厚生労働科学研究費補助金(循環器等総合研究事業)
分担研究報告書

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研究要旨
高齢糖尿病患者の認知機能と動脈硬化関連の臨床検査成績などとの関連を検討したところ、一部の認知機能検査の成績低下と脈圧の大きさに関連がある可能性が示唆され、高齢糖尿病患者の認知機能低下の機序の解明に有用な所見と考えられた。

A. 研究目的

近年、高齢者糖尿病患者においては、認知機能低下が存在することがよく知られるようになり、Diabetic Encephalopathy という概念が提唱されるようになってきた (1,2)。しかしながら、未だその発症機序、危険因子については明らかになっていない。糖尿病患者には脂質代謝異常、高血圧が合併することが多いが、非糖尿病の高齢者の認知機能低下や痴呆発症には、こうした動脈硬化の危険因子が関与している可能性が指摘されている (3)。しかしながら、糖尿病患者の認知機能低下と動脈硬化性因子との関わりについては、これまで十分な検討がなされていない。従って、今回我々は、高齢者糖尿病患者の認知機能と脂質、血圧の関連について横断的に検討を行った。

B. 研究方法

高齢糖尿病患者 48 名(男性 23 名、女性 25 名、登録時平均年齢 72.5 才)に認知機能検査を施行した。認知機能検査の項目は、Mini-Mental State Examination (MMSE)、ADAS 単語再生(直後、遅延)、物語り再生(直後、遅延)、Stroop test、Word fluency test(動物の名称、「か」ではじまる単語を1分間にあげられる数を測定する)、WAIS-R 符号である。臨床検査としては、空腹時血糖、IRI、HbA1c、血清コレステロール値(LDL,HDL,TG)、血圧、BMI などの測定を行った。

解析法としては、Pearson の単相関により有意となった因子を投入し、ステップワイズ(増加法)で独立変数を選択し、ロジスティック回帰分析を行った。

(倫理面への配慮)

参加者には研究内容につき充分な説明を行った後、文書による同意をいただいた。データについては、匿名で処理さ

れ個人が特定されないように配慮した。

C. 研究結果

今回は、Knopman らの報告で、糖尿病患者において低下しているとされた Word fluency test の得点と臨床検査項目との関連について検討した (4)。

年齢、教育歴、HDl、TG、BMI、FBS、血中インスリン値、脈圧、Word fluency test (動物の名称) の得点の単相関をとり、p 値が10%未満となった因子をロジスティック回帰分析に用いた。

Word fluency test (動物の名称) の得点を目的変数とし、説明変数として上記で選択された教育歴、HDL　TG、FBS、HbA1c、脈圧を投入した。

ステップワイズにより、教育歴と脈圧が選択され、R 二乗は 0.396 であった。

D. 考察

今回の検討は対象数も少なく、予備的なものであるが、認知機能の項目の1つである Word fluency test (動物の名称) の得点を目的変数とすると、有意な説明変数として教育歴と脈圧がステップワイズで選択された。教育歴が認知機能検査結果に有意に影響することは当然であるが、今回の検討で脈圧が有意な因子として抽出されたことは興味深い。

脈圧は動脈硬化の進行によって大きくなることがしられており、脈圧の増加と認知機能低下の関連は動脈硬化を介して関連している可能性がある。また、拡張期血圧が低いと痴呆発症のリスクがあがるとの報告もあり (5)、脳血流との関連も示唆される可能性があるものと考えられる。

今後さらに症例数を増やし検討を続ける必要がある。

E. 結論

高齢糖尿病患者の一部の認知機能検査の成績低下に脈圧の大きさが関与している可能性が示唆された。

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G. 知的財産の出願、登録状況

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表レイアウト

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Ⅳ. 研究成果の刊行物・別冊

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The effect of high glucose on NO and O_2^- through endothelial GTPCH1 and NADPH oxidase

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Abstract

Although endothelial dysfunction deteriorates diabetic angiopathy, the mechanisms are obscure. We revealed that high glucose augmented eNOS through stimulation of eNOS mRNA in cultured BAECs. NO was decreased and O_2^- was increased simultaneously. NOS inhibitor, inhibited O_2^- release, so did NADPH oxidase inhibitor. The effects were synergistic. Both intracellular BH_4 level and GTPCH1 activity were decreased by high glucose, in line with decrease of GTPCH1 mRNA. HMG-CoA reductase inhibitor, atorvastatin increased GTPCH1 mRNA and activity, and BH_4 level. Conclusively, high glucose leads to eNOS dysfunction by inhibiting BH_4 synthesis and atorvastatin stimulate BH_4 synthesis directly, and it may work as atherogenic process.

Keywords: eNOS dysfunction; Superoxide anion; L–NAME; Apocynin; Aminoguanidine; BH_4; GTPCH1 activity; HMG-CoA reductase inhibiters

Introduction

The acceleration of atherosclerosis in diabetes mellitus results in higher risks of cardiovascular events. Growing clues showed that an impairment of diabetic endothelial function exhibited crucial

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roles. Endothelial NOS plays an important role in vascular endothelium functions by producing NO, an important anti-atherosclerotic agent. Recent studies also showed that eNOS has a dual effect on atherosclerosis (Robert et al., 1998). eNOS itself could be an important source of endothelial superoxide production in hypercholesterolemia (Kirkwood et al., 1995). In diabetic vessels of human, the endothelium was found to be an additional net source of superoxide production because of eNOS dysfunction (Tomas et al., 2002). On the other hands, study of insulin-resistant rat aorta revealed a decreased aortic BH_4 contents as well as increased BH2 (7,8-dihydrobiopterin) levels, when compared with normal and non-insulin-resistant diabetic groups (Shinozaki et al., 1999). They reported that insulin resistance is the pathogenic factor of eNOS dysfunction and BH_4 deficiency. Other study showed that the balance between reduced and oxidized BH_4 is a key redox switch controlling superoxide formation from eNOS (Vasquez-Vivar et al., 2002). Exogenous administration of BH_4 leads to an acute amelioration of endothelium-dependant relaxation in DM rats (Pieper, 1997). Intravenous administration of sepiaptrin, which is an ancestor of BH_4, could improve the endothelial-dependent vasodilatation of diabetic patients clinically (Heitzer et al., 2000). There is little evidence that shows the relationship among high glucose, eNOS dysfunction and BH_4. Hyperglycemia is an independent risk factor for ischemic heart disease proved by clinical studies such as UKPDS. The current study is aimed to reveal the mechanisms of eNOS dysfunction leading by high glucose in an in vivo model.

