Reactive oxygen species mediate a cellular ‘memory’ of high glucose stress signalling

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Abstract

Aims/hypothesis A long-term ‘memory’ of hyperglycaemic stress, even when glycaemia is normalised, has been previously reported in endothelial cells. In this report we sought to duplicate and extend this finding.

Materials and methods HUVECs and ARPE-19 retinal cells were incubated in 5 or in 30 mmol/l glucose for 3 weeks or subjected to 1 week of normal glucose after being exposed for 2 weeks to continuous high glucose. HUVECs were also treated in this last condition with

several antioxidants. Similarly, four groups of rats were studied for 3 weeks: (1) normal rats; (2) diabetic rats not treated with insulin; (3) diabetic rats treated with insulin during the last week; and (4) diabetic rats treated with insulin plus α -lipoic acid in the last week.

Results In human endothelial cells and ARPE-19 retinal cells in culture, as well as in the retina of diabetic rats, levels of the following markers of high glucose stress remained induced for 1 week after levels of glucose had normalised: protein kinase C- β , NAD(P)H oxidase subunit p47phox, BCL-2-associated X protein, 3-nitrotyrosine, fibronectin, poly(ADP-ribose). Blockade of reactive species using different approaches, i.e. the mitochondrial antioxidant α -lipoic acid, overexpression of uncoupling protein 2, oxypurinol, apocynin and the poly(ADP-ribose) polymerase inhibitor PJ34, interrupted the induction both of high glucose stress markers and of the fluorescent reactive oxygen species (ROS) probe CM-H₂DCFDA in human endothelial cells. Similar results were obtained in the retina of diabetic rats with α -lipoic acid added to the last week of normalised glucose.

Conclusions/interpretation These results provide proof-of-principle of a ROS-mediated cellular persistence of vascular stress after glucose normalisation.

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Abbreviations

ALA	α -lipoic acid
Bax	Bcl-2-associated X protein
BCL-2	B cell lymphoma protein-2
3-NY	3-nitrotyrosine
PAR	poly(ADP-ribose)

PARP	poly(ADP-ribose) polymerase
PKC	protein kinase C
ROS	reactive oxygen species
UCP2	uncoupling protein 2

Introduction

The hallmark of diabetes is hyperglycaemia, which, evidence suggests, plays a major role in the pathogenesis of the disease's complications, both micro and macrovascular in nature [1].

Dysfunction of the endothelium is an integral cause of development of diabetic vascular disease [2], with many studies confirming that high glucose *in vitro* can directly affect endothelial cell function [3]. Recently, an excess of reactive oxygen species (ROS) generated in the endothelium and in target organs such as the retina, kidney and heart in response to hyperglycaemia has been shown to form a causative link between high glucose and diabetic complications [4, 5]. In particular, endothelial dysfunction in diabetes has been found to be mediated through an excess of cellular ROS [4] involving respiratory chain uncoupling [5], protein kinase C (PKC) activation [6], NAD(P)H oxidase activation [4], formation of single strand DNA breaks and activation of poly(ADP-ribose) (PAR) polymerase (PARP) [7], basement membrane thickening [8] and activation of pro-apoptotic proteins [9].

Almost 20 years ago, the laboratory of Lorenzi showed that there was a persistence or 'memory' of the induced expression of basement membrane mRNAs (fibronectin, collagen IV) long after high glucose levels were normalised in endothelial cells in culture [10, 11] and in diabetic rats [11], suggesting the possibility of a long-lasting deleterious effect of hyperglycaemia on these cells, independent of the actual level of glucose. These data are consistent with findings that retinopathy progression can persist in dogs even after the normalisation of glycaemic control [12].

The aim of this study was to duplicate the previous findings in endothelial cells, retinal cells and in the retina of diabetic animals, and to evaluate the possible role of the persistence of ROS production after glucose normalisation as a possible explanation for the phenomenon termed here 'memory.'

