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Impairing Effects of Acute Glucose Overload on Calcium Homeostasis in Vascular Endothelial Cells

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INTRODUCTION

The vascular endothelium lines the interior of vessels as large as the aorta to those as small as the capillary. These cells support many functions in response to various biochemical and biomechanical stimulations. As examples, endothelial cells regulate vascular tonus by the secretion of nitric oxide, endothelin or prostaglandin (for a review see (Vanhoutte et al, 1986)). By secretion of growth factors, the endothelium regulates vascular proliferation (Cowan & Langille, 1996; Kita et al, 1997). Angiogenesis, which consists of endothelial migration and proliferation, is an essential step for tumor growth and metastasis (Folkman, 1995). It is also known that blood coagulation is generated as a balance of platelet aggregation and anti-aggregating effect of endothelium (Luschor et al, 1993). Therefore, endothelial cell dysfunction has pathological significance in the development of hypertension (Cardillo et al, 1998), atherosclerosis (Cowan & Langille, 1996), malignant tumors (Folkman, 1995), thrombotic diseases (Bell et al, 1998) and diabetic microangiopathy (Mayhan & Patel, 1995; Tesfamariam & Cohen, 1992). In achieving these functions, the elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is necessary. For example, it has been reported that nitric oxide synthetase, which produces nitric oxide, is activated by elevated $[Ca^{2+}]_i$ (Lopez et al, 1990). Therefore the investigation of endothelial $[Ca^{2+}]_i$ has a significant importance in vascular biology.

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OVERVIEW OF ENDOTHELIAL Ca^{2+} HOMEOSTASIS

Vascular endothelium belongs to a non-excitable cell type. The most remarkable characteristics of a non-excitable cell is the absence of voltage dependent Ca^{2+} channel, meaning that the membrane depolarization does not alter cellular functions directly. Endothelial Ca^{2+} homeostasis is mainly regulated by the intracellular Ca^{2+} store sites instead. Ca^{2+} is released from intracellular store sites by IP_3 generated by various agonists (Oike & Ito, 1997). Thus depleted store sites activates store-operated Ca^{2+} entry pathway; i.e., Ca^{2+} release-activated Ca^{2+} entry (CRAC) (Berridge, 1995). This pathway cannot be recorded as a current by whole cell patch clamp method in vascular endothelium, because it is supposed to be a very small channel (Oike et al, 1994). These two pathways are main Ca^{2+} sources, but there are other Ca^{2+} pathways in endothelium such as Ca^{2+} release by arachidonic acid (Oike et al, 1994), Na^+/Ca^{2+} exchanger (Li & van Breemen, 1995) and Ca^{2+} entry through non-selective cation channel (Nilius et al, 1993).

These are known Ca^{2+} pathways so far in vascular endothelium (Fig. 1).

ENDOTHELIUM AS A TARGET OF HYPERGLYCEMIC CELL DAMAGE

Diabetes mellitus is a metabolic disease, but the most of its complications target vessels. So from the point of its pathological aspect, diabetes mellitus should be considered as a vascular disease as well.

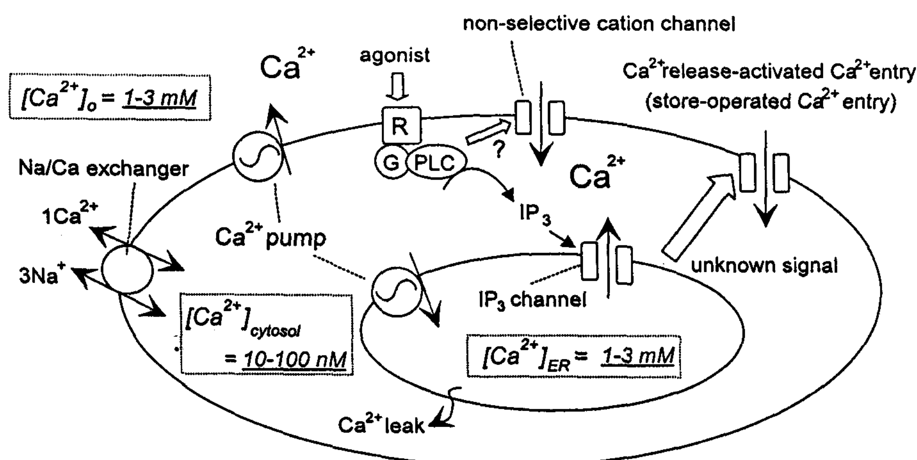

Fig. 1. Ca^{2+} mobilization pathways in vascular endothelium.

