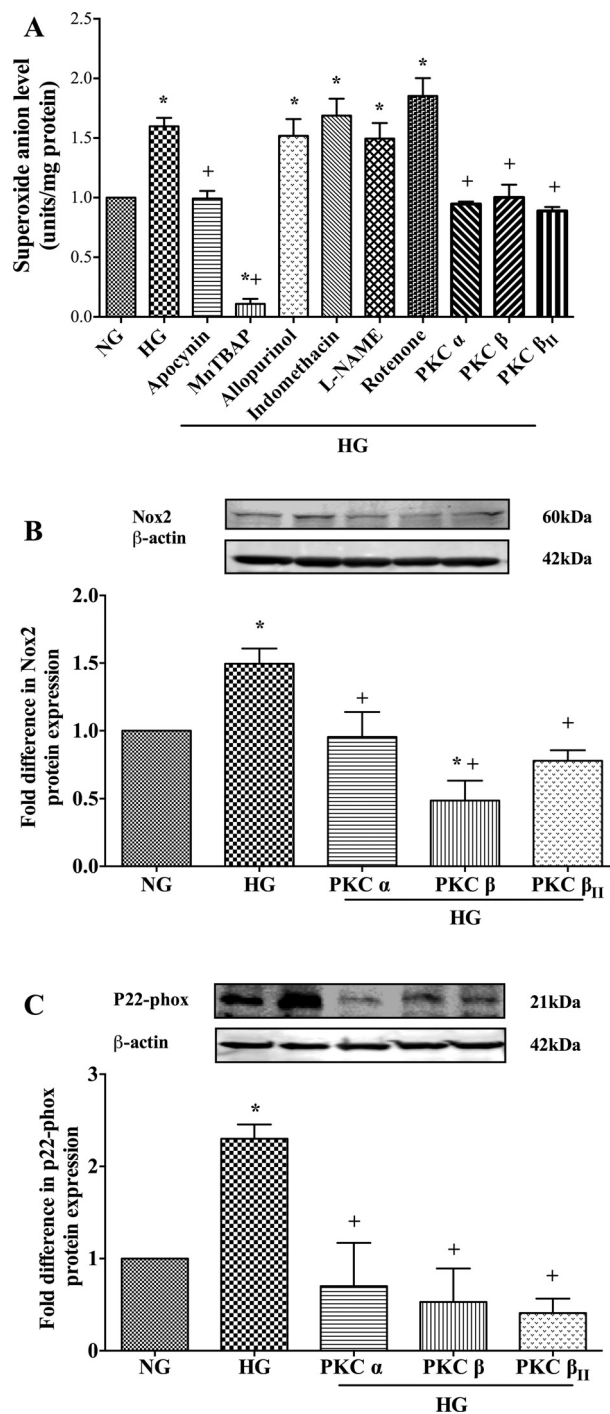


Fig. 1. (A) Effects of specific inhibitors targeting the main free radical-generating enzymes and protein kinase C (PKC)- α , PKC- β and PKC- β_{II} isoforms on $O_2^{\bullet-}$ production ($n \geq 3$) in human brain microvascular endothelial cells (HBMEC) subjected to hyperglycaemia (HG; 25 mM D-glucose) for 72 h. Effects of inhibitors for PKC- α , PKC- β and PKC- β_{II} on gp91-phox ($n = 3$) (B) and p22-phox ($n = 3$) (C) protein expressions in HBMEC exposed to HG. Data are expressed as mean \pm SEM. * $P < 0.05$ versus normoglycaemia (NG), + $P < 0.05$ versus HG.

of human cerebral barrier, we have recently shown that hyperglycaemia compromises the structural and functional capacities of BBB through a mechanism involving concurrent activations of PKC- β and NADPH oxidase [14]. In light of this, the present study investigated whether putative increases in apoptosis of BMEC, the most prominent cellular component of the cerebral barrier, may also contribute to the hyperglycaemia-evoked BBB dysfunction and is affected by the

