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Link between free radicals and protein kinase C in glucose-induced alteration of vascular dilation

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Abstract

Development of vascular complications in diabetes has been linked to the quality of glucose regulation and characterized by endothelial dysfunction. The exact mechanism behind vascular complications in diabetes is poorly understood. However, alteration of nitric oxide (NO) biosynthesis or bioactivity is strongly implicated and the mechanism behind such alterations is still a subject for research investigations. In the present study, we tested the hypothesis that glucose-induced attenuation of vascular relaxation involves protein kinase C (PKC)-linked generation of free radicals. Vascular relaxation to acetylcholine (ACh; 10^{-9} – 10^{-5} M), isoproterenol (10^{-9} – 10^{-5} M), or NO donor, sodium nitropruside (SNP; 10^{-9} – 10^{-6} M) was determined in phenylephrine (PE, 10^{-7} M) pre-constricted aortic rings from Sprague-Dawley rats in the presence or absence of 30 mM glucose (30 min), L-nitro-arginine methyl ester (L-NAME; 10^{-4} M for 15 min), a NO synthase inhibitor, or xanthine (10^{-5} M), a free radical generator. ACh dose-dependently caused relaxation that was attenuated by L-NAME, glucose, or xanthine. Pre-incubation (15 min) of the rings with vitamin C (10^{-4} M), an antioxidant or calphostin C (10^{-6} M), a PKC inhibitor, restored the ACh responses. However, high glucose had no significant effects on SNP or isoproterenol-induced relaxation. ACh-induced NO production by aortic ring was significantly reduced by glucose or xanthine. The reduced NO production was restored by pretreatment with vitamin C or calphostin C in the presence of glucose, but not xanthine. These data demonstrate that oxidants or PKC contribute to glucose-induced attenuation of vasorelaxation which could be mediated via impaired endothelial NO production and bioavailability. Thus, pathogenesis of glucose-induced vasculopathy involves PKC-coupled generation of oxygen free radicals which inhibit NO production and selectively inhibit NO-dependent relaxation.

Keywords

Glucose; Nitric oxide-dependent relaxation; Protein kinase C; Free radical; Antioxidant

Introduction

Endothelium is known to play significant role in the regulation of vascular tone in physiological and pathological conditions through the release of different endothelium-derived vasoactive agents (Cines et al., 1998; Cohen and Vanhoutte 1995; Ignarro 1990; Moncada et al., 1991; Rubanyi and Vanhoutte 1986). Principal among these endothelial factors is nitric oxide (NO). In the vascular system, NO is an important mediator of

vasodilation, it is released by various vasoactive agents and provides protection for the endothelium (Moncada et al., 1991). NO is synthesized by endothelial cells from L-arginine by a Ca^{2+} -dependent enzyme NO synthase (eNOS) (Moncada et al., 1991). Endothelial NOS is constitutively expressed in the endothelium and can be activated and/ or modulated by various stimuli (Moncada et al., 1991).

Endothelial dysfunction is a key feature of diabetes and is a major cause of associated vascular complications (Inoguchi et al., 2000; Koya and King, 1998). Endothelial cell damage with loss of vascular protective and dilator actions of NO is a likely step in the accelerated vascular dysfunction in diabetes. Impaired endothelium-dependent vasodilatation has been described in diabetes and was shown that the degree of impairment of relaxation correlated with glycemic control (Giugliano et al., 1996, 1997; Inoguchi et al., 2000). It has been reported that in diabetes, NO production and/or responsiveness of smooth muscle to NO are decreased (Sikorski et al., 1993; Taylor et al., 1995). Hyperglycemia-induced vascular dysfunction has been suggested to be mediated via several mechanisms. Enhanced NOS expression and increased superoxide anion generation in endothelial cells has been reported following exposure to high glucose (Cosentino et al., 1997; Tesfamariam and Cohen, 1992). The increased free radical generation can lead to reduction in NO availability and loss of NO-dependent vasodilation (Ganz and Seftel, 2000; Giugliano et al., 1996, 1997). The mechanism by which glucose impairs vascular dilatation via increased generation of free radical is not clearly defined. But increased de novo synthesis of diacylglycerol from glucose via acyl-dihydroxyacetone phosphate has been reported (Beckman et al., 2002; Dunlop and Larkns, 1985; Koya and King, 1998; Gutterman, 2002). Diacylglycerol is a known activator of PKC and increased activation of PKC by diacylglycerol may further stimulate the generation of free radicals. Therefore, PKC may play an important role in high glucose-induced alteration of vascular function via generation of free radicals. In the present study, we have investigated the link between free radical and PKC in high glucose-induced alteration of vascular responses to vasoactive agents in the rat aorta.