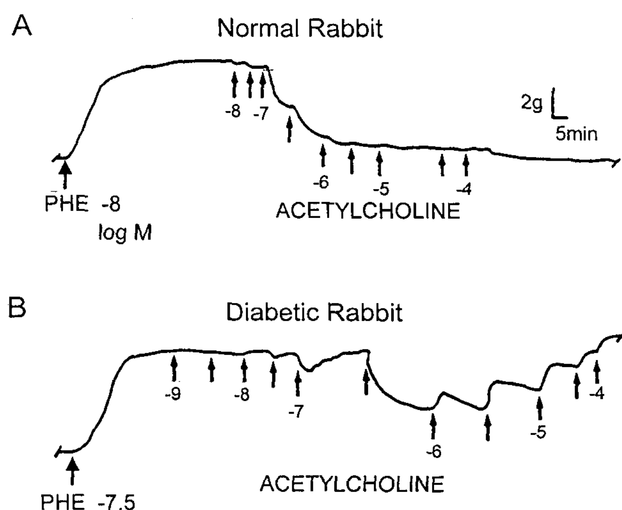

Fig. 2. Impairment of endothelium-dependent vasodilation in aorta from diabetic rabbit (B). The aortic ring from a normal rabbit showed endothelium-dependent vasodilation (A). Figure was taken from the reference (Tesfamariam & Cohen, 1992).

Fig. 2 shows an example of the impairing effect of diabetes on endothelium-dependent vasorelaxation. The aortic ring from a normal rabbit shows the endothelium-dependent relaxation (Fig. 2A). On the other hand, the aortic ring from diabetic rabbit failed to show it (Fig. 2B) (Tesfamariam & Cohen, 1992). This indicates that diabetic environment may inhibit endothelial function, and this may be related to the vascular complications of diabetes mellitus such as atherosclerosis, diabetic retinopathy and coronary disease.

Many investigators have studied cellular mechanisms of diabetic cell damages using various cell types. Firstly, excess amount of glucose is consumed by its collateral metabolic pathway, so called polyol pathway. The activation of this metabolic pathway reduces the cellular amount of coenzymes such as NADPH and NAD, and accumulates sorbitol and fructose in a cell. This results in the continuous elevation of the cellular osmolarity which may damage the cell. Protein kinase C is activated in hyperglycemic condition as a result of the activation of polyol pathway and also by a direct de novo synthesis of diacylglycerol. Many reports attribute diabetic cell damage to the activation of protein kinase C (Kimura et al, 1998; Tesfamariam et al, 1991). Accumulation of oxygen free radicals are another important candidate of the pathogenesis of hyperglycemic cell damage (Tesfamaariam, 1994). Auto-oxidation, glycation or altered co-enzyme ratios result in the overproduction or reduced clearance of free radicals including superoxide anion (O_2^-). Therefore, the impairment of endothelium-dependent relaxation in diabetic condition is probably brought by any of these changes.

EFFECT OF ACUTE GLUCOSE OVERLOAD ON RESTING $[Ca^{2+}]_i$ IN VASCULAR ENDOTHELIUM

We examined the effects of glucose overload on endothelial Ca^{2+} homeostasis. In this series of experiments, $[Ca^{2+}]_i$ was measured either from bovine

aortic or brain microvascular endothelial cells by using Ca^{2+} fluorescent dye, fura-2. The main reason for using two kinds of endothelium is that they showed different Ca^{2+} responses to agonists; i.e., the former showed typical IP_3-induced Ca^{2+} oscillation to ATP and histamine, and the latter showed gradual and sustained $[Ca^{2+}]_i$ elevation to histamine. Another purpose for suing two kinds of endothelium is the comparison of Ca^{2+} metabolism between macrovascular and microvascular endothelium.