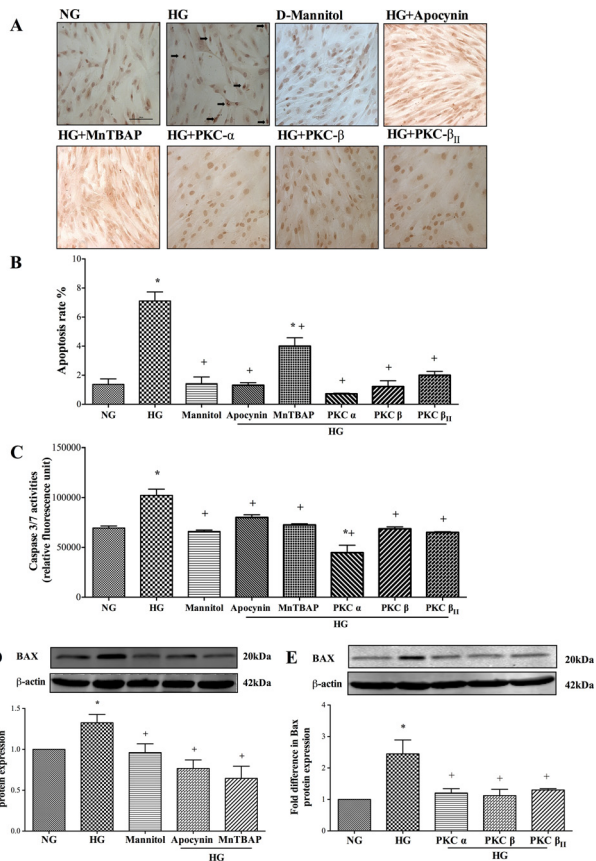


Fig. 2. Effects of inhibitors of NADPH oxidase (apocynin), superoxide anion (MnTBAP) and protein kinase C- α (PKC- α), PKC- β and PKC- β_{11} on (A) TUNEL staining (Bar, 100 μ m), (B) DNA fragmentation rates ($n \geq 3$), (C) caspase-3/7 activities ($n \geq 3$), (D, E) Bax protein expressions ($n = 4$ and 3) in human brain microvascular endothelial cells (HBMEC) exposed to hyperglycaemia (HG; 25 mM D-glucose) for 72 h. Data are expressed as mean \pm SEM. * $P < 0.05$ versus normoglycaemia (NG), + $P < 0.05$ versus HG.

activities of PKC and/or NADPH oxidase.

Accumulating evidence has displayed hyperglycaemia-mediated apoptosis as one of the key mediators of vascular complications, such as retinopathy, to which induction of NF- κ B, c-jun NH₂ terminal kinase, shp-1 and PKC- δ appear to contribute [12,15]. However, given that endothelial cells derive from different parts of the vascular tree respond to the effects of hyperglycaemia in a different manner, it was important to ascertain whether and to what extent hyperglycaemia alters BMEC viability and reveal the mechanisms involved [16]. To this end, the present study shows that hyperglycaemia evokes significant increases in HBMEC apoptosis as evidenced by increases in DNA fragmentation rates, caspase-3/7 activities and in expression of proapoptotic protein Bax. Marked increases in Bax or Bax/Bcl-2 ratio have previously been linked to hyperglycaemia-mediated apoptosis of human aortic endothelial cells [3]. Hyperglycaemia-evoked translocation of Bax protein into mitochondrial membrane leads to disruption of mitochondrial membrane integrity thereby facilitating the escape of cytochrome C from mitochondria and consequent activation of caspase-9 [3,17]. Caspase-9, in turn, activates several downstream caspases amongst which caspase-3 and caspase-7 were shown to be of particular importance in HBMEC.

