High glucose enhances IL-1β-induced cyclooxygenase-2 expression in rat vascular smooth muscle cells

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Abstract

The changes in vascular prostaglandin production are implicated in the derangement of vascular reactivity in diabetes. However, the mechanism of altered prostaglandin (PG) production in diabetes is largely unknown. In this study, we investigated the effect of high glucose on IL-1β-induced PG production and the possible underlying mechanism in cultured vascular smooth muscle cell (VSMC). High glucose evoked an augmentation of IL-1β-induced PG synthesis in a dose dependent manner and enhanced cyclooxygenase (COX) activity, which reached to maximum at 8-12 hours after stimulation. Western blot analysis supported the activity data. Protein kinase C (PKC) inhibitors, H-7 and chelerythrine, significantly inhibited the enhancement of IL-1β-induced COX-2 expression by high glucose. The activation of PKC by PMA resulted in marked increase of PG production in low glucose group, whilst this was not the case in high glucose group. Furthermore, glucose-enhancing effect was significantly suppressed by zopolrestat, an aldose reductase inhibitor, and sodium pyruvate. These results suggest that the augmenting effect of high glucose on IL-1β-induced PG production and COX-2 expression is, at least in part, due to increased glucose metabolism via sorbitol pathway following PKC activation. © 2000 Elsevier Science Inc. All rights reserved.

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Prostanoids are arachidonic acid-derived products of membrane phospholipids that act as short-lived local mediators that induce a variety of biological responses. Prostaglandin (PG) synthesis involves conversion of arachidonic acid by the cyclooxygenase (COX) to the common prostanoid precursor, PGH$_2$ [1]. So far, two types of COX isoforms are identified. COX-1 is constitutively expressed in many cell types and is thought to be responsible for the production of prostaglandins under physiological conditions, whereas COX-2 has low basal expression that is rapidly induced by pro-inflammatory stimuli [1]. Increasing evidence suggests that the induction and regulation of COX-2 may be a key element in the pathophysiological processes of inflammatory disorders, cancer, heart failure and atherosclerosis [1–4].

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The changes in vascular PG production have been implicated in the derangement of vascular reactivity in diabetes [5]. Recent studies showed that the instillation of high concentration of glucose not only changed the prostanooid profile which resulted in the alteration of vasomotor tone, but also enhanced eicosanoid production in vascular cells [6–8]. Although it has been reported that increase in arachidonic acid release is responsible for the enhanced production of PG under hyperglycemic condition [9,10], the mechanism of altered PG production in diabetes is largely unknown. In this context, it is worth to explore the possible role of COX for delineation of underlying mechanism of glucose enhancing effect.

In this study, we examined the modulation of the COX by high glucose in primary cultured vascular smooth muscle cells (VSMCs) by evaluating production of PG and COX-2 expression. In addition, we sought to identify the relevant signaling pathway that may be involved in the regulation of COX-2 by high glucose, particularly the contribution of protein kinase C (PKC) activation.

Materials and methods

Reagents

All reagents were from Sigma Chemical (St. Louis, MO, USA) unless otherwise noted. Murine IL-1β and PCR core kit were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Zopolrestat was kindly gifted from Dr. Pagani (Pfizer Inc., Central Research Division, Groton, CT, USA). COX-2 specific polyclonal antibody was prepared and characterized as previously described [11].

Cell culture

Rat aortic smooth muscle cells (SMC) were isolated by enzymatic digestion as described previously [12] and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml)-streptomycin (100 μg/ml). Cells between passages 2 and 5 were growth arrested at 70% to 80% confluence by incubation in 0.1% FBS/DMEM for 48 hours before relevant treatment.

