High glucose downregulates intercellular communication in retinal endothelial cells by enhancing degradation of connexin 43 by a proteasome-dependent mechanism

Rosa Fernandes, Henrique Girão and Paulo Pereira*

Centre of Ophthalmology, Biomedical Institute for Research in Light and Image (IBILI), Faculty of Medicine, University of Coimbra, 3000-354 Coimbra, Portugal

Phone: +351 239 480230  Fax: +351 239 480280

Email: rosa@ibili.uc.pt
     hgilao@ibili.uc.pt
     ppereira@ibili.uc.pt

*To whom correspondence should be addressed

Running title: Degradation of Cx43 by the proteasome in high glucose
SUMMARY

Intercellular communication through gap junctions (GJIC) is most likely to be relevant to maintain the integrity of blood-retinal barrier (BRB). In this study was investigated the mechanism whereby high glucose enhances degradation of connexin 43 (Cx43) contributing to decrease GJIC. The levels of Cx43 in bovine retinal endothelial cells (BREC) exposed to high glucose (25 mM) decreased about 50% as compared to controls (5.5 mM glucose). Consistently, the half-life of the protein decreased from 2.3 h to 1.9 h. The proteasome inhibitors MG132 and lactacystin prevented the loss of Cx43 induced by high glucose and extended Cx43 half-life. The amount of phosphorylated Cx43 increased in high glucose and following proteasome inhibition. Scrape-loading dye transfer experiments show that high glucose is associated to a decrease of 40% in GJIC. Significantly, this reduction can be reverted by proteasome inhibitors. The decreased GJIC in cells exposed to high glucose is associated with loss of Cx43 from the plasma membrane as demonstrated by immunofluorescence and biotinylation of cell surface proteins. Results indicate that increased phosphorylation of Cx43 under high glucose is the mechanism targeting Cx43 for degradation by a proteasome-dependent mechanism. Increased degradation of Cx43 and reduction of GJIC in high glucose may be of physiological importance by contributing to endothelial cell dysfunction associated with breakdown of BRB in diabetic retinopathy.
INTRODUCTION

Hyperglycemia and the consequent exposure of the intracellular milieu of the retinal capillary endothelial cells to elevated blood glucose concentrations have been implicated in the pathogenesis of vascular complications in diabetes, including the breakdown of the BRB\(^1\) (1,2).

The exact biochemical and molecular mechanisms that transduce chronic hyperglycemia into micro- and macrovascular complications of the retina are not clear. However, several mechanisms have been suggested to play a role in pathogenesis of diabetic retinopathy including increased nonenzymatic glycation (3), activation of aldose reductase (4), oxidative stress (3), increased production of diacylglycerol and stimulation of retinal protein kinase C (PKC) (5).

Despite the nature of such mechanisms early cell dysfunction related to diabetic vascular complications is often associated with abnormalities in cell-cell communication and maintenance of cell homeostasis.

Gap junctions are intercellular-channels that permit the passage of small molecules such as small metabolites, ions, and second messengers (6). These channels consist of two hemichannels, called connexons that are located in the plasma membrane of two adjacent cells. Each connexon is composed of six subunits of a protein termed connexin.

\(^1\) The abbreviations used are: GJIC, Intercellular communication through gap junctions; BRB, blood-retinal barrier; Cx, connexin; BREC, bovine retinal endothelial cells; LEC, lens epithelial cells; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol 13-acetate; DMSO, dimethyl sulphoxide; DOC, sodium deoxycholate; PMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline solution; FCS, fetal calf serum; HBC, Hank’s balanced salt solution containing 1% bovine serum albumin; P/NP, phosphorylated/ non phosphorylated; AP, alkaline phosphatase; PFA, paraformaldehyde.
Gap junctions have an important role in a variety of cellular processes including homeostasis, morphogenesis, cell differentiation and growth control (7).

Three connexin subtypes are expressed in the endothelium of the blood vessels, Cx37, Cx40 and Cx43 (8). Endothelial cells rely on a network of gap junctions for intercellular communication (9,10). Previous studies have shown that intercellular communication in endothelial cells is reduced under conditions of hyperglycemia (11). Such decrease in intercellular communication is most likely due to a reduction of the abundance of Cx43 in endothelial cells.

