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Antioxidant pyrrolidine dithiocarbamate prevents defective bradykinin-stimulated calcium accumulation and nitric oxide activity following exposure of endothelial cells to elevated glucose concentration

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Summary Previous studies from our laboratory suggest that reactive oxygen contributes to diminished bradykinin-stimulated calcium accumulation in endothelial cells exposed to elevated glucose concentrations. In this study, we evaluated the efficacy of the antioxidant pyrrolidine dithiocarbamate (PDTC), in preventing defects in intracellular calcium signalling and nitric oxide (NO) activity in endothelial cells exposed to elevated glucose concentration. We show that PDTC prevented the elevated glucose-induced impairment in bradykinin-stimulated calcium accumulation without changing the normal calcium accumulation in response to ionomycin. Furthermore, the impaired cyclic GMP in RFL-6 detector cells (an index of NO activity) generated by bradykinin-stimulation of high glucose-exposed endothelial cells was restored to normal by pretreatment with PDTC. These studies support a role of reactive oxygen in elevated glucose-induced defects in calcium signalling and NO activity by endothelial cells and that antioxidants may be useful in preventing this defect. [Diabetologia (1998) 41: 806–812]

Keywords Pyrrolidine dithiocarbamate, nitric oxide, endothelium, hydroxyl radical, intracellular calcium, diabetes mellitus, hyperglycaemia, antioxidants.

Endothelium-dependent relaxation is impaired in diabetes [1–4]. It is probable that chronic exposure of blood vessels to elevated glucose concentrations contribute to impaired endothelium-dependent relaxation in diabetes mellitus since restoration of glycaemic control by islet [5] or whole pancreas [6] transplantation restores relaxation to normal. This hypothesis is strengthened by studies showing that exposure of normal arteries to elevated glucose concentration in vitro [7–9] or in vivo [10–12] result in defective endothelium-dependent relaxation.

Release of agonist-stimulated NO from the vascular endothelium which contributes to endothelium-dependent relaxation is regulated by increases in intracellular calcium concentration [Ca^{2+}_i]. We have recently shown that exposure of cultured endothelial cells to elevated glucose concentrations (but not elevated mannose or sucrose concentrations) results in decreased bradykinin-stimulated [Ca^{2+}_i] and NO production [13].

While defective endothelium-dependent relaxation after exposure of intact blood vessels to elevated glucose concentration is believed to be mediated via oxygen-derived free radicals, the role of oxygen-derived free radicals contributing to impaired Ca^{2+}_i signalling and NO production in isolated endothelial cells exposed to elevated glucose concentration has yet to be ascertained. In the present study, we evaluated the efficacy of PDTC, an antioxidant and metal chelator [14, 15], in preventing glucose-induced defects in Ca^{2+}_i signalling in bovine aortic endothelial

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Abbreviations: PDTC, Pyrrolidine dithiocarbamate; NO, nitric oxide; •OH, hydroxyl radical; NF-κB, nuclear factor kappa B; IBMX, isobutyl-1-methylxanthine; HEPES, N-2-hydroxyethylpiperazine-N′-2ethanesulphonic acid; SOD, superoxide dismutase.