Materials and methods

Experimental animals

Male Sprague-Dawley rats (250–350 gm) were used for this study. The protocol for this study was approved by the Animal Care Committee of the Texas Southern University, Houston. Rats were treated according to the NIH Guidelines on Care for Animal Use in Research. Rats were anesthetized with pentobarbital sodium (50 mg/kg; ip). The chest cavity was opened, and the thoracic aorta removed and placed in a petri-dish containing cold Krebs' (4°C) solution of the following composition (mM): NaCl 113, KCl 4.7, NaHCO_3 25.0, CaCl_2 2.5, KH_2PO_4 1.2, MgSO_4 1.2, and glucose 5, pH 7.4, and continuously aerated with 95% O_2 , 5% CO_2 . The aorta was cleansed of excess fat and connective tissue and cut into 3–4 mm rings. The aortic ring was then mounted in a 10 mL jacketed bath (World Precision Instruments-WPI, Sarasota, FL) at 37°C. The ring was suspended in bath solution by means of two hooks, the lower one fixed to the bottom of the bath while the upper one was connected via a transbridge (model TBM4, World Precision Instruments, Sarasota, FL) data-acquisition system (DataQ Instruments, Akron, OH) for recording of isometric tension developed to application of vasoactive agents. The rings were subjected to a resting tension of 2 g, this tension have been shown to be sufficient for aortic ring of 3–4 mm length (Sofola et al., 2003). The ring under this tension was allowed to equilibrate for a period of 90 min while being rinsed every 15 min. During the equilibration period, the rings were subjected to two challenges of 10^{-7} M phenylephrine (PE), 30 min apart.

Cumulative dose-response curves to ACh

Following the equilibration period, relaxation responses to cumulative doses of ACh (10^{-9} – 10^{-5} M) were determined in aortic rings pre-constricted with PE (10^{-7} M). The concentration of PE used was shown in preliminary experiments to produce about 70% of maximal contraction. The contraction to PE was allowed to reach a plateau and stabilize (about 5–7 min) before relaxation studies commenced.

To investigate the role of NO in the ACh-induced relaxation, aortic rings were incubated with L-NAME (10^{-4} M) for 15 min before pre-constriction with PE and concentration-response relationship to ACh determined.

Effects of high glucose or xanthine on NO-mediated vascular relaxation

To investigate the effects of high glucose or xanthine, a free radical generator, on ACh-induced relaxation, aortic rings were incubated in 30 mM glucose or xanthine (10^{-5} M) for 30 min before dose-response to ACh was determined.

Effects of oxidant or PKC

We determined the effects of antioxidant, vitamin C or calphostin C, a PKC inhibitor, on glucose or xanthine-induced blunting of ACh relaxation by incubating aortic rings with vitamin C (10^{-4} M) or calphostin C (10^{-6} M) for 15 min before the addition of glucose (30 mM) or xanthine (10^{-5} M). Incubation was continued for additional 30 min after which, dose-response relationship to ACh was determined.

In another study, we investigated effects of acute (30 min) or chronic (20 hr) activation of PKC on ACh-induced relaxation. Following incubation of the ring with PMA (1 μ M) for 30 min or 20 hr (PKC down regulation), vascular reactivity to ACh was determined.