One of the most remarkable aspects of gap junctions biosynthesis is the exceptional metabolic lability of connexins. While the half-life of the great majority of plasma membrane proteins exceed 24 hours, connexins present a rapid turnover rate, with half-life of only 1.5-5 hours (12-14). To the present date, two proteolytic pathways have been implicated in connexin turnover, the lysosome (14,15) and the proteasome (13-17). The mechanisms and upstream events that lead to the choice of a degradation pathway over the other are largely unknown. It has however been shown that both pathways coexist and are active in a number of cell types and in a variety of experimental and pathological conditions (18-20). For example, we have previously shown that in response to specific stimuli proteasome is involved in degradation of phosphorylated Cx43 in lens epithelial cells (LEC) (17).

It has been widely shown that phosphorylation of Cx43 results in decreased GJIC, probably due to a reduction of abundance at the plasma membrane (21-24). For example, it was shown that in smooth muscle cells high glucose induced the inhibition of GJIC activity through hyperphosphorylation of Cx43 by PKC (25). On the other hand, a recent study has shown that high glucose inhibited GJIC activity by downregulating Cx43 synthesis in rat microvascular endothelial cells (11). We have shown before that Cx43 is remarkably labile and that the
ubiquitin proteasome pathway is involved in degradation of Cx43 in LEC (17). We have further suggested that the combined action of phosphorylation and protein degradation by the proteasome pathway act as a mechanism of decreasing intercellular communication in the LEC (17). By analogy, it is thus conceivable that a similar mechanism may occur in retinal endothelial cells.

To investigate the mechanism whereby hyperglycemia leads to decreased intercellular communication in endothelial cells we have hypothesised that hyperglycemia may create the conditions that favour increased degradation of Cx43, presumably involving increased phosphorylation of the protein. A reduction in Cx43 half-life and a decreased abundance of Cx43 at the plasma membrane would thus account for the reduced intercellular communication observed under conditions of hyperglycemia.
EXPERIMENTAL PROCEDURES

Cell Cultures

Bovine retinas were the source of capillaries used to isolate cells for primary culture. Cow eyes were obtained from a local slaughterhouse. Primary bovine retinal endothelial cells (BREC) cultures were established from fresh calf eyes. Under sterile conditions, the retinas were isolated and washed in Dulbecco’s modified Eagle’s medium (DMEM) and pieces of adherent retinal pigment epithelial cells were removed. The retinas were transferred to an enzyme solution containing pronase (100 μg/ml), collagenase (500 μg/ml) and DNase (70 μg/ml) and incubated with shaking at 37 °C for 20 minutes. After incubation, the retinal digest was passed through 210- and 50-μm nylon mesh and the microvessels trapped on top of the 50-μm mesh were collected in DMEM by centrifugation. The fragments were resuspended in DMEM with 15% fetal calf serum (FCS), 20 μg/ml endothelial growth supplement (Roche, Mannheim, Germany), heparin (100 μg/ml) and antibiotic-antimycotic solution (Sigma), plated and grown on fibronectin-coated dishes in low glucose DMEM, at 37°C with 5% CO2. Cultures were passaged every 7 to 10 days. For all experiments, cultures from passages three to five were used.

To determine the effect of high glucose on Cx43 expression and GJIC activity, BREC were grown in low (5.5 mM) or high (25 mM) D-glucose medium for 8 days to confluence.

Antibodies and Reagents

The rabbit polyclonal and mouse monoclonal anti-Cx43 antibodies were obtained from Zymed (San Francisco, CA, USA). The mouse anti-Cx43 monoclonal antibody obtained from Zymed was raised against a peptide sequence that represents amino acid residues 360-376 of Cx43.
Unless otherwise noted, all other reagents were from Sigma, except MG132 that was obtained from Calbiochem (Darmstadt, Germany). TPA (12-O-tetradecanoylphorbol 13-acetate) and MG132 were dissolved in dimethyl sulphoxide (DMSO).