Oxidative stress, associated with excessive availability of O₂^{•-} may account for hyperglycaemia-evoked apoptosis. Using specific inhibitors of the major prooxidant enzymes, the current study has shown NADPH oxidase as the main source of O₂^{•-} in hyperglycaemic endothelial cells. Indeed, specific inhibition of this oxidase protected

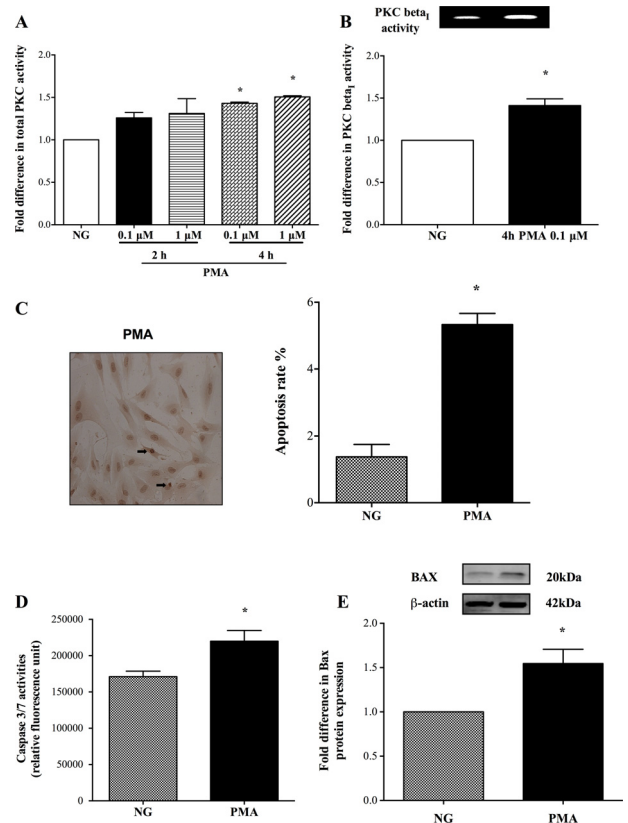


Fig. 3. Effect of phorbol myristate acetate (PMA; 0.1–1 μ M) on total protein kinase C (PKC) ($n = 4$) (A) and PKC- β_{11} ($n = 4$) (B) activities in human brain microvascular endothelial cells (HBMEC). Effect of PMA (4 h, 100 nM) on HBMEC (C) DNA fragmentation ($n \geq 3$), (D) caspase-3/7 activities ($n = 4$) and (E) Bax protein expression ($n = 4$). Data are expressed as mean \pm SEM. * $P < 0.05$ versus normoglycaemia (NG).

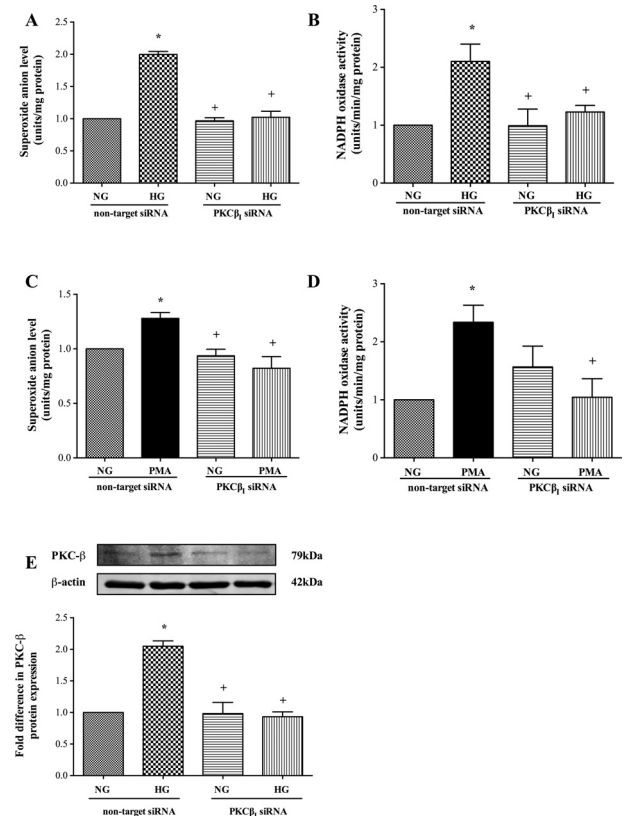


Fig. 4. Knockdown of protein kinase C (PKC)- β_{11} gene normalises the effects of hyperglycaemia (HG) (A, B) ($n = 3$ and 4) and PMA (C, D) (both $n = 3$) on superoxide anion generation and NADPH oxidase activity and reduces PKC- β_{11} protein expression ($n = 3$) (E) in human brain microvascular endothelial cells. Data are expressed as mean \pm SEM. * $P < 0.05$ versus normoglycaemia (NG), + $P < 0.05$ versus HG or PMA (4 h, 100 nM).