Materials and methods

'Memory' experiments in cells HUVECs were grown in MCDB-131 medium (GIBCO Life Technologies, Grand Island, NY, USA) with 15% cosmic calf serum (HyClone, Logan, UT), epidermal growth factor (10 ng/ml; Peprotech, Rocky Hill, NJ, USA) and penicillin/streptomycin. ARPE-

19 cells (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM with 10% cosmic calf serum (HyClone) and penicillin/streptomycin. As previously described [11], cells were incubated in 5 mmol/l glucose, with 25 mmol/l mannitol (Sigma Chemical, St Louis, MO, USA) for osmolarity normalisation, or in 30 mmol/l glucose for 3 weeks or up to 1 week in normal glucose (5 mmol/l) after being exposed for 2 weeks to continuous high glucose (30 mmol/l). Fresh medium was added to cells every other day and 24 h before the end of the experiment.

Effects of ROS inhibition on the 'memory' phenomenon in HUVECs In the last week of normal glucose in HUVECs, glucose was given with or without agents capable of inhibiting ROS production as follows: 62.5 μ mol/l α -lipoic acid (ALA), an antioxidant working also at the mitochondria level [13]; 10 μ mol/l apocynin [14]; 10 μ mol/l oxypurinol [15]; 1 μ mol/l PJ34 [16]; and 25 to 100 plaque-forming units per cell of an uncoupling protein 2 (UCP2) overexpressing adenovirus, used to specifically inhibit mitochondrial ROS production [17]. Inhibitors were replaced every other day and 24 h before the end of the experiment. Inhibitors were purchased from EMD Bioscience (San Diego, CA, USA), except oxypurinol and ALA, which were from Sigma Chemical and the UCP2 adenovirus, which was a kind gift of M. H. Zou (Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma, OK, USA). Cells were grown at 25 to 30% confluence in 25 or 75 cm² flasks until 100% confluent.

Rat streptozotocin-induced hyperglycaemia model All animal studies were conducted in accordance with guidelines approved by the Association for Assessment and Accreditation of Laboratory Animal Care International using a protocol approved by the Institutional Animal Care and Use Committee at the University of Oklahoma. To make 6- to 8-week-old female Brown Norway rats (Charles River Laboratories, Wilmington, MA, USA) hyperglycaemic, a single intraperitoneal injection of 50 mg/kg streptozotocin (Sigma Chemical) in 10 mmol/l citrate buffer, pH 4.5, was given as previously described [18]. Similarly to cell experiments, four groups of rats were studied for 3 weeks: (1) normal rats; (2) diabetic rats not treated with insulin; (3) diabetic rats treated with insulin during the last week; and (4) diabetic rats treated with insulin plus ALA during the last week. Rats receiving insulin were implanted with a continuous-release insulin implant (LinShin, Scarborough, ONT, Canada) [19] under the skin of the upper abdomen. Blood glucose levels were in the following ranges: 5.8–8.0 mmol/l in non-diabetic rats, 17–22.8 mmol/l in diabetic rats and 4.8–5.6 mmol/l in the last week of treatment in insulin-treated animals. ALA (racemic mixture; Sigma Chemical) was prepared in 0.9% sodium chloride, pH 7.4, snap-frozen, stored at –80°C and aliquots

Fig. 1 Evidence of a persistent induction of the high glucose stress markers fibronectin (a), phospho PKC α / β II (b), p47phox (c), Bax (d), 3-NY (e) and PAR (f) in HUVECs.

HUVECs were cultured in normal (5 mmol/l; N) or high (30 mmol/l; H) glucose for 3 weeks or for 2 weeks in high (30 mmol/l) glucose followed by normal (5 mmol/l) glucose for 1 week (H $\rightarrow$ N). Whole-cell lysates were made and western blots run against high glucose stress proteins as described (Materials and methods). Data are from two separate experiments, $n=3$ –5 flasks of cells. Data represent the mean $\pm$ SEM of the densitometry of bands as a percentage of cells incubated in N for 3 weeks. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs HUVECs cultured in 5 mmol/l glucose for 3 weeks

thawed daily just before injection [20]. Animals receiving ALA were injected intraperitoneally with 75 mg/kg ALA each day, while all other animals received daily injections of 0.9% sodium chloride, pH 7.4. Blood glucose of animals receiving insulin and ALA during the last week was 16.0–22.2 mmol/l during the first 2 weeks and 6.0–8.8 mmol/l in the last week.