Effects of high glucose on non endothelium-dependent relaxation

Effects of high glucose on sodium nitroprusside (SNP; 10^{-9} – 10^{-6} M), an NO-donor, or isoproterenol (10^{-9} – 10^{-5}) a cAMP-dependent vasodilator, were investigated following a 30 min incubation of the aortic ring with glucose (30 mM) or xanthine (10^{-5} M).

Determination of nitrite

For determination of NO production, samples (0.5 mL of Kreb's solution) were collected from the organ bath under control conditions, following ACh dose-responses, and following tissue exposure to glucose or xanthine alone or in the presence of calphostin C or vitamin C. NO₂ was measured by the Griess method as an indication of NO production by the aortic rings. Briefly, assay samples were mixed with an equal volume of the Griess reagent [0.1% *N* (1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 3% H₃PO₄] and incubated to yield a chromophore. Using Plate Reader (Model EL808UV, Bio-Tek Instruments, Uniooski, VT), the absorbance at 540 nm was measured, and nitrite concentration was determined using a sodium nitrite standard curve. The efficiency was at least 95%. Nitrite level was expressed as nmol/mg protein.

Statistical analysis

Vascular relaxation responses are presented as % change in relaxation of aortic ring from pre-constricted values. Data are reported as mean $\pm$ SEM and subjected to analysis of variance (ANOVA) followed by Student Newman-Keul's post-hoc test. $P < 0.05$ was considered significant.

Results

PE-induced tension was not significantly affected by incubation with glucose or xanthine. PE-induced tensions were 0.71 ± 0.1 , 0.75 ± 0.1 , and 0.72 ± 0.2 gram for control, glucose, and xanthine respectively.

In Fig. 1, ACh (10^{-9} – 10^{-5} M) dose-dependently relaxed aortic ring pre-constricted with PE (10^{-7} M). L-NAME (10^{-4} M) virtually abolished ACh-induced relaxation producing about 95% inhibition of the relaxation at the highest concentration of ACh (10^{-5} M) employed and abolishing relaxation at the lower concentrations. Incubation of aortic rings with 30 mM glucose attenuated ACh-induced relaxation ($P < 0.05$; $n = 9$). The attenuation of ACh-induced relaxation in the presence of L-NAME and high glucose was not greater than that in the presence of L-NAME alone.

Fig. 2 depicts the effect of vitamin C (10^{-4} M) on high glucose-induced attenuation of ACh relaxation. Vitamin C inhibited the attenuation by glucose of ACh-induced relaxation ($P < 0.05$; $n = 8$). The effects of vitamin C were such that there were no significant differences between control rings or glucose plus vitamin C-treated rings.

Fig. 3 shows that xanthine (10^{-5} M), a free radical generator, attenuated ACh-induced relaxation as did high glucose ($P < 0.05$; $n = 6$). Pretreatment of aortic rings with vitamin C (10^{-4} M) abolished xanthine-induced attenuation of ACh relaxation such that the relaxation to ACh was similar to that observed in control rings.

Pretreatment of aortic rings with PKC inhibitor, calphostin C (10^{-6} M), abolished the effects of high glucose (Fig. 4A), or xanthine (Fig. 4B) on ACh-induced relaxation of the aortic ring ($P < 0.05$; $n = 6$). Calphostin C alone enhanced ACh-induced relaxation compared to the control ($P < 0.05$; $n = 8$). This enhancement was still present in rings exposed to combined calphostin C and xanthine (Fig. 4B) but not with glucose (Fig. 4A).

Fig. 5A and B depicts the effect of glucose on SNP or isoproterenol-induced relaxation. Unlike the effects on ACh-induced relaxation, 30 mM glucose did not have significant effect on SNP- or isoproterenol-induced relaxation. Similarly, L-NAME 10^{-4} M had no effects on SNP or isoproterenol-induced relaxation, (Fig. 5A and 5B) ($n=8$). In additional experiments, xanthine also did not have a significant effect on SNP- or isoproterenol-induced relaxation of aortic ring (results not shown).