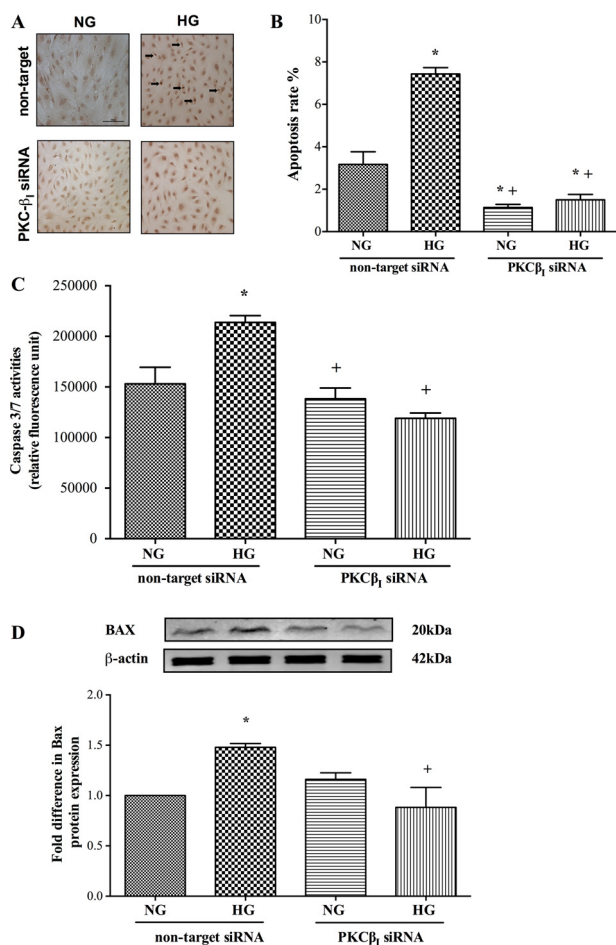


Fig. 5. Effect of protein kinase C-β₁ (PKC-β₁) gene knockdown on TUNEL staining (Bar, 100 μm) (A), DNA fragmentation rates ($n = 3$) (B), caspase-3/7 activities ($n \geq 6$) (C) and Bax protein expression ($n = 3$) (D) in human brain microvascular endothelial cells exposed to hyperglycaemia (HG). Data are expressed as mean \pm SEM. * $P < 0.05$ versus normoglycaemia (NG), + $P < 0.05$ versus HG.