Western blot analysis Whole-cell lysates were made using M-PER lysis buffer (Pierce Chemical, Rockford, IL, USA) for endothelial cells with 300 mmol/l sodium chloride and containing protease inhibitor cocktail (EMD Bioscience) and phosphatase inhibitors (Sigma Chemical). Equal amounts of protein lysates (30 or 50 μ g; determined by Pierce micro BCA protein assay) were separated using 8–12% SDS-PAGE gels, transferred to 0.2 μ mol/l nitrocellu-

lose (Schleicher and Schuell, Keene, NH, USA), blocked in SuperBlock (Pierce Chemical), incubated with primary antibodies, either overnight at 4°C, or for 2 h at room temperature, washed three times in Tris-buffered saline to which 0.25% Tween-20 secondary antibodies (Pierce Chemical) was added, washed again with 0.25% Tween-20, to which SuperSignal Dura chemiluminescence substrate (Pierce Chemical) was added and subjected to digital imaging using a charge-coupled device camera (ImageStation 4000; Kodak, New Haven, CT, USA). Densitometry of bands was measured using Image J 1.30 analysis software (<http://rsb.info.nih.gov/nih-image/>). In all cases, confirmation of equal loading was performed by Memcode staining (Pierce Chemical) and by probing blots against vinculin [21]. Primary antibodies and dilutions used were

Fig. 2 Evidence of a persistent induction of the high glucose stress markers fibronectin (a), phospho PKC α / β II (b), p47phox (c), Bax (d), 3-NY (e) and PAR (f) in ARPE-19 cells. ARPE-19 cells were cultured in normal (5 mmol/l; N) or high (30 mmol/l; H) glucose for 3 weeks or for 2 weeks in high (30 mmol/l) glucose followed by normal (5 mmol/l) glucose for 1 week (H→N). Whole-cell lysates were made and treated

as in Fig. 1. Data are from two separate experiments, $n=3-4$ flasks of cells. Data represent the mean $\pm$ SEM of the densitometry of bands as a percentage of cells incubated in N for 3 weeks. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs ARPE-19 cells cultured in 5 mmol/l glucose for 3 weeks

as follows: 3-nitrotyrosine (3-NY) (1:100; Upstate Biotech, Lake Placid, NY, USA); B cell lymphoma protein-2 (BCL-2)-associated X protein (Bax) (1:1,000; Cell Signaling Technology, Danvers, MA, USA); fibronectin human (H-300, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA); manganese superoxide dismutase (1:650; Abcam, Cambridge, MA, USA); p47phox human (1:500; Upstate Biotech); PAR (1:400; Alexis Biochemicals, San Diego, CA, USA); phospho PKC- α / β II (1:1,000; Cell Signaling); PKC- β II (1:200; Santa Cruz).

CM-H₂DCFDA ROS fluorescence assay HUVECs were exposed for 3 weeks to 5 or 30 mmol/l glucose or for 2 weeks to 30 mmol/l glucose, followed by 1 week in 5 mmol/l glucose with or without inhibitors as described above. The day preceding the end of the experiment, HUVECs were plated at a density of 7,000 cells (70% confluence) on to white 96-well microplates with clear bottoms (3610; Costar, Cambridge, MA, USA) in 5 mmol/l with or without inhibitors. At the end of the experiment, medium was removed from the cells and 2 μ g/ml of the cell-permeable CM-H₂DCFDA probe (Molec-

ular Probes-Invitrogen, Eugene, OR, USA) in Hank's Balanced Salt Solution was added to each well of the plates and incubated for 15 min at 37°C as previously described [22]. Fluorescence intensity was then measured using a plate reader (BMG Labtech, Durham, NC, USA) using 488 nm excitation and 530 nm emission filters.

Statistics Data were analysed using one-way ANOVA to compare the means of all the groups. The Neuman-Keuls multiple comparisons procedure was used to determine which pairs of means were different. Differences were considered significant at $p<0.05$.