Fig. 6 show results of NO production presented as NO_2 production in nanomol/mg protein. In the control group, 30 mM glucose (Fig. 6A) or 10^{-4} M xanthine (Fig. 6C) significantly ($P < 0.05$; $n = 5-9$) reduced NO_2 concentration when compared to the control but not to vitamin C or calphostin C, alone or in combination with glucose. Following cumulative dose-responses to ACh, NO production was significantly reduced in the glucose- or xanthine-treated group (Fig. 6A–D; $P < 0.05$; $n = 6-10$). This reduction was restored in the presence of vitamin C or calphostin C alone (Fig. 6A–D) and partially in combination with glucose (Fig. 6A and B). Co-incubation of xanthine with vitamin C or calphostin C further reduced NO production following cumulative dose-response to ACh (Fig. 6C and D).

Fig. 7 shows effects of pretreatment with PMA (10^{-6} M) on ACh-induced relaxation. Acute treatment with PMA for 30 min significantly ($P < 0.05$; $n = 4-5$) attenuated ACh-induced relaxation. The attenuation observed is similar to that observed in the presence of glucose or xanthine. Down regulations of PKC by chronic (20 hr) treatment did not have any effects on ACh-induced relaxation. The relaxation observed following chronic PMA was similar to that observed following inhibition of PKC by calphostin C.

Discussion

In the present study, we observed that (1) acute high glucose-induced attenuation of ACh relaxation involves free radical linked activation of PKC, (2) free radical-PKC mediated actions of glucose is selective for NO-sensitive relaxation mechanism, (3) NO production by aortic ring following cumulative action of ACh was reduced in the presence of high glucose and free radical generator, these reductions were restored by antioxidant as well as by PKC inhibition.

Compromise of endothelium-mediated vasodilatation has been reported in different vascular beds of several animal species following conditions of hyperglycemia (Brodsky et al., 2001; Chan et al., 2000; Sobrevia and Mann, 1997; Taylor et al., 1995; Tesfamariam et al., 1991; Tesfamariam and Cohen, 1992). However, the mechanism by which such attenuation occurs is still not well understood. In this study, ACh-induced relaxation was significantly attenuated by L-NAME, a NO synthase inhibitor, confirming that ACh relaxation of aortic ring is mediated via the release of NO (Moncada et al., 1991; Taylor et al., 1995). In high glucose as well as in the presence of xanthine, ACh-induced relaxation was attenuated and this attenuation was blocked by treatment with vitamin C, antioxidant or calphostin C, a PKC inhibitor. This is an indication that the deleterious actions of high glucose on vascular relaxation involves the generation of free radicals. We posit that the mechanism that links high glucose and increased free radical generation to attenuation of ACh-induced relaxation involves possible activation of PKC as PKC inhibition blunted the effect of high glucose on vascular dilation. Alterations of vascular signal transduction pathways involving activation of PKC via increased generation of free radicals may therefore be affected by high glucose. In addition to generation of free radicals, glucose can independently and directly activate PKC via increased generation of diacylglycerol (Beckman et al., 2002; Koya and King, 1998; Gutterman, 2002). PKC activation has been reported to cause generation of free radicals furthering the cyclic effects of high glucose-induced alteration of vascular relaxation (Beckman et al., 2002; Gutterman, 2002). These processes can result in alteration of endothelium-dependent relaxation of the vascular system. However, the mechanisms involved in such interactions on the vascular system are not clear. In this study, we have shown that pretreatment with calphostin C, a PKC inhibitor, attenuated high glucose- or xanthine-induced attenuation of ACh relaxation. This is consistent with our working hypothesis that glucose-induced attenuation of NO-dependent relaxation mediated by ACh involves activation of PKC. Furthermore, prevention of such interactions by pretreatment of the ring with a PKC inhibitor or an antioxidant prevented the glucose and free radical-induced alteration of vascular relaxation. We hypothesize that the increase in free radical generation and PKC activation may contribute to the loss of ACh-induced and NO-dependent relaxation observed. This is supported by the increase ACh-induced relaxation in aortic rings exposed to vitamin C. This observation is consistent with the inhibition of endogenous NO production by free radicals as pretreatment with antioxidant leads to decreased breakdown of NO and hence increased dilation to ACh. Also consistent with this observation is the blunted attenuation of NO production by glucose but not xanthine following treatments with antioxidant or PKC inhibitor. The reasons behind the lack of effect by xanthine on NO production following antioxidant or PKC inhibition is not known especially since xanthine-induced attenuation of vascular relaxations to ACh was restored in the presence of antioxidant or PKC inhibition. There is the possibility that xanthine may have other effects on NOS enzyme and/ or activity. Despite the presence of antioxidant or PKC inhibition, xanthine-induced reduction in NO production was not restored though attenuated relaxation to ACh was improved. This observation suggests that ACh-induced relaxation may be mediated via other mechanism(s) that is independent of NO for example via endothelium-dependent hyperpolarizing factor, or epoxides (Cohen and Vanhoutte, 1995; Hoepfl et al., 2002). However, this speculation is beyond the scope of this study.