**Gel electrophoresis and Western Blotting**

Cells exposed to normal and high-glucose medium were washed, collected in phosphate buffered saline solution (PBS) by centrifugation and cell pellets were frozen at −80°C until use. The cells were resuspended in lysis buffer containing 10 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100 and 0.5% sodium deoxycholate (DOC), supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM iodoacetamide, 10 mM NaF, 500 μM Na₃VO₄. The lysates were sonicated 6 times for 3 seconds in order to disrupt completely all cells and then centrifuged at 16,000 x g for 15 min. The supernatants were used to determine the protein concentration by BCA reagent (Pierce) and then were denaturated with Laemmli buffer. For studies involving total cell lysates, electrophoresis was performed on 50-μg aliquots of each sample on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were probed with rabbit polyclonal or mouse monoclonal antibodies against connexin 43. The signals were detected by ECL system (Amersham). Films were scanned and the optical density of the bands was measured with appropriate software. All results are representative of at least three independent experiments.
**Metabolic Labelling and Immunoprecipitation**

For metabolic labelling with $^{35}$S, cells were rinsed with methionine-free medium prior incubation with $[^{35}S]$-Methionine (Amersham) 100 $\mu$Ci/ml for 1 hour. The cells were then chased in DMEM supplemented with 0.5 mM unlabelled methionine, in the presence or absence of MG132, for 1, 2 and 4 hours. The samples were resuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 2.5% Triton X-100, 0.2% SDS, pH 8.3) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), protein phosphatase inhibitor set II (Calbiochem, La Jolla, CA, USA), 2 mM PMSF, 10 mM iodacetamide, 50 mM NaF, 500 $\mu$M Na$_3$VO$_4$. Cells were lysed by sonication and supernatants were used for immunoprecipitation using polyclonal antibodies directed against Cx43. After centrifugation at 16,000 x g, the supernatants were incubated with antibodies directed against Cx43, for 3 hours, at 4°C, followed by incubation with protein A-sepharose, for 1 hour. The samples were then centrifuged and the protein A-sepharose sediments resuspended in Laemmli buffer and denatured at 37°C, for 30 min. Proteins were resolved by polyacrylamide gel electrophoresis (SDS-PAGE) and the gels were dried and autoradiographed.

**Semi-quantitative RT-PCR analyses**

RNA extracted from BREC grown in low- and high-glucose medium was simultaneously determined by semi-quantitative reverse transcription-PCR, using the forward primer 5’-TTAAGGATCGGTGAAGGAAGAG -3’ and the reverse primer 5’–CTAGATCTCTAGGTATCAGGCCG -3’ for amplification of Cx43, and the forward primer 5’-AAGGAGAAGCTGTGCTACGTCGCCCTGG - 3’ and the reverse primer 5’-GATCTGTATATCATTGTGCTGGTGTCG -3’ for amplification of actin. For simultaneous
amplification of Cx43 and β-actin, cDNAs were generated in the same RT reaction. To confirm that amplification was in the linear region separate tubes containing increasing volumes of the RT reaction were used. Amplification products were electrophoresed on 1% agarose gel and stained with ethidium bromide. Densitometry was performed at nonsaturating exposures and the Cx43/β-actin ratios were determined.

**Biotinylation of cell surface proteins**

The cell surface proteins of BREC were biotinylated. Dishes of confluent BREC cells (10 cm diameter) were rinsed twice with 5 ml of ice-cold PBS containing 0.5 mM MgCl₂ and 1 mM CaCl₂, followed by the addition of 3 ml of the same cold solution containing 1 mg/ml freshly added SULFO-NHS-SS-biotin (Pierce, Rockford, IL). After 30 min at 4º C, the medium was removed and the plates were washed 3 times with PBS containing 0.5 mM MgCl₂, 1 mM CaCl₂, and 100 mM glycine. The cells were scraped in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA containing 1% Triton, 0.5% DOC, 0.1% SDS and supplemented with protease inhibitor cocktail, 2 mM PMSF, 10 mM iodoacetamide, 10 mM NaF and 500 μM Na₃VO₄; pH 7.5). After 10-20 min on ice the cells were sonicated and the homogenates were centrifuged at 16 000 x g, for 10 minutes. The protein content of the supernatants was determined and the same quantity of protein was transferred to 1.5 ml Eppendorf microfuge tubes containing 200 μl of Neutravidin (Pierce, Rockford, IL). After 2 h of incubation at 4º C with agitation, the beads were washed four times with RIPA buffer. The final pellets were resuspended in 150 μl 2x Laemmli buffer and incubated 1 h at 37º C. The beads were pelleted and the proteins resolved by SDS/PAGE, transferred to PVDF membranes and blotted against Cx43 antibodies.
**Immunofluorescence**