Determination of COX activity

Spent media were removed and cells were rinsed with phosphate buffered saline (PBS, 0.01M, pH7.4) prior to addition of fresh media containing arachidonic acid (10 μM). After incubation for 10 minutes at 37°C, media were removed and subjected to enzyme immunoassay for the measurement of 6-keto-PGF1α, a stable degradation product of PGI₂. Levels of 6-keto-PGF1α were determined using enzyme immunoassay kit from Cayman chemical (Ann Arbor, MI, USA). Release of 6-keto-PGF1α from exogenous arachidonic acid was taken as an index of COX activity [11].

Western blotting for COX-2

Cells were lysed in 200μl of Tris buffer (50 mM, pH 7.4) containing 1 mM DDTC-Na, 10 mM EDTA, 1% Tween-20, 10 μM leupeptin, and 1 mM phenylmethylsulfonylfluoride.
Equal amounts of protein were subjected to 10% SDS-PAGE and separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes. The blot was blocked with 5% non-fat dried milk, incubated with the anti-COX-2 antibody (1:600) overnight and treated with goat anti-rabbit IgG conjugated with alkaline phosphatase. Color development was made with alkaline phosphatase color reagent (Bio-Rad, Hercules, CA, USA) containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from cells using an RNAzol B (Tel-test, USA), and reverse transcribed to cDNA by AMV reverse transcriptase (Boeringer Mannheim, Germany). cDNA samples were subjected to 28 cycles of PCR reaction for COX-2 and 30 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences for oligonucleotide primers used were as follows: for COX-2, sense 5'-ACTCACTCAGTTTGGAGTGTCATT-3', antisense 5'-TTTGATAGTACTGTAGGGTTAG-3'; for GAPDH, sense 5'-GTGAAGGGTCGTTGGAACGGATT-3', antisense 5'-CACAGTCTTCTGAGTGCAATGAT-3'. The reaction cycles were as follows: denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min for COX-2, and denaturing at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 1 min for GAPDH.

Statistical analysis

Data are expressed as mean±S.E. The significance of differences was evaluated by the Student’s t-test.

Results

Enhanced production of prostaglandin by high glucose

To determine whether the high glucose affects PG production in VSMC, cells were made quiescent for 48 hours and then incubated with different glucose concentrations in the absence or presence of IL-1β (100 U/ml) for 48 hours. As depicted in Fig. 1, glucose increased the release of PGI$_2$ from VSMCs stimulated with IL-1β in a concentration dependent manner. Maximal effect was observed at 25 mM of glucose concentration, showing 3.7 fold increase in PG production compared with that of control. This was not an osmotic effect as the addition of 20 mM mannitol to 5 mM glucose to attain the same osmolarity as high glucose (25 mM) did not reproduce the high glucose effect. The time course study showed that difference in PG production between high- and low-glucose treated group could be observed after 4 hours incubation (Fig. 2A). Changes of COX activity in cells treated with IL-1β showed different time course depending on the glucose concentration, that is, COX activity reached to maximum in 8 hours under high glucose condition, whereas the maximal activity was seen after 12 hours in low glucose group (Fig. 2B).

Effects of PKC modulators on PGI$_2$ production in IL-1β-stimulated VSMCs

A high concentration of glucose activates PKC in VSMCs by increasing de novo synthesis of diacylglycerol (DAG) [13]. Therefore, we next examined whether PKC activation is in-
volved in high glucose effect on IL-1β-stimulated PG production. Concurrent incubation of cells with IL-1β and H-7, a PKC inhibitor, (25 μM), resulted in decrease of PGJ2 production to 26.0% in low glucose group and 48.7% in high glucose group. Chelerythrine (5 μM), a selective PKC inhibitor, also showed a similar diminution in PG production (Fig. 3). The addition of phorbol myristic acetate (PMA, 100 ng/ml), which can substitute for DAG to activate PKC, significantly increased PG production in low glucose group, whereas this maneuver did not evoke any significant effect in high glucose group. Thus, these results suggest that PKC activation may be a potential mechanism for the enhanced PG production under high glucose conditions.