Results

Persistence of high glucose stress markers after glucose normalisation in endothelial and retinal cells We first wished to examine whether there was a persistence or a

Fig. 3 Effect of inhibitors given during the normalisation period on the persistence of induction of the high glucose stress markers fibronectin (a), phospho PKC α / β II (b), p47phox (c), Bax (d), 3-NY (e) and PAR (f). Human endothelial cells were incubated ($n=8-9$) with or without the following inhibitors during the last week of normalised glucose: 62.5 μ mol/l ALA, 25–100 plaque-forming units/cell UCP2, 10 μ mol/l apocynin (APO), 10 μ mol/l oxypurinol (OXY) or 1 μ mol/l PJ34. Western blots were performed on cellular lysates. Data represent the mean $\pm$ SEM of the densitometry of bands as a percentage of cells incubated in high glucose (H) for 2 weeks followed by normal glucose (N) for 1 week (H $\rightarrow$ N). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs cells incubated in high glucose for 2 weeks followed by 1 week of normal glucose

cellular ‘memory’ of hyperglycaemic stress after glucose levels normalised. Six different markers of high glucose stress were then measured (Fig. 1), compared with exposure to continuous normal glucose or high glucose. The markers chosen were: (1) the basement membrane protein fibronectin, shown to be overexpressed in the vessels of diabetic patients [23]; (2) the signalling kinase PKC- β , stimulated by high glucose through a cofactor, diacylglycerol [24, 25]; (3) the mitochondrial pro-apoptotic protein BCL-2 family member Bax, indicative of mitochondrial stress [26] and associated with vascular diabetic complications [27]; (4) the DNA damage protein PAR, a product of PARP, shown to be

a critical factor in the development of vascular diabetic complications [7]; (5) p47phox, an inducible subunit of the enzyme NAD(P)H oxidase, shown to be a source of ROS in the endothelium of diabetic patients [28]; and (6) the protein adduct 3-NY, a marker of oxidative stress and vascular diabetic complications [29–32]. As has been shown previously, chronic high glucose resulted in significantly increased levels of: fibronectin [10]; phospho(activated) PKC- α / β II [25]; p47phox [33]; and 3-NY [31], while the increase in Bax was not statistically significant [27] (Fig. 1). As previously reported, fibronectin levels remained increased 1 week after glucose normal-

Fig. 4 Evidence of a persistent ROS build-up in HUVECs after glucose normalisation. HUVECs were cultured in normal (5 mmol/l; N) or high (30 mmol/l; H) glucose for 3 weeks or for 2 weeks in high (30 mmol/l) glucose followed by normal (5 mmol/l) glucose for 1 week (H→N) with or without 62.5 μ mol/l ALA, 25–100 plaque-forming units/cell UCP2 or 10 μ mol/l apocynin (APO). Cells were loaded with 2 μ g/ml CM-H₂DCFDA for 15 min at 37°C and fluorescence measured as in the Methods. Data are from two separate experiments, $n=3$ –6 flasks of cells. Data represent the mean $\pm$ SEM of the percentage of fluorescence of cells incubated in normal glucose for 3 weeks. ** $p<0.01$; *** $p<0.001$ vs HUVECs cultured in 5 mmol/l glucose for 3 weeks. ## $p<0.01$ vs H→N

isation [10]. In addition, levels of all the stress proteins measured remained significantly induced 1 week after levels of glucose were normalised (Fig. 1). Similarly, five of these six stress proteins remained induced in ARPE-19 human retinal pigment epithelial cells exposed for 2 weeks to 30 mmol/l glucose, followed by 1 week of 5 mmol/l glucose (Fig. 2a, b, d–f), whereas no significant induction was observed with p47phox (Fig. 2c).