BREC grown on fibronectin-coated glass coverslips were fixed with 4% paraformaldehyde (PFA) in PBS. The samples were then washed with PBS, permeabilised with 1% v/v Triton X-100 in PBS, and blocked with goat serum (1:10) for 20 minutes prior to incubation with primary mouse monoclonal antibody against connexin (1:25) for 1 h at room temperature. The specimens were rinsed in PBS and mounted with Glycergel (Dako, Carpinteria, CA). All solutions were made up in 0.02% w/v BSA (Sigma) containing 0.02% sodium azide (Sigma) in PBS. For controls, primary antibodies were omitted.

**Alkaline phosphatase treatment**

The dephosphorylation reactions were carried out in lysis buffer following overnight incubation at 37°C in the presence of alkaline phosphatase (5 units) from *E. coli* (Sigma, St Louis, MO, USA)

**Cell-cell communication assay**

BREC grown on fibronectin-coated glass coverslips were assessed for gap junction-mediated intercellular coupling as described by Musil (14). Briefly, the culture medium from confluent cells was removed and saved. The cells were rinsed three times with Hank’s balanced salt solution containing 1% bovine serum albumin (HBC), after which a 27-gauge needle was used to create multiple scrapes through the cell monolayer in the presence of Dulbecco’s phosphate buffered saline containing 0.5% rhodamine-dextran and 0.5% Lucifer yellow. After 3 minutes of incubation at room temperature, the culture was rinsed three times with HBC and then incubated for an additional 8 min in the saved culture medium to allow the loaded dye to transfer to
adjoining cells. The cells were then rinsed and fixed with 4% paraformaldehyde and viewed using a fluorescence microscope with a UV light source.

**Statistical analysis**

Data is expressed as mean ± SD or SE. Comparison between groups was performed with Student’s *t* test.

**RESULTS**

**High glucose leads to a decrease in the amount of Cx43 by a proteasome dependent mechanism**

It has previously been shown that hyperglycemia leads to a decrease in total Cx43 in various cell types (11,25,26). To investigate the mechanisms whereby high glucose leads to decreased Cx43 in BREC, the amount of total Cx43 was determined by western blot (Fig. 1A). The levels of Cx43 decreased about 50% in cells exposed to 25 mM glucose for 8 days as compared to controls (cells incubated in 5.5 mM glucose) (Fig. 1B). Since the proteasome was reported to be involved in Cx43 degradation (13-17), we further evaluated the participation of the proteasome on Cx43 degradation in high glucose by treating BREC with the proteasome inhibitors MG132 (Fig. 1A, 1B) or lactacystin (data not shown). Data shown in Fig. 1 clearly indicates that proteasome inhibitors prevent to a significant extent, the loss of Cx43 induced by high glucose. In fact, the levels of Cx43 in cells exposed to high glucose were restored to about 85% of its initial levels by proteasome inhibitors (Fig. 1B).
**Cx43 is stabilised by proteasome inhibitors under high glucose**

Since RT-PCR experiments suggest that the levels of mRNA for Cx43 are not remarkably altered following exposure of BREC to high glucose (data not shown) the decreased amounts of Cx43 observed under high glucose are likely to be related to an increased turnover of the protein. To determine the half-life of Cx43, cells were metabolically labelled with $^{35}$S-methionine for 1 hour and then chased for 1, 2 or 4 hours. The data presented in Fig. 2 shows that the half-life of Cx43 in cells exposed to high glucose decreased from 2.3 hours, in control cells, to 1.9 hours. To further determine if the increased turnover of Cx43 in high glucose was associated with increased degradation of the protein by a proteasome dependent mechanism, proteins in cells were metabolically labelled with $^{35}$S-methionine for 1 hour and chased with unlabelled medium in the presence of the proteasome inhibitor MG132. Data shows that Cx43 degradation under high glucose is, at least in part, prevented by the proteasome inhibitor MG132 (Fig 2) and by the more specific inhibitor lactacystin (data not shown). The half-life of Cx43 in cells grown in high glucose increased from 1.9 hours to 3.4 hours when the proteasome was inhibited. The fact that proteasome inhibitors extended Cx43 half-life beyond the half-life of the protein in controls, indicates that proteasome has a role in constitutive degradation of Cx43 and that high glucose may create the conditions that favour increased degradation of the protein by a proteasome dependent mechanism.