Effects of zopolrestat and sodium pyruvate

It is well known that the activation of sorbitol pathway in hyperglycemia can increase the NADH/NAD+ ratio in the cytosol, which favors de novo synthesis of DAG, and in turn, activates PKC [14,15]. Therefore, in order to determine the possible involvement of sorbitol pathway in glucose enhancing effect, cells were treated with zopolrestat, an aldose reductase inhibitor, or sodium pyruvate, which is expected to normalize the altered NADH/NAD+ ratio in the cells exposed to high glucose [5]. As shown in Fig. 4, both zopolrestat (100 μM) and sodium pyruvate abolished the augmentation of IL-1β-induced PG production by high glucose. These data provide the evidence that glucose enhancing effect is mediated by the activation of sorbitol pathway.

Effects of glucose on IL-1β stimulated COX-2 expression

Western blotting was performed to address whether the glucose effect was operating at the level of the expression of COX-2 protein (Fig. 5A). VSMCs with high glucose (25 mM) and IL-1β (100 U/ml) showed the marked increase in the expression of COX-2, which was in
parallel with PG production. Without IL-1β, COX-2 protein was not detected in both low (5 mM) and high glucose group (25 mM). The addition of zopolrestat (100 μM) resulted in the diminution of the enhancing effect of high glucose on IL-1β-induced COX-2 expression. Next, the effect of high glucose on transcript level of COX-2 was examined by RT-PCR. Incubation of VSMCs with high glucose (25 mM) and IL-1β (100 U/ml) for 6 hours led to significant increase in the accumulation of COX-2 mRNA by 1.45 fold compared with low glucose group (Fig. 5B). Zopolrestat (100 μM) diminished the augmentation effect of high glucose on IL-1β-induced COX-2 mRNA expression.

From these data, it is suggested that the enhancement of IL-1β-induced PGI₂ production by high glucose in VSMC is due to, if not all, the increased expression of COX-2 and the activation of sorbitol pathway is involved in these effects.
Discussion

It is well documented that diabetes mellitus is associated with the development of various vascular complications and hyperglycemia is regarded as the primary causative factor for accelerated cardiovascular disease in diabetes [16]. It is likely that glucose and its metabolites mediate their adverse effects by altering the various bioactive mediators, which are used by vascular cells to perform their functions and to maintain cellular integrity. Indeed, multiple cellular consequences of glucose-induced activation of VSMCs have been described including increases in transforming growth factor-β [17], osteopontin [18] and extracellular matrix proteins [19], activation of cytosolic phospholipase A2 and inhibition of Na\(^+\)-K\(^+\) ATPase activities [20]. In this study, we also observed the enhancement of IL-1β-induced prostaglandin production and COX-2 expression in VSMCs under hyperglycemic condition, adding another example for the glucose mediated alterations in bioresponses.

Amongst multiple biochemical mechanisms that have been proposed to explain so called glucose toxicity, the activation of sorbitol pathway has been the theory studied most intensively. Glucose metabolism via sorbitol pathway is known to increase the NADH/NAD\(^+\) ratio in the cytosol, which favors the de novo synthesis of DAG, and in turn, activates PKC [5, 13–15]. Several lines of evidences have implicated increased PKC activity as a key player in the pathophysiological effects of high glucose [21]. Functionally, it was shown that aldose reductase inhibitors, which inhibit the glucose metabolism via sorbitol pathway, prevented the
high glucose-induced PKC activation in VSMC [22]. And also, it was reported that the elevation of tissue pyruvate levels, which was known to normalize the altered NADH/NAD$^+$ ratio in the cells, prevented glucose-induced vascular dysfunction [5]. Based on these reports, we postulated that the activation of PKC via sorbitol pathway could explain the glucose-enhancing effect on IL-1β-induced PG production and COX-2 expression in VSMCs. In order to validate this hypothesis, firstly, pharmacological modulation of PKC activity was adopted. The addition of PKC inhibitors, H-7 and chelerythrine diminished IL-1β-induced PG synthesis. The activation of PKC by PMA resulted in significant increase of PG production in low glucose group, but this was not the case in high glucose group (Fig. 3). And also, glucose-enhancing effect was significantly suppressed when the aforementioned glucose metabolic scheme leading to the activation of PKC was modulated by zopolrestat, an aldose reductase inhibitor, and sodium pyruvate (Fig. 4). These results support our hypothesis that the activation of sorbitol pathway by high glucose and following elevation of NADH/NAD$^+$ ratio in cytosol is involved in the enhanced COX-2 expression and thereby increase of PG production in IL-1β-stimulated VSMCs.