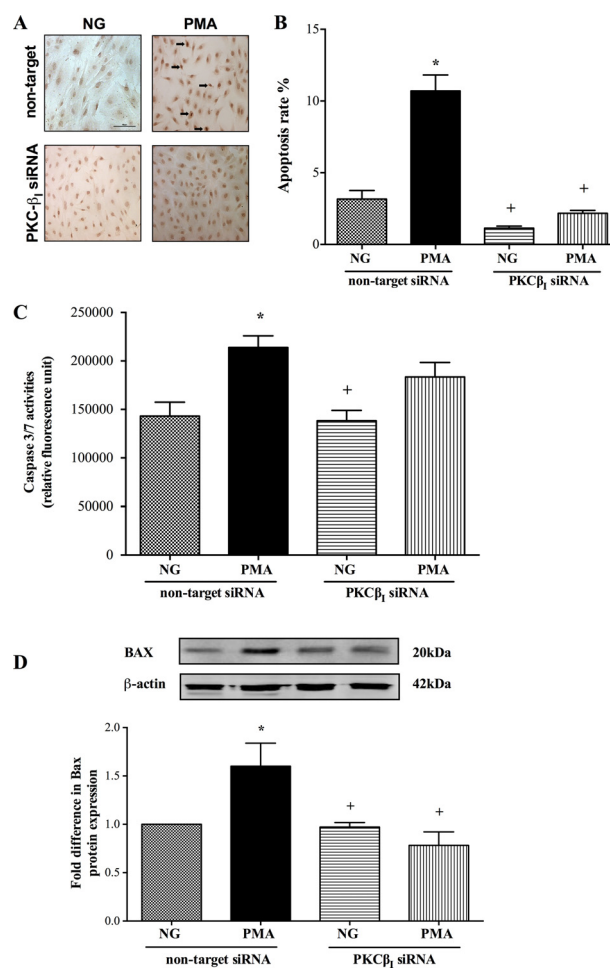


Fig. 6. Effect of protein kinase C-β₁ (PKC-β₁) gene silencing on TUNEL staining (Bar, 100 μm) (A), DNA fragmentation rates ($n = 3$) (B), caspase-3/7 activities ($n \geq 6$) (C) and Bax protein expression ($n = 3$) (D) in human brain microvascular endothelial cells exposed to PMA (100 nM) for 4 h. Data are expressed as mean \pm SEM. * $P < 0.05$ versus normoglycaemia (NG), + $P < 0.05$ versus PMA.

HBMEC from apoptosis as evidenced by marked decreases in all apoptotic parameters. Interestingly, despite almost completely eradicating the availability of O₂^{•-}, MnTBAP, a cell-permeable superoxide dismutase mimetic failed to normalise HG-mediated elevations in DNA fragmentation rates. Taken together, these data ascribe additional benefits to inhibition of vascular NADPH oxidase beyond its O₂^{•-}-related effects.

NADPH oxidases make up a dedicated family of O₂^{•-}-forming enzymes. In general, they are activated by coupling of Nox2, the catalytic subunit, with other subunits, p22-phox, p47-phox, p40-phox and p67-phox. Although seven isoforms of Nox have been identified to date, only Nox1, Nox2, Nox4 and Nox5 are known to be expressed in vascular cells [18,19]. In light of our past and present studies proving Nox2-derived O₂^{•-} as the key regulator of blood–brain barrier integrity, endothelial function and microvascular endothelial cell growth, we specifically focused on this particular isoform in the current study [20–23]. Discovery of considerably smaller cerebral infarcts in Nox2-deficient mice subjected to middle cerebral artery occlusion further corroborate the correlation between Nox2 availability and cerebral homeostasis [24].

Despite constituting the main Nox isoform in colon epithelial cells, Nox1 is also associated with production of low levels of O₂^{•-} in vasculature [25,26]. However, through a complex reaction involving concomitant induction of PKC-, MAPK- and PKA-dependent mechanisms, the vascular pathologies appear to elevate Nox1-mediated

release of O₂^{•-} [27–29] which in turn may trigger BMEC apoptosis to elicit barrier permeability. In this context, the hyperglycaemia-evoked apoptosis of a murine BMEC line, bEnd3 has recently been attributed to NF-κB-dependent upregulation of p22-phox and Nox1 isoforms. However, negation of apoptosis by agents that inhibit the activity of NADPH oxidase complex, namely apocynin and resveratrol, a polyphenolic antioxidant suggest the involvement of other Nox isoforms, in particular Nox2, in this phenomenon [30].

Unlike other isoforms of Nox, Nox4 predominantly generates H₂O₂ [31]. As it is mainly implicated in cellular senescence and vascular and renal complications of diabetes, its inhibition, in various models of diabetes, has unsurprisingly led to reduced oxidative stress, inflammation and availability of profibrotic markers and thus protected renovascular function as evidenced by attenuation of albuminuria and glomerular macrophage infiltration [32–36]. Conversely, other studies reported endogenous Nox4 as a pivotal stabiliser of vascular integrity owing to its anti-apoptotic effects which seem to be mediated, in part, by activation of akonitase in various cell types including microvascular endothelial cells exposed to diabetic, ischaemic or inflammatory stress [37–41]. Considering the alleged protective effects exerted by Nox4 itself, it is unlikely that its inhibition can contribute to apocynin-mediated BBB protection observed in this study.