**Increased degradation of Cx43 under high glucose is associated with increased phosphorylation of the protein**
High glucose was shown to induce PKC-mediated phosphorylation of Cx43, which in turn may stimulate protein degradation (11,25,26).

The effect of high glucose on phosphorylation of Cx43 induced by PKC was confirmed by incubating cells with staurosporine, an inhibitor of PKC. The decrease in the levels of Cx43 in cells exposed to high glucose was prevented, at least in part, by incubating cells with staurosporine (Fig. 3A, 3B). Consistently, incubation with TPA, an activator of PKC, resulted in a decrease of about 85% in the content of Cx43 (Fig. 3B). These results show that high glucose may activate PKC which in turn may hyperphosphorylate Cx43 leading to its increased degradation. Results obtained in our laboratory showed that the phosphorylated forms of Cx43 are preferentially degraded by a mechanism that involves the proteasome (17). In order to investigate if proteasome is the mechanism leading to increased degradation of phosphorylated Cx43 under high glucose Cx43 was immunoprecipitated from lysates of cells incubated under high glucose in the presence of proteasome inhibitors. Proteins were separated by SDS-PAGE, transferred to PVDF membranes that were probed with antibodies directed against the phosphoserine/ phosphothreonine residues (Fig. 4A) or polyclonal antibodies directed against Cx43 (Fig. 4B). The ratio phosphorylated/ non phosphorylated (P/NP) Cx43 was calculated and plotted in a graph (Fig. 4D). The results show that the ratio P/NP Cx43 increased 2 fold in cells incubated under high glucose and, more importantly, inhibition of the proteasome led to a 3 fold increase of the ratio P/NP Cx43, as compared to control cells incubated under low glucose. On the other hand, the ratio P/NP Cx43 was not significantly altered when the PKC inhibitor staurosporine was added to cells exposed to high glucose. These results suggest that phosphorylation of Cx43 in cells exposed to high glucose may act as a signal for degradation of the protein by a proteasome dependent mechanism.
High glucose leads to decreased intercellular communication by a proteasome dependent mechanism

The functional implications of increased Cx43 degradation in high glucose was evaluated by scrape loading-dye transfer experiments. The intercellular communication was quantified as the distance travelled by the dye Lucifer yellow, following scrape loading (Fig. 5A). The GJIC decreased about 40% in cells exposed to high glucose (Fig. 5B). Conversely, but not surprisingly, proteasome inhibitors had an opposite effect in GJIC leading to an increase of about 20% in the distance travelled by the dye (Fig. 5B). However, under high glucose, proteasome inhibitors not only restored the GJIC to control levels, but rather increased intercellular communication two fold as compared to controls incubated under low glucose. Taken together these results suggest that high glucose has a direct impact in GJIC by a mechanism that involves increased degradation of Cx43 in a proteasome dependent way.

Treatment of BREC, grown in high glucose, with the PKC inhibitor staurosporine resulted in an increase of GJIC of about 36%. On the other hand, and as predicted, treatment with TPA resulted in a dramatic inhibition (about 80%) of GJIC.

Inhibition of GJIC is the direct result of decreased abundance of Cx43 at the plasma membrane