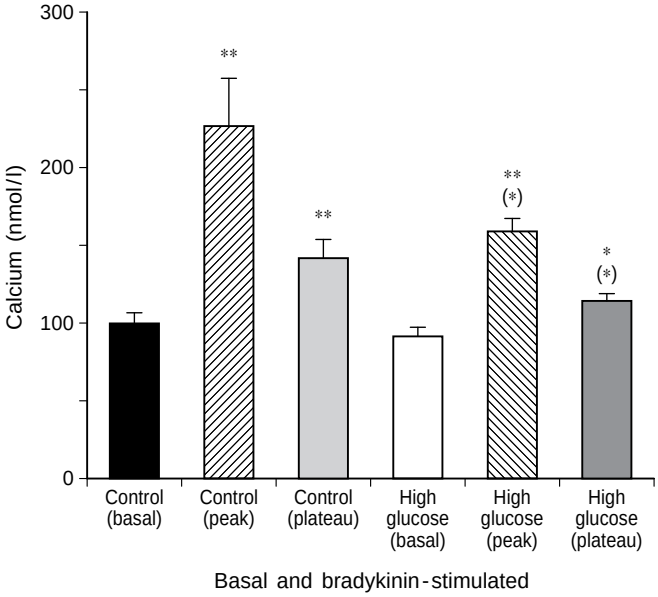

Fig. 1. Effects of previous incubation for 24 h with high glucose (35 mmol/l) concentration versus control glucose (5.5 mmol/l) in media on $[Ca^{2+}_i]$ at baseline and at peak and plateau after stimulation of bovine aortic endothelial cells stimulated with 200 nmol/l bradykinin. Results reflect the mean ± SEM of $n = 7$ paired experiments. $*p < 0.05$ and $** p < 0.01$ compared with basal response in the same cells; $(*)p < 0.05$ compared with pair-matched response in control cells

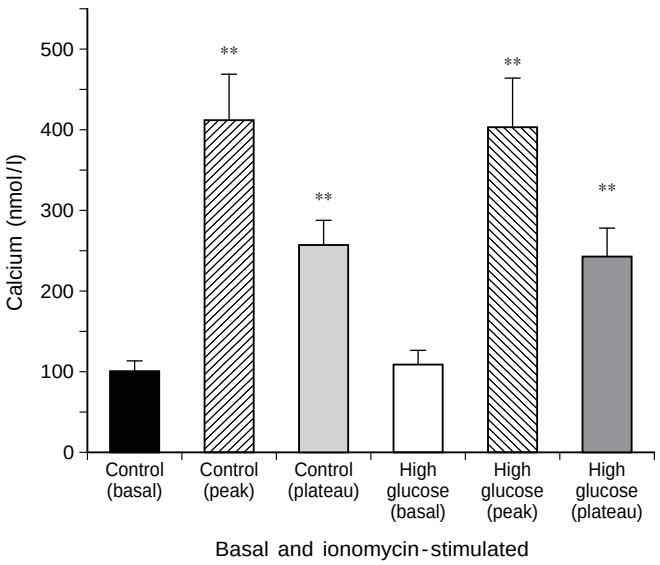

Fig. 2. Exposure to 35 mmol/l glucose for 24 h does not alter the peak and plateau $[Ca^{2+}_i]$ in endothelial cells stimulated by 190 nmol/l ionomycin. Results reflect the mean ± SEM of $n = 7$ paired experiments. $** p < 0.01$ compared with $[Ca^{2+}_i]$ under basal, unstimulated conditions

cells using fluorescence spectroscopy. In addition, we investigated whether improvements in Ca^{2+}_i signalling by PDTC pretreatment resulted in enhanced NO activity.

Materials and methods

Bovine aortic endothelial cells (passage no. 2) were obtained from N.I.A. Cell Culture Repository (Coriell Institute for Medical Research, Camden, N.J., USA). Cells were grown in monolayers in MEM (minimum essential media) media supplemented with 10% fetal calf serum, 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamicin, 8 μg/ml tylosin and 0.25 μg/ml amphotericin in humidified 95% air and 5% CO_2. Endothelial cells after passages 5–9 were used for these studies.

At 24 h prior to Ca^{2+}_i determinations, the culture media was removed and replaced with glutamine-free, serum-free media containing normal glucose concentration (i.e. 5.5 mmol/l) or high glucose (i.e. 35 mmol/l glucose). In pair-matched cultures, cells were preincubated with 50 μmol/l PDTC for 30 min before and during 24 h in media containing control or high glucose. Cells were harvested by adding 0.03% trypsin in Puck's media, centrifuged and re-suspended in N-2-hydroxyethylpiperazine-N′-2ethanesulphonic acid (HEPES)-buffer (pH 7.4) consisting of (in mmol/l): 137 NaCl, 5.3 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES.