In contrast to other Noxs, Nox5 does not require p22-phox for activation and is regulated in a Ca²⁺-sensitive manner [42,43]. Although the biological importance of vascular Nox5 is largely unknown, its

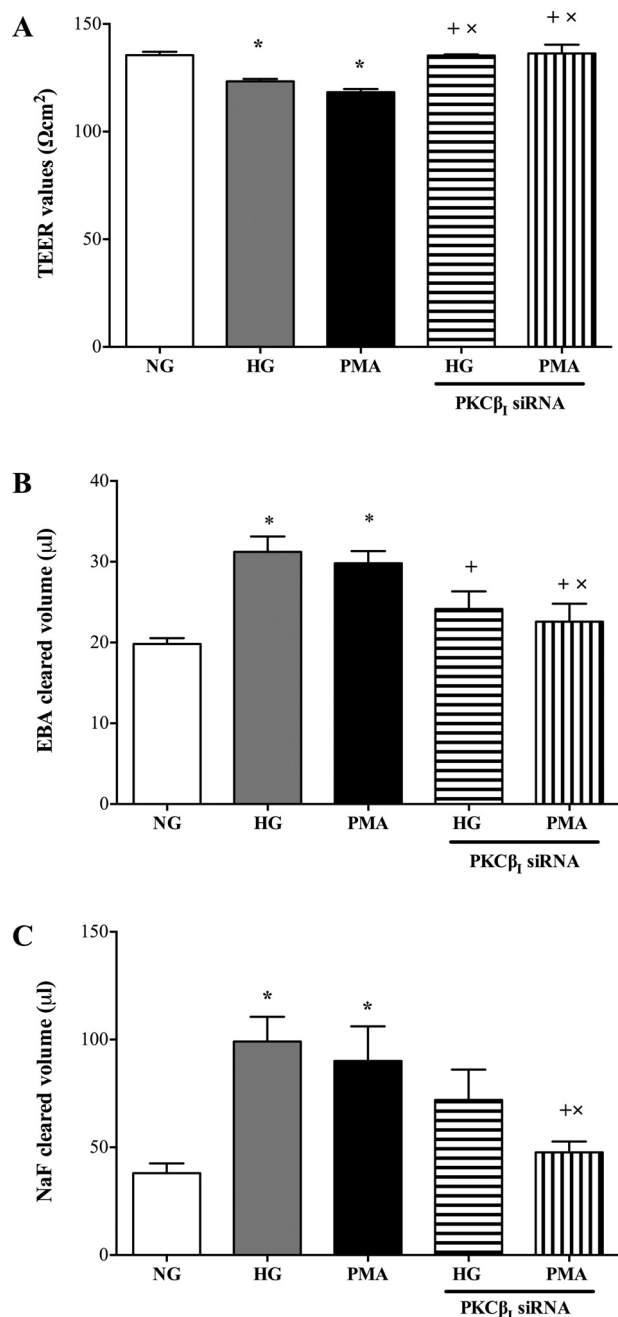


Fig. 7. Effects of protein kinase C- β_1 (PKC- β_1) gene knockdown on (A) transendothelial electrical resistance (TEER) ($n \geq 3$) and paracellular flux of high and low molecular weight permeability markers, namely Evan's Blue-labelled albumin ($n \geq 3$) (B) and sodium fluorescein ($n \geq 3$) (C) across an in vitro model of human blood-brain barrier composed of human brain microvascular endothelial cells, astrocytes and pericytes in the absence or presence of PMA (100 nM, 4 h) or hyperglycaemia (HG; 25 mM D-glucose). Data are expressed as mean \pm SEM from at least three different experiments. * $P < 0.05$ versus normoglycaemia (NG), + $P < 0.05$ versus HG, × $P < 0.05$ versus PMA.

involvement in endothelial cell proliferation and oxidative stress-mediated atherosclerotic damage imply a putative role for this isoform diabetic cerebral barrier injury [44,45].