Intercellular communication is usually associated with the abundance of functional gap junction plaques, at cell-cell interfaces. Thus, alteration on intercellular communication is either associated with changes in gap junction activity or with the levels of Cx43, at the plasma membrane, available to form gap junctions. To investigate whether the decrease of GJIC in high
glucose is related to alterations in the subcellular distribution of Cx43, BREC were immunostained with antibodies directed against Cx43 and imaged by confocal microscopy. In control cells, Cx43 is localised both to the plasma membrane, to cell-cell contacts, and intracellularly. On cells exposed to high glucose there is a clear decrease in the abundance of Cx43 detected at the plasma membrane (Fig. 6A). The amount of Cx43 at the plasma membrane was determined as the fluorescent punctate at the site of contact between adjacent cells (Fig. 6B). The involvement of the proteasome on Cx43 redistribution in high glucose was determined by incubating cells with the proteasome inhibitor MG132. Results presented in Fig. 6A show that proteasome inhibition reverted the redistribution induced by high glucose, with the amount of Cx43, at the plasma membrane, being similar to that observed in control cells. These results indicate that inhibition of GJIC in high glucose is directly related to the amount of Cx43 available to form gap junctions at the plasma membrane. Moreover, the extraction of Cx43 from plasma membrane occurs through a proteasome dependent mechanism and results in an increased degradation of Cx43 in retinal capillary endothelial cells. The amount of Cx43 at the plasma membrane was further assessed by biotinylation of cell surface proteins. The amount of biotinylated Cx43 decreased in cells exposed to high glucose, as compared to control cells (Fig. 6C). However, treatment with MG132 resulted in a significant increase on the amount of biotinylated Cx43 recovered in lysates of BREC exposed to high glucose (Fig. 6C).

Taken together these results support a model in which hyperglycemia creates the conditions that favour targeting of membrane Cx43 to degradation by a proteasome-dependent mechanism. The results presented in this study, together with previous reports, suggest that phosphorylation of Cx43 is the stimuli targeting Cx43 for degradation by a proteasome dependent mechanism.
Moreover, increased degradation of Cx43 results in a decrease in the amount of Cx43 available to form gap junctions at the plasma membrane leading to a reduction in intercellular communication in endothelial cells.
DISCUSSION

Previous studies demonstrated that high glucose induces a reduction of GJIC (25-28). More recently, Sato et al, showed that the decreased levels of Cx43 were partially associated with a down-regulation of Cx43 in response to hyperglycemia (11). Endothelial cells exposed to high glucose showed a decrease of about 30% in Cx43 mRNA, while the total amount of the protein decrease 45%, what suggests that Cx43 degradation might also be enhanced in hyperglycemia (11). Data reported in this study shows that the amount of Cx43 in BREC decreased about 50% in high glucose whereas the levels of Cx43 mRNA do not vary significantly. We have previously shown in other cell types that, in addition to the lysosome, Cx43 abundance can be regulated by an ubiquitin-proteasome dependent mechanism. We thus hypothesised that reduction of Cx43 induced by high glucose might be associated to an increase in the turnover of the protein. In support of this hypothesis is the observation that the half-life of Cx43 in BREC exposed to high glucose decreased about 20% (this study). Most likely the lysosome is the major site for constitutive degradation of Cx43. However, it has been well established that proteasome is also involved in degradation of Cx43 in a number of cell types (13-17). Data obtained in this study shows, for the first time, that the proteasome is involved in degradation of Cx43 in blood retinal endothelial cells in high glucose. Significantly, we have further shown that degradation of Cx43 by a proteasome dependent mechanism can be enhanced by protein phosphorylation and results in decreased GJIC (17). The evidence for proteasomal degradation of Cx43 is such that it has been considered a novel mechanism for regulating intercellular communication (14,17). It has been shown that phosphorylation of Cx43 by various kinases, including PKC (23,34), mitogen-activated protein kinase (24,29) and the v-Src tyrosine protein kinase (29,30) all result in
decreased intercellular communication. We have suggested before that phosphorylation of Cx43 is the signal that targets Cx43 for degradation by a proteasome dependent mechanism (17). The pathways that transduce hyperglycemia into increased degradation of Cx43 are largely unknown, as are the triggering signals and mechanism for extraction of Cx43 from plasma membrane. However, by analogy to what has been observed in other cell systems and based on accepted models for regulation of GJIC it is possible to suggest that phosphorylation of Cx43 is the upstream signalling event that triggers the degradation of the protein by a proteasome-dependent mechanism. Indeed, we show in the present report that activation of PKC is required to phosphorylate Cx43 in cells exposed to high glucose, as revealed by the inhibitory effect of staurosporine. There is ample evidence in the literature to suggest that hyperglycemia and diabetes are associated with increased activity of PKC (25,26,31-33). Moreover, it has been widely demonstrated hyperphosphorylation of Cx43 in conditions of hyperglycemia (25,26,32). Multiple isoforms of PKC were shown to be activated in response to high glucose including PKC \( \alpha, \beta_1, \beta_2 \) and \( \delta \) (34-36). More importantly, activation of various isoforms of PKC is implicated in vascular disfunction associated with diabetes (35-37). At least part of these isoforms were shown to phosphorylate Cx43 (38-40), presumably targeting the protein for degradation (17). At present the specific isoforms that lead to increased phosphorylation of Cx43 in high glucose, and presumably in diabetes, remain to be elucidated. However, data presented in this report clearly show an association between exposure of retinal endothelial cells to high glucose, hyperphosphorylation of Cx43 and increased degradation of the protein by a proteasome dependent mechanism.