Endothelial cells were loaded with FURA-2AM (20 μmol final concentration) for 30 min in the dark, diluted with 10 × volumes of buffer and incubated for 30 min. After loading with indicator, the cells were centrifuged, washed and re-suspended to 3.2×10^5 cells/ml using HEPES-buffered saline (pH 7.4). $[Ca^{2+}_i]$ was determined using a Perkin-Elmer Model LS50 spectrofluorometer. Fluorescence was measured at excitation wavelengths of 333 and 375 nm and emission wavelength of 510 nm and corrected for autofluorescence as described in our previous study [13]. $[Ca^{2+}_i]$ was determined under resting conditions and after stimulation with 200 nmol/l bradykinin or 190 nmol/l ionomycin representing receptor-dependent and receptor-independent agonists for NO release.

Additional studies were performed to evaluate the change in NO production by monolayers of endothelial cells exposed to elevated glucose in the presence or absence of PDTC using the cyclic GMP generated in RFL-6 detector cells [16] and described previously in our laboratory [13]. In addition to the use of detector cells to improve sensitivity and detection of released NO activity, endothelial cells were stimulated with either bradykinin or ionomycin at 1 μmol. Media (containing 100 U/ml superoxide dismutase (SOD) and 100 μmol IBMX) from basal and agonist-stimulated endothelial cells was transferred after 2 min to RFL-6 cells in the presence of SOD and IBMX and the reaction terminated using ice cold TCA after 2 min. Using this protocol, we have shown that agonist-stimulated cyclic GMP generation is specific for NO since it is blocked by the NO synthase inhibitor, L-nitroarginine [13].

For each experiment, the $[Ca^{2+}_i]$ and cyclic GMP measurements were performed in at least duplicate, repeated on individual days and the results of several days averaged. Data were analysed by analysis of variable (ANOVA) for paired or unpaired observations, where appropriate, with a $p < 0.05$ chosen to denote statistical significance.

Results

Exposure to elevated glucose had no effect on basal $[Ca^{2+}_i]$ (Fig. 1). Exposure to elevated glucose concentration produced a decrease in bradykinin-stimulated

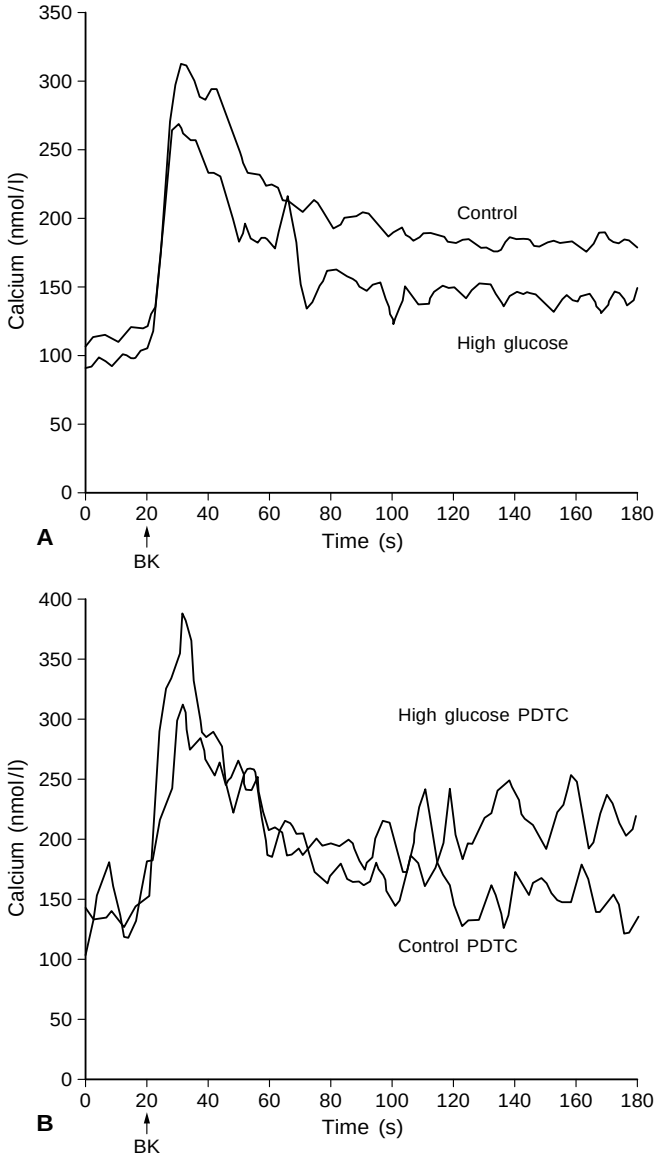

Fig. 3. Example showing peak and plateau $[Ca^{2+}_i]$ in endothelial cells stimulated by bradykinin but exposed previously for 24 h in the presence of either 5.5 mmol/l glucose (control) or 35 mmol/l glucose (high glucose) in the absence (panel **A**) or presence (panel **B**) of the antioxidant, PTDC (50 μmol/l)