Taken together, these results suggest that high glucose and xanthine-induced attenuation of vascular relaxation by ACh involves the generation of free radicals that is coupled to PKC activation.

In this study, we have noticed a strong independent effect of Calphostin C, a PKC inhibitor, on ACh-induced relaxation. Relaxation in the presence of PKC inhibition was significantly enhanced even in the absence of any other treatment (Fig. 4). This strongly suggests that PKC activation may be involved in attenuation of ACh-induced relaxation. We therefore investigated the possible role of PKC activation in ACh-induced relaxation. Acute activation of PKC by PMA (30 min) significantly attenuated the ACh-induced relaxation; while, chronic treatment of the ring with PMA (20 hr) to down regulate PKC tended to enhance relaxation to ACh similarly to that observed following PKC inhibition by Calphostin C. Is a further indication that activation of PKC plays a prominent role in the alteration of vascular function especially relaxation produced by ACh.

Modulation of ACh-induced relaxation can occur at several points. In the present study, three possible steps could be involved in such modulation; namely, de novo synthesis of NO, stimulation and/or release of NO, and NO-interaction with smooth muscle cells through the activation of cGMP, its second messenger. The latter step does not appear to be involved in high glucose- and free radical-mediated attenuation of ACh-induced relaxation in as much as relaxation of aortic ring by NO donor, SNP, did not appear to be affected by acute high glucose. Similarly, relaxation of aortic ring by isoproterenol, a non NO-dependent agent, does not appear to be affected by high glucose or xanthine. The acute (30 min) exposure of the aorta to high glucose does not rule out the possibility that there was alteration in NO synthesis since effects of classical NOS inhibitors such as L-NAME manifests in a short period of time (15–30 min) (Sikorski et al., 1993). Therefore, the mechanism involved in glucose- and xanthine-induced attenuation of NO-dependent vascular relaxation is most likely due to inhibition of NO synthesis and/ or release from the endothelium. The results from this study indicate that the effect of high glucose and free radical on NO-dependent relaxation appear to be mediated possibly via NO release.

The acute nature of the exposure of vasculature to high glucose probably does not involve any vascular compensation via altered protein synthesis and might reflect a different pattern of effects than seen in arteries exposed to chronic hyperglycemia. Following chronic hyperglycemia, alteration in vascular responses may be wide spread, involving alterations of numerous vascular dilatory mechanisms as we have seen in STZ diabetic rats (Yakubu et al., 2003). Such may result from vascular remodeling, altered vascular smooth muscle cell sensitivity to vasoactive agents due to prolonged perturbation of the vascular system by enhanced oxidative stress (Taylor et al., 1995; Tesfamariam et al., 1991).

In summary, we have demonstrated that high glucose attenuated ACh-induced relaxation of aortic ring via mechanisms that involve free radical generation and is coupled to activation of PKC. The attenuation of relaxation does not seem to involve interference with post NO release mechanisms since the effect of SNP was not affected by high glucose or xanthine. Thus, pathogenesis of glucose-induced vasculopathy involves generation of oxygen free radicals which selectively affect NO production and NO-dependent relaxation via PKC signaling pathway.

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Fig. 1.