Consistent with the above model we also demonstrate that inhibition of the proteasome leads to an accumulation of phosphorylated Cx43. These data together with the observation that
staurosporine (an inhibitor of PKC) and TPA (an activator of PKC) have an opposite effect on half-life of the protein, strongly suggests that phosphorylation of Cx43 leads to its extraction from the plasma membrane and increased degradation.

The physiological implications of hyperglycemia in GJIC have been extensively demonstrated in various cell types and systems. For example in retinal pigment epithelium (41), epididymal endothelial cells (11,27) and smooth muscle cells (25) it was shown that high glucose reduced the intercellular communication. Significantly, the decrease in intercellular communication induced by high glucose, in most studies, can be reverted by PKC inhibitors (25,28,32), thus indicating that phosphorylation is the mechanism whereby hyperglycemia reduces intercellular communication.

Data presented in this study consistently shows that high glucose reduces GJIC and also that such reduction involves activation of PKC and most likely other kinases (since the intercellular communication is not completely reverted by PKC inhibitor). More importantly, we propose an integrated model in which increased phosphorylation of Cx43 (mostly by PKC) under high glucose leads extraction of Cx43 from the plasma membrane and increased degradation of Cx43 with the consequent reduction of GJIC. To demonstrate that reduction of GJIC is the direct result of decreased abundance of Cx43 available to form gap junction plaques and not the result of alterations on the activity of gap junctions, we have performed biotinylation of membrane proteins and have imaged cell cultures by confocal microscopy. Data obtained confirms that high glucose leads to a reduction on the abundance of Cx43 forming gap junctions at the plasma membrane and that such reduction can be prevented by inhibitors of the proteasome.

Taken together, data presented in this report support a model in which hyperphosphorylation of Cx43 in hyperglycemia constitutes the triggering signal for Cx43 degradation by a proteasome.
dependent mechanism. Impairment of gap junction intercellular communication activity between vascular endothelial cells is suggested to be involved in breakdown of BRB in chronic hyperglycemia (11). Increased degradation of Cx43 and reduction of GJIC may thus contribute to endothelial cell dysfunction associated with breakdown of BRB associated with diabetic retinopathy.

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**FIGURE LEGENDS**

**FIG. 1. Effect of high glucose and proteasome inhibition on the amount of Cx43.** BREC were incubated in medium containing 5.5 mM (low glucose) or 25 mM glucose (high glucose) for 8 days. During the last 4 h of incubation cells were exposed to 40 µM MG132. After the treatments, the proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with monoclonal antibodies directed against Cx43; calnexin on the same samples is included to demonstrate comparable loading of the lanes (A). The intensity of the bands was determined by laser scanning of the films followed by quantitative densitometric analysis and the results plotted in a graph (B). Data are means ± SE (n=3 per group). Asterisk (*) denotes a significant difference (P<0.05) from control incubated in low glucose, as determined by unpaired two-tailed Student’s *t* test.
FIG. 2. Effect of proteasome inhibitors in half-life of Cx43 under high glucose. BREC were labelled with [35S]-methionine for one hour either in the presence or absence of 40 µM MG132. The cells were then chased for 1, 2 and 4 hours with non-labelled medium and Cx43 was immunoprecipitated using a polyclonal antibody. Immunoprecipitated proteins were separated by SDS-PAGE and autoradiographed. (○, BREC grown in low glucose, in the absence of MG132; □, BREC grown in low glucose, in the presence of MG132; ●, BREC grown in high glucose, in the absence of MG132; ■, BREC grown in high glucose, in the presence of MG132). Quantification of the bands was performed using a PhosphorImage analyser, storm 860. The data presented is representative of two independent experiments.