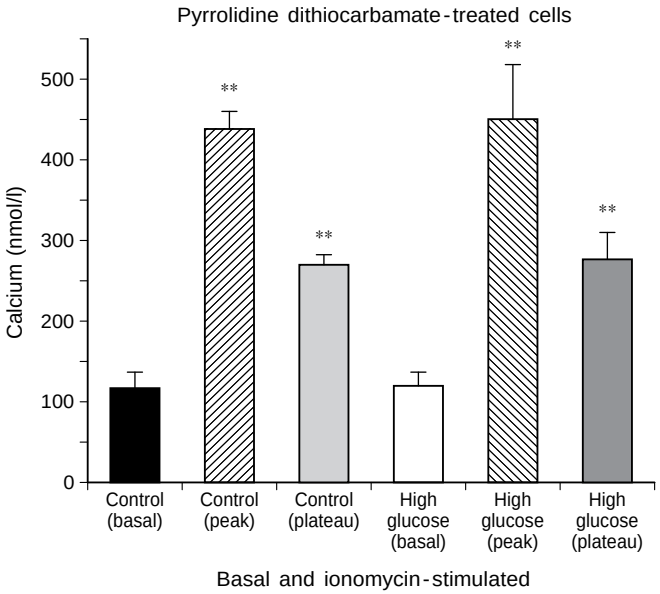

Fig. 4. Pair-matched experiments showing the lack of attenuated bradykinin-stimulated $[Ca^{2+}_i]$ during peak and plateau phases in endothelial cells exposed previously for 24 h with 35 mmol/l vs 5.5 mmol/l glucose but pretreated with the antioxidant, PDTC (50 μmol/l). Results reflect the mean ± SEM of $n = 10$ paired experiments. ** $p < 0.01$ compared with $[Ca^{2+}_i]$ under basal, unstimulated conditions

$[Ca^{2+}_i]$ assessed at the peak response (1 min) or at the plateau phase (3 min) following bradykinin. In contrast, ionomycin-stimulated $[Ca^{2+}_i]$ at 1 and 3 min post-stimulation was unaltered by elevated glucose exposure (Fig. 2). Pretreatment with PDTC prevented the attenuated bradykinin-stimulated Ca^{2+}_i at both peak and plateau phases in cells exposed to elevated glucose concentration (example shown in Fig. 3) while having no effect on cells exposed to normal media glucose concentration or on basal $[Ca^{2+}_i]$ in either group (Fig. 4). In cells exposed to elevated glucose concentration, pretreatment with PDTC did not alter the peak or plateau $[Ca^{2+}_i]$ in response to ionomycin (Fig. 5).

Previous exposure to elevated glucose conditions produced no significant change ($p > 0.05$) in the cyclic GMP generated within RFL-6 detector cells in endothelial cells stimulated with ionomycin (i. e. 5.5 mmol/l glucose: 4.92 ± 0.34 pmol/mg protein; 35 mmol/l glucose: 5.60 ± 0.47 pmol/mg protein, $n = 7$ paired experiments). In contrast, cyclic GMP generation was diminished by bradykinin-stimulated endothelial cells exposed to elevated glucose concentration (Fig. 6). Furthermore, treatment with PDTC normalized the cyclic GMP production elicited by bradykinin-stimulated endothelial cells exposed to elevated glucose concentration.