Because it was technically difficult to culture cells under a constant level of glucose for days, we examined the effect of acute glucose overload up to four hours. At first we examined the effect of changing extracellular glucose concentration on the resting level of $[Ca^{2+}]_i$. We measured $[Ca^{2+}]_i$ with high (23 mM; two times higher than normal) or low (1.1 mM and 2.3 mM; one tenth and fifth normal) concentrations of extracellular glucose in Krebs solution. Cells were incubated with these solutions for four hours before experiment and the resting $[Ca^{2+}]_i$ was measured in each solution. The solutions with these different concentration of glucose were made by replacing with NaCl, so that the osmolarity of the solutions can be regarded as constant. We could not find any difference in resting $[Ca^{2+}]_i$ either by decreasing or increasing extracellular glucose concentration in bovine brain microvascular endothelial cells. There was also no significant difference in resting $[Ca^{2+}]_i$ between normal and elevated glucose condition in bovine aortic endothelial cells. Therefore it can be concluded that altering glucose concentration dose not change resting level of $[Ca^{2+}]_i$ during a period of four hours (Kimura et al, 1998).

EFFECTS OF ACUTE GLUCOSE OVERLOAD ON ATP-INDUCED, IP_3-MEDIATED Ca^{2+} OSCILLATION IN BOVINE AORTIC ENDOTHELIAL CELLS

Endothelial cells play various physiological roles in response to biochemical stimulations, so we then examined the effect of glucose overload on the agonists-stimulated Ca^{2+} mobilization. Firstly we examined the effect of acute glucose overload on IP_3-mediated Ca^{2+} oscillation in bovine aortic endothelial cells. When IP_3-genereating agonists such as ATP are applied to the endothelium, oscillatory increase of $[Ca^{2+}]_i$ is observed in a control condition (Fig. 3A). The frequency of Ca^{2+} oscillation depends on the concentration of extracellular ATP (Kimura et al, 1998), therefore, the ability of the cell to generate Ca^{2+} osicillation would have physiological significance in endothelial functions. Actually, in T-lymphocyte, it has been reported that the expression level of genes depends on the frequency of Ca^{2+} oscillation (Dolmetsch et al, 1998).

Three hours after incubation with high D-glucose solution (23 mM), the threshold concentration of ATP (0.01 μM) to induce Ca^{2+} transient was not different from the control value. However, application of ATP (0.1 μM) did not induce Ca^{2+} oscillation but induced only a phasic followed by a sustained increase in $[Ca^{2+}]_i$ (Fig. 3B). The elevation of glucose concentration with L-glucose did not abolish ATP-induced Ca^{2+} oscillation, indicating that metabolism of D-glucose is responsible for its impairing action.

As described previously, endothelial $[Ca^{2+}]_i$ is maintained by various Ca^{2+} pathways, and it has been suggested that intact functions of these pathways are necessary to generate Ca^{2+} oscillation (Sneyd et al, 1995). We then tried to clarify which Ca^{2+} pathways are impaired by glucose overload. So the disappearance of Ca^{2+} oscillation indicates one or more Ca^{2+} pathways are affected. When supramaximal concentration of ATP (10 μM) was applied, the whole stored Ca^{2+} was released at the first Ca^{2+} releasing event and no Ca^{2+} oscillation was observed in a normal glucose level. When cells were pretreated with high D-glucose solution, a single steep elevation of $[Ca^{2+}]_i$ was observed as in control cells. However, the falling phase of the Ca^{2+} transient was markedly prolonged (Fig. 3C). Therefore, the Ca^{2+} extrusion mechanism, Ca^{2+} pump, is probably impaired by glucose overload.