The role of ROS in the ‘memory’ of high glucose stress in endothelial cells To explore whether decreasing levels of intracellular ROS can interrupt the cellular ‘memory’ of high glucose stress, an antioxidant and ROS inhibitors were added during the last week of normal glucose levels subsequent to 2 weeks of continuous high glucose. Because of the difficulty of transfecting endothelial cells and the requirement for inhibitors to be added chronically (for 1 week) after 2 weeks of high glucose, well-characterised pharmacological agents were chosen in preference to inducible genetic manipulations for expressing inhibitors. It was first found that addition of the antioxidant ALA to the last week of normal glucose resulted in significant decreases in the induction of all markers of high glucose stress (Fig. 3). Blockade of NAD(P)H oxidase by apocynin [14] or of xanthine oxidoreductase by oxypurinol [15] during the glucose normalisation period interrupted the induction of several high glucose stress markers, but not all (Fig. 3), while overexpression of the mitochondrial respi-

ratory chain uncoupling protein UCP2 [17] during the normalisation period reduced the induction of all the stress proteins (Fig. 3). Finally, inhibiting the DNA damage inducible enzyme PARP during the last week of normalised glucose using PJ34 [16] interrupted the persistence of several high glucose stress markers (Fig. 3).

To examine whether free ROS remained increased after glucose normalisation, HUVECs, exposed as above, were loaded with the cell-permeable ROS-sensitive fluorescent dye CM-H₂DCFDA and fluorescence measured (Fig. 4). Exposure to 30 mmol/l continuous high glucose for 3 weeks resulted in significant induction of fluorescence compared with cells exposed to 5 mmol/l glucose, with levels of fluorescence remaining induced 1 week after glucose normalisation subsequent to 2 weeks of 30 mmol/l glucose (Fig. 4). Further, it was shown that addition of the mitochondrial uncoupling protein UCP2, the antioxidant ALA and the NAD(P)H oxidase inhibitor apocynin to the last week of normalised glucose reduced ROS-related CM-H₂DCFDA fluorescence (Fig. 4).

‘Memory’ of hyperglycaemic stress in the retina of diabetic animals As with the isolated endothelial cells, levels of fibronectin, activated PKC- β , p47phox, Bax, 3-NY and PAR were assessed in the retina of rats that were (1) normoglycaemic for 3 weeks or (2) hyperglycaemic for 3 weeks or (3) hyperglycaemic for 2 weeks followed by normoglycaemic for 1 week (‘memory’). With the exception of Bax, the levels of these hyperglycaemic stress markers were increased in the retina of diabetic animals (Fig. 5a–c, e, f). The levels of these markers, including Bax, remained elevated for 1 week after normalisation of glucose levels (Fig. 5).

The role of ROS in ‘memory’ of high glucose stress in diabetic retina The next experiment was to determine whether the persistence of hyperglycaemic stress could be interrupted in the rat diabetic model in vivo, using the mitochondrial antioxidant nutritional supplement, ALA, which has previously been shown to interrupt target organ damage when given chronically to diabetic animals [20]. The addition of ALA to the last week of normalised glucose resulted in significant decreases in most of the hyperglycaemic stress markers in the retina of diabetic animals (Fig. 5).

Discussion

In this study we were able to confirm that a persistence or cellular ‘memory’ of high glucose stress exists after glucose levels are normalised in endothelial cells. However, for the first time, we have demonstrated that persisting overproduction of ROS may explain this phenomenon. Also, for the first time, we have found that the inhibition of

Fig. 5 Effect of glucose normalisation and ALA on the markers of hyperglycaemic stress fibronectin (a), phospho PKC α / β II (b), p47phox (c), Bax (d), 3-NY (e) and PAR (f) in retina of streptozotocin-induced diabetic rats. Data, expressed as mean $\pm$ SEM, are representative of

two separate experiments, $n=7$ –11 rat retinas. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs organs of rats made hyperglycaemic for 2 weeks followed by glucose normalisation for 1 week (H→N). # $p<0.05$ vs H→N; ## $p<0.01$ vs H→N. N, normal glucose; H, high glucose

mitochondrial ROS production with ALA [13] or UCP2 transfection [17] can interrupt aspects of this ‘memory’ phenomenon. Moreover, inhibiting extra-mitochondrial ROS production or PARP activity also reduced several, but not all, of the persistently induced stress markers.