Discussion

Chronic treatment of experimental diabetic animals with antioxidants such as dimethylthiourea [17] or vitamin E [18] has been shown to prevent the impairment of endothelium-dependent relaxation. The observation that scavengers of reactive oxygen can also prevent the impairment of endothelium-dependent relaxation initiated by acute exposure of normal arteries to elevated glucose concentrations [7–10] suggests that reactive oxygen generated under high glucose conditions may contribute to the etiology of defective relaxation.

Indeed, a significant increase in reactive oxygen production has been demonstrated previously during

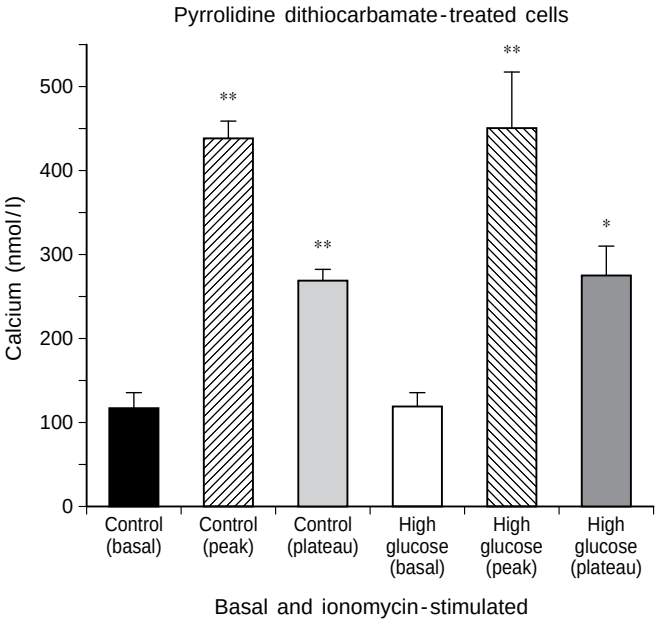

Fig. 5. Pretreatment with 50 μmol/l PDTC does not alter the ionomycin-stimulated $[Ca^{2+}_i]$ in endothelial cells exposed for 24 h to 35 mmol/l glucose vs 5.5 mmol/l glucose. Results reflect the mean ± SEM of $n = 7$ paired experiments. ** $p < 0.01$ compared with $[Ca^{2+}_i]$ under basal, unstimulated conditions

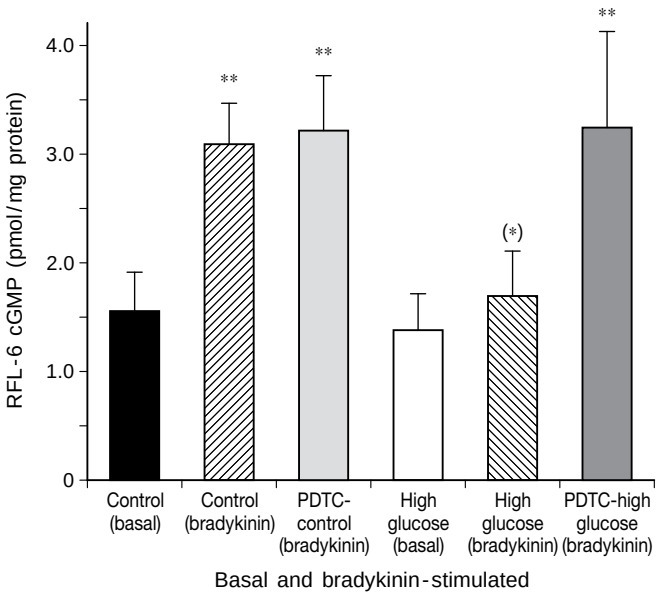

Fig. 6. Exposure to 35 mmol/l glucose for 24 h impairs and pretreatment with 50 μmol/l PDTC reverses bradykinin-stimulated nitric oxide activity arising from endothelial cells as assessed by cGMP generation in RFL-6 detector cells. Results reflect the mean ± SEM. Untreated cells ($n = 7$); treated cells ($n = 5$). ** $p < 0.01$ compared with cGMP generated under the corresponding basal, unstimulated conditions; (*)$p < 0.05$ compared with bradykinin-stimulated response in control cells

the actual exposure period of bovine endothelial cells to elevated glucose concentration using fluorescence spectroscopic techniques [13, 19]. In other studies, prior exposure to elevated glucose concentration and subsequent removal also produced a latent increase in the endogenous rate of superoxide radical release by murine or porcine endothelial cells as determined by the ferricytochrome C technique [20, 21]. Taken together, these observations suggest two separate effects of high glucose concentrations on reactive oxygen production from endothelial cells: a) direct stimulation of reactive oxygen co-incidental with glucose incubations and; b) indirect modification in the intrinsic rate of spontaneous reactive oxygen production following prolonged exposure to elevated glucose.