We then used thapsigargin, and inhibitor of endoplasmic Ca^{2+}-ATPase, to examine the effect of glucose overload on Ca^{2+} leak from intracellular store sites and the following Ca^{2+} entry (CRAC), Thapsigargin (1 μM) induced a transient followed by a sustained increase of $[Ca^{2+}]_i$ in control cells. The former is by Ca^{2+} leak from intracellular Ca^{2+} store sites, and the latter is by the store depletion-induced activation of CRAC. In the cells pretreated with high D-glucose solution for three hours, thapsigargin evoked an initial transient elevation of $[Ca^{2+}]_i$, but it was not followed by a sustained elevation of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}, suggesting that CRAC was inhibited by glucose overload. In

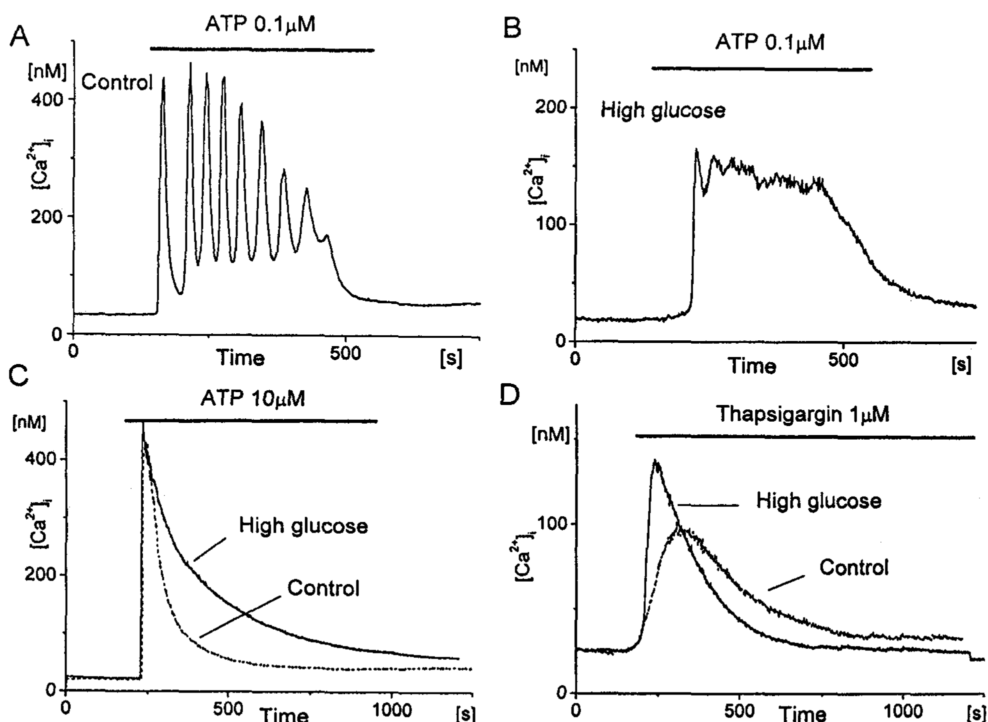

Fig. 3. A: ATP (0.1 μM) induced a typical Ca^{2+} oscillation accompanied by a gradual elevation of basal level of $[Ca^{2+}]_i$ in bovine aortic endothelial cells. B: Oscillatory increase in $[Ca^{2+}]_i$ was not observed in response to 0.1 μM ATP in a high D-glucose-treated cell. C: Effect of glucose overload on high concentration of ATP (10 μM)-induced Ca^{2+} transient. Broken line indicates a trace from a control cell. D: Thapsigargin (1 μM) was applied to control (broken line) and high glucose-treated cell (continuous line). Note that glucose overload abolishes Ca^{2+} release-activated Ca^{2+} entry and accelerates the initial Ca^{2+} leak velocity. Figures were taken from the reference (Kimura et al, 1998).

addition, the maximum rate of rise of $[Ca^{2+}]_i$ increase induced by thapsigargin was significantly increased after the glucose overload, suggesting that the maximal leak velocity was accelerated by high glucose (Fig. 3D). These changes were also not induced by glucose overload with L-glucose.