Recent studies have shown that the activation cytosolic phospholipase A$_2$ (cPLA$_2$) is responsible for enhanced prostanoid production by high glucose and this could be completely inhibited by the addition of LY333531, a selective PKC β2 inhibitor [20, 23]. Thus, together with these reports, our data suggested that increase in PG production under hyperglycemic condition is not only due to activation of cPLA$_2$ but also enhanced expression of COX-2 in VSMCs, especially in case of pro-inflammatory stimulation.
At present, signaling pathway downstream to PKC leading to enhancement in COX-2 expression is not clarified, but several recent studies have provided the clues to this question. Mitogen activated protein kinase (MAPK) activity increased time-dependently under high glucose conditions, the rate of increase being consistent with those in cPLA2 activity and PG production [10, 24]. Furthermore, the activation of MAPK cascade was repressed by PKC inhibitor [10, 25]. And also, the activation of p38 MAPK signaling pathway has been known to mediate IL-1β signal amplification and modulation that results in the expression of COX-2 [26]. These reports suggest that the possible role of MAPK pathway in high glucose-enhanced COX-2 expression. Besides this, the activation of nuclear factor-κB (NF-κB) is noteworthy as an another possible explanation for glucose-enhancing effect. In VSMC, it was reported that the high glucose could activate the NF-κB in PKC dependent manner [27]. It is
well known that the NF-κB has the major role in the regulation of the IL-1β-induced COX-2 expression [28]. However, despite of the activation of MAPK and NF-κB by high glucose and their suggestive roles in COX-2 expression, these do not necessarily mean that the activation of NF-κB and/or MAPK pathways can sufficiently explain the augmentation effect of high glucose on the IL-1β-stimulated COX-2 expression. Indeed, recent report showed that the activation of NF-κB does not always mediate the induction of COX-2 in VSMCs [29]. Thus, further studies are required to determine the involvement of these signaling pathways.

At this time, it is difficult to specify what the consequences of increased COX-2 expression and PG production by IL-1β in VSMC under hyperglycemic condition would be. However, there are several reports showing modification of functional vasoactive responses in blood vessels by cytokines and LPS [30–32], and the localization of the COX-2 enzyme in pathological specimens from patients with heart failure [3], atherosclerosis [4] and septic shock [30], all of which have elevated levels of pro-inflammatory cytokines. And also, it is well known that hyperglycemia is an important risk factor for accelerated development of cardiovascular diseases [33, 34]. Thus, it is quite conceivable that the induction of COX-2 and thereby enhanced production of PGs may be an important mechanism in aforementioned pathological processes and may give us one of the clues to the answer for the increased prevalence of cardiovascular disorders among diabetic patients. It is hoped that documentation of the conditions required to induce COX-2 will allow future studies to examine the regulation of this important pathway in the vasculature and these findings will help in further understanding the role of COX-2 in different disease processes, and possibly lead to new ways of treatment.

In conclusion, our data suggest that glucose enhances the PG production via mechanisms dependent of PKC activation via sorbitol pathway in IL-1β-stimulated VSMC, and also, the increase in PG production under hyperglycemic condition is, if not all, due to the enhanced expression of COX-2.

References