In our study, we found that pretreatment with the antioxidant, PDTC, prevented the reduction in both bradykinin-stimulated Ca^{2+}_i and NO activity as a result of exposure to elevated glucose concentration. This effect could not be explained by a generalized improvement in Ca^{2+}_i signalling since the response to the receptor-independent agonist, ionomycin, was not enhanced by PDTC treatment. Furthermore, the restoration of bradykinin-stimulated $[Ca^{2+}_i]$ was associated with an improvement in NO activity as judged by the improved cyclic GMP generated in RFL-6 detector cells. This is consistent with a role of Ca^{2+}_i signalling for NO release.

Previous studies have shown that elevated glucose decreases agonist-stimulated Ca^{2+}_i production or NO activity or both in porcine [22, 23] and rat endothelial cells [24]. Furthermore, previous exposure of endothelial cells to exogenous reactive oxygen (e. g. peroxides) also diminishes receptor-dependent Ca^{2+}_i signalling [25–27]. The finding that both oxidants [28] and high glucose concentrations [9, 11] selectively impair receptor-mediated endothelium-dependent relaxation of normal blood vessels, taken together with the findings in isolated endothelial cell preparations, suggests a defect in receptor coupling of Ca^{2+}_i signalling to NO synthesis and that this is not due to a generalized defect in endothelial cell function.

The precise location of this defect is not known. Our previous work using the G-protein activators, NaF [13] and mastoparan (unpublished observations) in endothelial cells suggests that there is also no direct intrinsic defect in G-protein initiated Ca^{2+}_i increases and that a site proximal to G-protein activation may be important in this differential effect of elevated glucose. According to previous studies using differing endothelial cell types [29–31], ionomycin stimulates intracellular Ca^{2+}_i accumulation by release from intracellular stores followed by entry from extracellular sources. This entry is believed to occur via bradykinin-like entry pathways but to be elicited via a receptor-independent mechanism. Accordingly, our results suggest the possibility that the glucose-induced defect arises from altered Ca^{2+} release while indirectly impacting on extracellular Ca^{2+} entry. Since

the plateau phase of ionomycin was not altered by glucose, this suggests a defect that is unique to receptor-mediated coupling of intracellular Ca^{2+} release and extracellular Ca^{2+} entry.

As opposed to the findings of defective Ca^{2+}_i signalling and NO activity by elevated glucose exposure of bovine (this study), porcine [22, 23] and rat [24] endothelial cells, other investigators have shown that elevated glucose exposure enhanced basal or agonist-stimulated Ca^{2+}_i signalling in porcine [21] and human umbilical vein endothelial cells [32]. These discrepancies may arise from variances in culturing conditions and media, type of endothelial cells, glucose concentration and exposure time, and/or the conditions of measurement of Ca^{2+}_i and NO/cyclic GMP. As to the Ca^{2+}_i measurements, it is interesting to note that in some studies [21, 29] the actual Ca^{2+}_i measurements appear to have been conducted under substrate-free conditions (i.e. without any glucose in the media). Also, in contrast to all previous studies in this area, our studies evaluated NO production using RFL-6 detector cells rather than detection of cyclic GMP generated within the endothelial cell.

We used this RFL-6 detector system in order to negate any potential influences of variability in endogenous guanylate cyclase within endothelial cells and to obtain a more true measure of the bioactive NO released by the endothelium under these conditions. The provision of extracellular SOD is designed to inhibit interaction of superoxide anion radicals released from endothelial cells with NO released from endothelial cells. Without SOD this interaction could have produced diminished bioactive NO in the media and, thus, NO dectected by the RFL-6 cells. This design does not eliminate the possibility that intracellularly generated superoxide might interact with intracellularly generated NO especially after exposure to high-glucose resulting in diminished bioactive NO. It is possible that PDTC returns NO production to normal by preventing changes induced by glucose exposure in intrinsic superoxide generation.