It has been reported that O_2^- was responsible for the impairment of endothelial function in diabetic aorta (Tesfamariam & Cohen, 1992). So we then examined the effect of superoxide dismutase (SOD) on glucose overload- induced impairment of Ca^{2+} oscillation in aortic endothelium. When endothelium was co-incubated with high glucose solution and SOD (150 IU/ml), Ca^{2+} oscillation as weell as the falling phase of elevated Ca^{2+}, CRAC and Ca^{2+} leak were observed as in control cells. On the other hand, scavengers of other reactive oxygen such as catalase and deferoxamine failed to restore the impairing actions of glucose overload. These observation was confirmed by the fact that xanthine and xanthine oxidase, which generates O_2^-, mimicked the all the changes of glucose overload-induced Ca^{2+} mobilization (Kimura et al, 1998).

These results indicate that, in aortic endothelium, glucose overload accumulates O_2^-, which then inhibits Ca^{2+} oscillation by affecting Ca^{2+} extrusion, CRAC and Ca^{2+} leak. These results also coincide with previous reports showing that O_2^- is responsible for the impairment of endothelium-derived vasodilation in aorta (Tesfamariam & Cohen, 1992).

EFFECTS OF ACUTE GLUCOSE OVERLOAD ON HISTAMINE-INDUCED, cAMP-MEDIATED Ca^{2+} MOBILIZATION IN BOVINE CEREBRAL ENDOTHELIAL CELLS

Endothelium possesses Ca^{2+} releasing mechanism other than IP_3-mediated one such as arachidonic acid-mediated one (Oike et al, 1994). We then examined the effects of glucose overload on histamine-induced, cAMP-mediated Ca^{2+} release from intracellular Ca^{2+} store sites in bovine brain microvascular endothelial cells. Brain microvascular endothelium is an important component of blood-brain barrier, and its impairment would result in various pathological conditions such as brain edema (Wahl et al, 1993). By the application of 10 μM histamine, Ca^{2+} was gradually increased both in Ca^{2+} free and Ca^{2+} containing solutions (Fig. 4A), thereby indicating that Ca^{2+} was released from intracellular store sites.

This histamine-induced Ca^{2+} transient was inhibited by H_2 receptor antagonists but not by H_1 receptor antagonists. It is known that H_2 receptor is coupled with adenylate cyclase and that its activation generates cyclic AMP (Hill, 1990), and we confirmed that externally applied membrane permeable cyclic AMP analogue, dibutyryl cyclic AMP, also induced gradually occurring $[Ca^{2+}]_i$ elevation in brain microvascular endothelium. Therefore, histamine induces gradual Ca^{2+} release from intercellular Ca^{2+} store sites in bovine brain microvascular endothelial cells by the production of cyclic AMP via H_2 activation.

We then examined the effect of acute glucose overload on histamine-induced $[Ca^{2+}]_i$ increase in bovine brain microvascular endothelium, and found that it was almost completely abolished (Fig. 4B). It has been reported in cerebral microvessel that protein kinase C is responsible to glucose overload-induced impairment of endothelial function (Mayhan & Patel, 1995). Furthermore, it is known that the production of cyclic AMP is inhibited by protein kinase C (Teitelbaum, 1993). So then we examined the efects of protein kinase C inhibitors, staurosporine and calphostin C, on the effect of glucose overload, and confirmed that these inhibitors resstored the impairing effect of glucose overload. Furthermore, the activator of protein kinase C, PDBu, mimicked the effect of glucose eoverload on histamine-induced $[Ca^{2+}]_i$ elevation in the presence of normal concentration of glucose. On the other hand, when dibutyryl cyclic AMP was applied to high glucose solution-pretreated cell, it showed $[Ca^{2+}]_i$ increase as in control cells. These results indicate that glucose overload inhibits histamine-induced Ca^{2+} release by the activation of protein kinase C, which suppresses the production of cyclic AMP (Kimura et al, 1998).