There is convincing evidence for the notion that a persisting increase of ROS generation provides the mechanistic basis underlying the hyperglycaemic ‘memory’ of high glucose stress marker induction. The fact that the general antioxidant ALA, which works at the mitochondrial level [13], was capable of disrupting the ‘memory’ for all of the high glucose stress markers in isolated endothelial cells and rat retinas as well as the induction of free ROS in endothelial cells, supports this hypothesis. Moreover, the differential effects of inhibiting mitochondrial ROS compared with extra-mitochondrial ROS in endothelial cells suggest that the persistence of the ‘memory’ phenomenon is related to a mitochondrial effect of hyperglycaemia. This is

in keeping with previous findings, which have demonstrated that in conditions of high glucose mitochondrial overproduction of ROS accounts for the activation of all the other pathways involved in damaging endothelial cells, including those involved in amplifying the production of ROS, such as PKC and NAD(P)H excess [33–35].

Our data confirm the existence of a ‘memory’ of hyperglycaemia-induced damage in retinal cells in culture and in the retina of diabetic rats, suggesting that this phenomenon is linked to persistence of the oxidative stress. This hypothesis is consistent with a previous finding that the return to good glycaemic control in rats after 6 months of very poor control was ineffective in decreasing 3-NY levels or other markers of oxidative stress in the retina of these diabetic animals [12]. Moreover, the effect of ALA in our study supports the finding of Lin et al., showing protective effects of ALA in experimental diabetic retinopathy through the inhibition of oxidative and nitrosative stress [36].

The 1 week duration of the ‘memory’ of high glucose stress is too long to be explained by simple signalling mechanisms or the half-lives of messenger RNA or protein. Thus there must be something propagating this ‘memory’ phenomenon. Based on the excess of free ROS observed after glucose normalisation and the ability of different mechanisms to inhibit ROS and interrupt aspects of the persistence of high glucose stress, an excess of intracellular ROS in target cells is a likely candidate. In addition, although the particular regulatory mechanisms of the high glucose stress markers assessed in this paper differ, all of these markers are directly or indirectly induced by ROS [34, 37–42], again implicating an excess of cellular ROS in the ‘memory’ phenomenon. Interestingly, ALA and in particular overexpression of UCP2 were able to reduce the ‘memory’ effect for all the studied parameters, suggesting that persistent mitochondrial generation of ROS plays a key role in producing this phenomenon. A purely speculative explanation is the possibility that mitochondrial proteins are glycated during the 2 week exposure to high glucose. These premises are important because a recent study, for the first time, has described a direct relationship between the formation of intracellular AGE on mitochondrial respiratory chain proteins, the decline in mitochondrial function and excess formation of reactive species [43, 44]. Thus, AGE-modified mitochondrial respiratory chain proteins are prone to produce more ROS, independently of the level of glucose [43, 44], and could therefore also be propagating the ‘memory.’ Another possible alternative explanation is persistent activation of AGE receptors (RAGE) [45]. Further studies will be needed to explore these hypotheses.

In our study, only the inhibition of mitochondrial production of ROS seemed to completely abolish the ‘memory’ effect of hyperglycaemia, while the inhibition of cytoplasmic free radicals only partially interrupted this effect. This is not surprising because mitochondrial generation of free radicals is considered to be only the first step of ROS generation, which can proceed through several other intracellular sources [46–48].

It is also intriguing that several markers not only failed to normalise or remain at the level reached during high glucose exposure, but in fact increased further after glucose normalisation. However, because expression of these markers has been shown to be regulated by free radical generation, the persistence of ROS production in the cells may also account for this phenomenon.

In summary, our results provide a proof-of-principle of cellular ‘memory’ of induction of six high glucose stress markers of basement membrane thickening, oxidative stress, cell death, cell signalling and DNA damage, as well as of free ROS in endothelial and retinal pigment epithelial cells and in the retina of diabetic rats long after glucose normalisation. Furthermore, antioxidants and inhibitors of

mitochondrial ROS and to a lesser extent extra-mitochondrial ROS production were shown to interrupt aspects of this ‘memory’ phenomenon. These data support and extend the hypothesis that an excess of ROS is the causal link between hyperglycaemia and complications of diabetes [49]. Although the length of the experiment was very short, our data still show that 1 week of tight glucose control is not sufficient to normalise the damage induced by hyperglycaemia. Our data therefore confirm the need for early tight glycaemic control to avoid diabetic complications. At the same time, they also further strengthen the use of antioxidant agents, together with glucose normalisation, to mitigate the progression of these complications.

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