FIG. 3. Effect of phosphorylation on the amount of Cx43. BREC were incubated with a medium containing 5.5 or 25 mM glucose for 8 days. During the last 1 h or 40 min of incubation cells were exposed to 100 nM staurosporine or 80 nM TPA, respectively. After the treatments, the proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with monoclonal antibodies directed against Cx43; the amount of calnexin on the same sample is included to demonstrate comparable loading of the lanes (A). The intensity of the bands was determined by laser scanning of the films followed by quantitative densitometric analysis and the results plotted in a graph (B). Data are means ± SE (n=3 per group). Asterisk (*) denotes a significant difference (P<0.05) from control incubated in low glucose, as determined by unpaired two-tailed Student’s t test.

FIG. 4. Effect of the proteasome inhibitors on phosphorylation of Cx43. Cx43 was immunoprecipitated from cell lysates of BREC incubated with MG132 or staurosporine using
polyclonal antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with antibodies directed against phosphorylated residues of serine/threonine (A) or polyclonal antibodies to Cx43 (B). Enzymatic dephosphorylation of Cx43. Cell lysates were incubated in the absence or presence of alkaline phosphatase (AP) from *E. coli* at 37°C overnight (C). The intensity of the bands was determined by laser scanning of the films followed by quantitative densitometric analysis. The ratio phosphorylated/non phosphorylated (P/NP) Cx43 was calculated and plotted in a graph (D). Data are means ± SE (n=3 per group). Asterisk (*) denotes a significant difference (P<0.05) from control incubated in low glucose, as determined by unpaired two-tailed Student’s *t* test.

**FIG. 5. Effect of high glucose and proteasome inhibition on GJIC activity.** BREC exposed to 5.5 or 25 mM glucose for 8 days were incubated with 40 μM MG132, 100 nM staurosporine or 80 nM TPA. The cells were then assayed for intercellular communication by Lucifer yellow dye transfer, after scrape loading (A). The intercellular communication was evaluated as the average distance travelled by the dye Lucifer yellow along the monolayer and is represented as an histogram (B). Data are means ±SD (n= 3 per group). Asterisk (*) denotes a significant difference (P<0.05) from control incubated in low glucose, as determined by unpaired two-tailed Student’s *t* test.

**FIG. 6. Effect of high glucose and proteasome inhibition on distribution of Cx43 at the plasma membrane.** BREC exposed to 5.5 or 25 mM glucose for 8 days were incubated with 40 μM MG132, 100 nM staurosporine or 80 nM TPA. The cells were then fixed and stained with antibodies directed against Cx43 and imaged by confocal microscopy (A). The amount of Cx43
at the plasma membrane was determined as the fluorescent punctate at the site of contact between adjacent cells and the results obtained plotted in a graph (B). BREC were incubated with a medium containing 5.5 or 25 mM glucose for 8 days. During the last 4 h of incubation cells were exposed to 40 μM MG132. The cells were then surface-labelled with biotin and the biotinylated proteins were isolated with neutravidin beads. The isolated proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with monoclonal antibodies to Cx43 (C). Data are means ±SD (n= 3 per group). Asterisk (*) denotes a significant difference (P<0.05) from control incubated in low glucose, as determined by unpaired two-tailed Student’s t test.
FIGURES

FIG. 1
FIG. 2
FIG. 3

A

[Glucose], mM | 5.5 | 25 | 25 | 5.5
---|---|---|---|---
Cx43 |  |  |  |  
calnexin |  |  |  |  

B

Total Cx43 (% of control)

5.5 mM Glucose |  |  |  |  
25 mM Glucose |  |  |  |  
25 mM Glucose + Stauro |  |  |  |  
25 mM Glucose + TPA |  |  |  |  

*
FIG. 4
FIG. 5
FIG. 6
High glucose downregulates intercellular communication in retinal endothelial cells by enhancing degradation of connexin 43 by a proteasome-dependent mechanism

Rosa Fernandes, Henrique Girao and Paulo Pereira

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