It is interesting that antioxidants including the metal-chelator, desferal, inhibited the enhanced Ca^{2+}_i signalling and NO activity subsequent to glucose exposure of porcine endothelial cells [21, 33]. Despite these opposing observations of the beneficial effects of antioxidants on enhanced NO activity, no other laboratory has evaluated whether antioxidants can prevent glucose-induced impairment in Ca^{2+}_i signalling and NO activity from isolated endothelial cells. Thus, this study together with our previous study [13] provides important new information regarding the efficacy of antioxidants in preventing glucose-induced signal transduction defects. It also provides the first known application and efficacy of the antioxidant, PDTC, under conditions of elevated glucose concentration.

We concluded previously that intracellular generation of the hydroxyl radical (•OH) was probably the molecular species responsible for the attenuation in bradykinin-stimulated Ca^{2+}_i and NO production by exposure of endothelial cells to elevated glucose concentration, since these defects were prevented by dimethylthiourea (an intracellular •OH scavenger) but not by either mannitol (an extracellular •OH scavenger), SOD (an extracellular enzyme which converts superoxide anion to H_2O_2) or catalase (an extracellular enzyme which detoxifies H_2O_2). Our findings support and extend the conclusion that the •OH plays a significant role in endothelial cell dysfunction produced by elevated glucose concentration based upon the understanding that the dithiocarbamate PDTC (unlike dimethylthiourea) is both a metal chelator and an •OH scavenger [14, 15]. Thus, the present study provides new information that metal ion-catalysed •OH plays a role in defective Ca^{2+}_i signalling and NO release in endothelial cells exposed to high glucose conditions. This conclusion is consistent with our recent study showing that chronic treatment in vivo with a conjugated form of deferoxamine prevented the impaired endothelium-dependent relaxation in diabetic rats [34]. Collectively, these findings support the hypothesis that elevated glucose conditions generate reactive oxygen via a metal-catalysed process [35].

In addition, we suggest for various reasons that PDTC is probably acting via an intracellular process to prevent glucose-induced defects. First, we indicated previously a requirement for intracellular glucose action since no defects in $[Ca^{2+}_i]$ were seen if glucose uptake was inhibited using cytochalasin B [13]. Second, PDTC has been used frequently as a classical inhibitor of cytokine-induced activation of the transcription factor, nuclear factor kappa B (NF-κB). This activation requires an intracellular signal which is believed to be mediated by reactive oxygen in particular •OH [15]. NF-κB activation may play a key role in etiology of endothelial cell dysfunction in diabetes since it binds to the promoter region of several genes including those which encode various adhesion proteins.

Recent studies have shown that glycated albumin produced by incubation of albumin with elevated glucose concentrations produces NF-κB activation in endothelial cells [36]. Independent of this, we have demonstrated recently that oxidative stress produced by elevations in glucose concentration per se may also be a stimulus for NF-κB activation since glucose produced a time-dependent and concentration-dependent increase in NF-κB activation in endothelial cells [37]. Consistent with the present study, we have observed that NF-κB activation occurs as early as a 2 h after exposure to 35 mmol/l glucose and that this activation was prevented completely by incubation with PDTC (unpublished observations). Since the ac-

tivation of NF-κB is an intracellular event, the ability of PDTC to block glucose-induced NF-κB expression implies that PDTC can act via an intracellular mechanism. Taken together with the present study, these findings indicate that PDTC may be effective in interrupting a variety of biological processes activated by increases in glucose concentration which could impact on endothelial cell function in hyperglycaemic states.

In summary, our results suggest that the antioxidant, PDTC, was effective in preventing glucose-induced defects in Ca^{2+}_i and NO production by endothelial cells. These studies support the notion that antioxidants may be effective therapeutic interventions to prevent defects in endothelial cell function associated with hyperglycaemic states such as diabetes mellitus.

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