Hyperglycaemia-mediated activation of PKC represents a key step in vasculopathies and is documented in organs that display hyperglycaemia-mediated vascular leakage such as heart and retina [46,47]. In keeping with these findings, stimulation of total PKC activity in HBMEC (by PMA) increased the rates of apoptosis in this study and consequently compromised the integrity and function of

an in vitro model of human BBB. Time-dependent increases in the expression and activities of specific PKC isoforms have previously been correlated with the severity of diabetic microvascular complications, endothelial barrier breakdown and apoptosis during cardiac ischaemia [48–50]. Naturally, suppression of PKC- α , PKC- β or PKC- β_{II} in the current study prevented BMEC apoptosis. This appeared to be modulated in part by curtailment of NADPH oxidase activity deriving from reductions in its p22-phox and Nox2 subunits, membrane-bound components that determine oxidase activity and stability and O₂^{•-} bioavailability. Decreased phosphorylation of cytosolic components, notably p47-phox stemming from PKC inhibition may also contribute to the diminished oxidase activity [14].

Increases in PKC- β and PKC- β_{II} activities have been shown to account for much of the elevated total PKC activity in hyperglycaemic HBMEC. However, despite abating total PKC activity, the inhibition of PKC- β_{II} failed to improve BBB integrity and conferred a critical role on PKC- β_I in this process and possibly in apoptosis [14]. Indeed, silencing of PKC- β_I gene in HBMEC supported this notion by radically diminishing the levels of all apoptotic parameters in cells subjected to hyperglycaemia. Concurrent suppressions of NADPH oxidase activity and O₂^{•-} release in the same cells reveal that PKC- β_I acts upstream to NADPH oxidase and regulation of oxidative stress is a crucial factor in its barrier-protective effects. Since knockdown of PKC- β_I failed to normalise the PMA-evoked increases observed in caspase-3/7 activities despite preventing DNA fragmentation and Bax expression in an efficacious fashion, it is likely that other PKC isoforms and associated downstream mechanisms possibly involving other Noxs may also contribute to the regulation of endothelial cell apoptosis. In support of this notion, hyperglycaemia- and PMA-induced production of O₂^{•-} in human lung microvascular endothelial cells has recently been ascribed to PKC- α -evoked activation of Nox5. Interestingly, while genetic silencing of PKC- ϵ also reduced PMA-stimulated Nox5 activity in these cells, suppression of PKC- δ elevated activity [51]. Furthermore, activation of PKC- ζ by disturbed blood flow has been shown to induce endothelial apoptosis by modulating p53 and thus contribute to the early events in atherosclerosis [52].

It is possible that other mechanisms such as hyperglycaemia-induced activation of matrix metalloproteinases (MMPs) may also be responsible for BMEC apoptosis similar to this reported in diabetic mice retinas [53]. Given that hyperglycaemia substantially increases MMP-2 release in HBMEC through a process involving both NADPH oxidase and PKC- β , this hypothesis warrants further investigation [14]. Activation of small GTP-binding protein RhoA by high glucose levels may also be implicated in hyperglycaemia-evoked HBMEC apoptosis due to its roles in cytoskeletal reorganisation, proapoptotic Bax expression and pro-survival phosphatidylinositol-3-kinase/Akt/endothelial nitric oxide synthase pathway [54,55].

In conclusion, the present study reveals that hyperglycaemia perturbs BBB integrity, in part, through promotion of HBMEC apoptosis by sequential activations of PKC and NADPH oxidase where targeting of PKC- β_I may be of therapeutic value. Studies with PMA have revealed that activation of PKC per se compromise endothelial cell viability and cerebral barrier integrity in equal measures and therefore justify targeting of its specific isoform(s) as efficacious therapeutic options in negating hyperglycaemic barrier damage. Exploration of growth profile of cells that make up the BBB, notably HBMEC, astrocytes and pericytes in normal versus hyperglycaemic conditions would be beneficial in unravelling the precise contribution of each cell line to hyperglycaemia-mediated BBB hyperpermeability.

Conflicts of interest

B.S. performed the study, collected and analysed the data and wrote the first draft of the manuscript. U.B. designed and supervised the study, interpreted the data and wrote the manuscript. The authors have no conflict of interest to declare in relation to this manuscript.

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