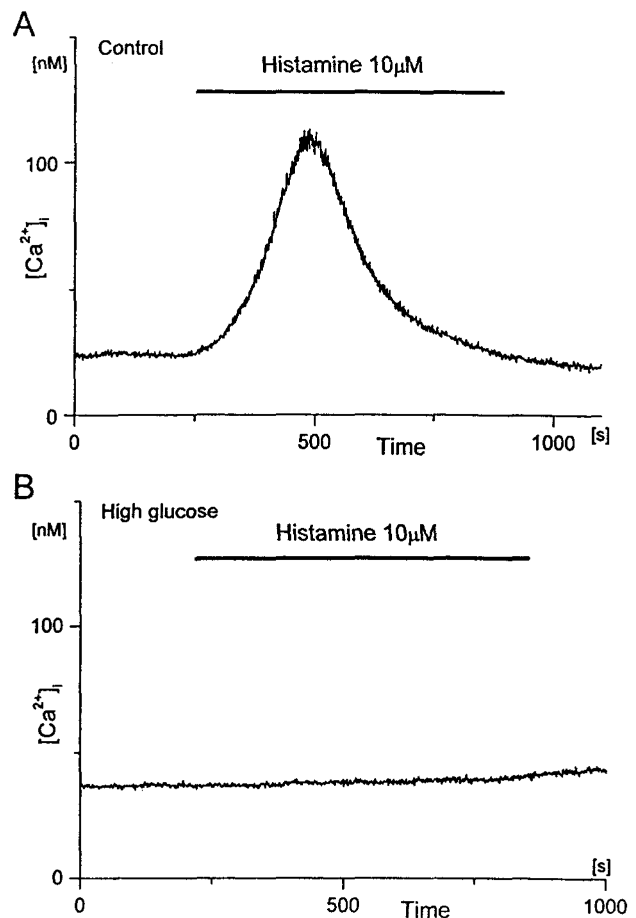

Fig. 4. A: Histamine (10 μM) induced a gradual $[Ca^{2+}]_i$ elevation in Ca^{2+} free solution in bovine brain microvascular endothelial cells. B: When histamine was applied to the high glucose-treated cell, it failed to show $[Ca^{2+}]_i$ transient. Figures were taken fthe reference (Kimura et al, 1998).

SUMMARY AND PERSPECTIVE

We demonstrated two kinds of impairing effect of glucose overload on endothelial Ca^{2+} mobilization; i.e., O_2^--mediated and protein kinase C-mediated ones. As already mentioned in the previous sections, endothelium-dependent vasodilation was impaired in

aorta by the hyperglycemia-induced production of O_2^- (Tesfamariam & Cohen, 1992). In contrast, vasodilation in response to agonists such as acetylcholine and histamine was impaired by hyperglycemic condition in cerebral microvessels by the production of protein kinase C (Mayhan & Patel, 1995). Our observations happened to support these reports; i.e., O_2^- was responsible for glucose overload-induced impairment of Ca^{2+} mobilization in aortic endothelium and protein kinase C in brain microvascular endotheluim. However, because each mechanism affects Ca^{2+} mobilization in a quite different manner, we suppose that this does not simply imply the site-specificity of the impairing action of glycose overload, but is due to the difference of Ca^{2+} mobilization mechanism. In other words, O_2^- mainly affects Ca^{2+} pathways such as channels and pumps, and protein kinase C affects the signaling cascade which is related to Ca^{2+} mobilization.

As summarized above, many Ca^{2+} mobilizing pathways, which are regulated by various biochemical and biomechanical stimulation, are involved in the regulation of endothelial $[Ca^{2+}]_i$. However, the details of such Ca^{2+} mobilizing mechanism are not fully clarified. For instance, it is not known whether the cyclic AMP-mediated Ca^{2+} release observed in brain microvascular endothelium plays a significant role also in other vessels such as aortic endothelium. Therefore, the detailed clarification of the mechanisms of Ca^{2+} mobilization in vascular endothelium has an essential importance in vascular biology not only for physiological reason but also for pathophysiological reason.

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