Effects of glucose (30 mM), glucose plus L-NAME, or L-NAME (10^{-4} M) alone on ACh-induced relaxation of aortic ring pre-constricted with PE (10^{-7} M). Rings were incubated with Krebs' solution (control) or Krebs' containing glucose, glucose + L-NAME or L-NAME alone for 30 min before dose-dependent relaxation to ACh was determined. Data are presented as mean $\pm$ sem; * $P < 0.05$ compared to the control, ** $P < 0.05$ compared to control and glucose, ANOVA, $n = 9$ from different rats.

Fig. 2.

Effects of Vitamin C (10^{-5} M) on glucose (30 mM)-induced attenuation of ACh relaxation on ACh-induced relaxation of aortic ring pre-constricted with PE (10^{-7} M). Rings were incubated with Krebs' solution (control) or Krebs' containing glucose or glucose plus vitamin C for 30 min before dose-dependent relaxation to ACh was determined. Data are presented as mean $\pm$ sem; * $P < 0.05$ compared to the control, ANOVA, $n = 8$ different rats.

Fig. 3.

Effects of xanthine (10^{-5} M) on ACh-induced relaxation of aortic ring pre-constricted with PE (10^{-7} M). Rings were incubated with Krebs' solution (control) or Krebs' containing xanthine, or xanthine plus vitamin C (10^{-5} M) for 30 min before dose-dependent relaxation to ACh was determined. Data are presented as mean $\pm$ sem; * $P < 0.05$ compared to control, # $P < 0.05$ to control and xanthine: ANOVA, $n = 6$ from different rats.

Fig. 4.

Effects of PKC inhibitor calphostin C (10^{-6} M) on glucose (30 mM) (A) or xanthine (10^{-5} M) (B) -induced attenuation of ACh relaxation of aortic ring pre-constricted with PE (10^{-7} M). Rings were incubated in Krebs' solution (control) or Krebs' containing glucose, or glucose plus calphostin C for 30 min before dose-dependent relaxation to ACh was determined. Data are presented as mean $\pm$ sem; * $P < 0.05$, compared to control and glucose plus Calphostin C, ** compared to control and glucose plus calphostin C; (A), * $P < 0.05$ compared to control, ** compared to control and xanthine (B) respectively, ANOVA, $n = 6-8$ different rats.

Fig. 5.

(A) Effect of glucose (30 mM), glucose plus Vitamin C (10^{-4} M), or L-NAME on SNP-induced relaxation of aortic ring pre-constricted with PE (10^{-7} M). Rings were incubated in Krebs' solution (control) or Krebs' containing glucose, glucose plus Vitamin C (10^{-4} M), or L-NAME alone for 30 min before dose-dependent relaxation to SNP was determined. Data presented as mean $\pm$ sem; $n = 8$ from different rats. (B) Effects of acute glucose (30 mM), glucose plus L-NAME (10^{-4} M), or L-NAME alone on isoproterenol-induced relaxation of aortic ring pre-constricted with PE (10^{-7} M). Rings were incubated in Krebs' solution (control), glucose, glucose + L-NAME or L-NAME alone for 30 min before dose-dependent relaxation to isoproterenol was determined. Data presented as mean $\pm$ sem; ANOVA, $n = 8$ from different rats.

Fig. 6.

Effects of acute glucose (A) or Xanthine (B) on NO production by aortic ring in control or following cumulative dose responses to ACh and following pretreatment with vitamin C or calphostin C alone or in combination with either glucose or xanthine. Data presented as mean $\pm$ sem; ANOVA, * $P < 0.05$ compared to control, $n = 5-10$ from different rats.

Fig. 7.

Effects of acute (30 min) and chronic (20 hr) activation of PKC on ACh-induced relaxation of aortic ring pre-constricted with PE (10^{-7} M). Rings were incubated with Krebs' solution (control) or Krebs' containing PMA (10^{-6} M) for 30 min for acute treatment or 20 hr for chronic treatment before dose-dependent relaxation to ACh was determined. Data are presented as mean $\pm$ sem; * $P < 0.05$ compared to control, ANOVA, $n = 3-5$ from different rats.