Materials and methods

Cells

BAECs were isolated from fetal calf as described previously (Hayashi et al., 1995a) and cultured in DMEM with 10% (v/v) of CS, 100 u/ml of penicillin, 100 μg/ml of streptomycin, 2 mM glutamine. Cells were allowed to the confluent of 80%, and then stimulated with different concentration of D-glucose (5.5, 12.5, 25 and 50 mM) as well as other reagents in DMEM with 2% CS and phenol red free for 24 hours. Mannitol was used as control to rule out the effect of osmotic pressure.

Measurement of NOx (nitrite and nitrate)

Measurement of NOx (nitrite and nitrate) in supernatant was performed as described in previous study (Yamada and Nabeshima, 1997). Briefly, the supernatant were taken for the measurement of NOx by HPLC (ENO10, Eicom Co, Kyoto, Japan), where nitrate was converted to nitrite in an in-line copper coated cadmium reduction column (NO–RED), and then nitrite was detected based on Griess reaction.

Western blot analysis of eNOS protein

Determination of eNOS protein expression were performed as described in our previous study (Hayashi et al., 1995b). Protein concentration was determined by Dc protein assay kit (Bio-Rad, CA). 15 μg protein was loaded. Primary anti-eNOS monoclonal antibody (Anti-mouse IgG1

monoclonal antibody, Transduction Laboratories, CA) was incubated in the ratio of 1:2000, overnight. HRP-linked anti-mouse IgG antibody (Cell signaling) was used as second a antibody. Bands of eNOS protein were developed in dark on the film (Fuji Medical X-ray Film, Japan). Band densities were analyzed densitometrically by the National Institutes of Health IMAGE program.

RT–PCR analysis of eNOS and GTPCH1 mRNA

Total RNA was isolated from BAECs with TRIZOL reagent according to the manufacture's protocol (GIBCO BRL, Life Technologies). eNOS mRNA were analyzed by reactions with RNA PCR kit (One step RNA PCR Kit, Takara, Japan) as described in our previous study (Kano et al., 1999). The programmed cycles for eNOS RT PCR were as follows: 1 cycle of 50 °C × 30 minutes and 94 °C × 2 minutes; 30 cycles of 94 °C × 30 seconds, 60 °C × 30 seconds, and 72 °C × 30 seconds. Bands were visualized on dual intensity transluminator. RT–PCR of GTPCH1 mRNA were carried on such a programmed cycles: 1 cycle of 50 °C × 30 minutes and 94 °C × 2 minutes; 30 cycles of 94 °C × 30 seconds, 60 °C × 30 seconds, and 72 °C × 1 minutes. Sequence of bovine GTPCH1 primer is as follows: sense: 5′ CCGCCTACTCGTCCATCCTGA 3′, antisense: 3′ACCTCGCATTACCATACACAT 5′.

Measurement of intracellular superoxide by FACS

At the end of treatment period, cells were washed with PBS, 2 μl of 5 mM DCFH-DA was added and then incubated in 37 °C for 30 minutes. Cells were detached with trypsin, and centrifuged at 4 °C, 15000 rpm for 5 minutes. Cell suspensions in PBS were transferred into 5 ml polystyrene round-bottom tubes with cell-strainer caps (Becton Dickinson lab ware, Becton Dickinson and company, France). And they were kept on ice for immediate measurement by FACS (Fluorescence-activated cell sorter, BD Biosciences).

Determination of intracellular BH4 level and GTPCH1 activity

Cells were harvested with trypsin and pelleted by centrifugation and frozen at −80 °C. BH_4 measurements were performed by HPLC procedure described by Fukushima and Nixon (Consrtino et al., 1997). Intracellular BH_4 levels were expressed in terms of pmoles per mg protein of the cell pellet. GTPCH1 activity was assayed based on the quantification of D-erythro-neopterin by HPLC after conversion of enzymatically formed D-erythro-7,8-dihydroneopterin triphosphate into D-erythroneopterin by sequential reaction of iodine oxidation and dephosphorylation.

Statistics

Data were reported as mean ± SD, and represent three independent experiments. Comparisons between the two groups were made based on the nonparametric Mann-Whitney *U* test. Significant differences were accepted when $P < 0.05$.

Results

Effects of high glucose on eNOS protein and mRNA expression

After exposure to high glucose for 24 hours, eNOS proteins were increased significantly, and in accordance with it, expression of eNOS mRNA were also enhanced (Fig. 1A, B). As mannitol treatment did not affect the expression of eNOS protein or eNOS mRNA, these results attributed to high glucose itself, not to osmolality.

Effects of high glucose on NOx produced by eNOS

After stimulated by high glucose (12.5 mM, 25 mM) for 24 hours, NOx production was significantly decreased compared with control (5.5 mM), but there were no significant different between the two high glucose groups (12.5 mM, 25 mM) (Fig. 2A). As mannitol treatment did not affect the concentration of NOx, the effect attributed to high glucose itself, not to osmotic pressure.

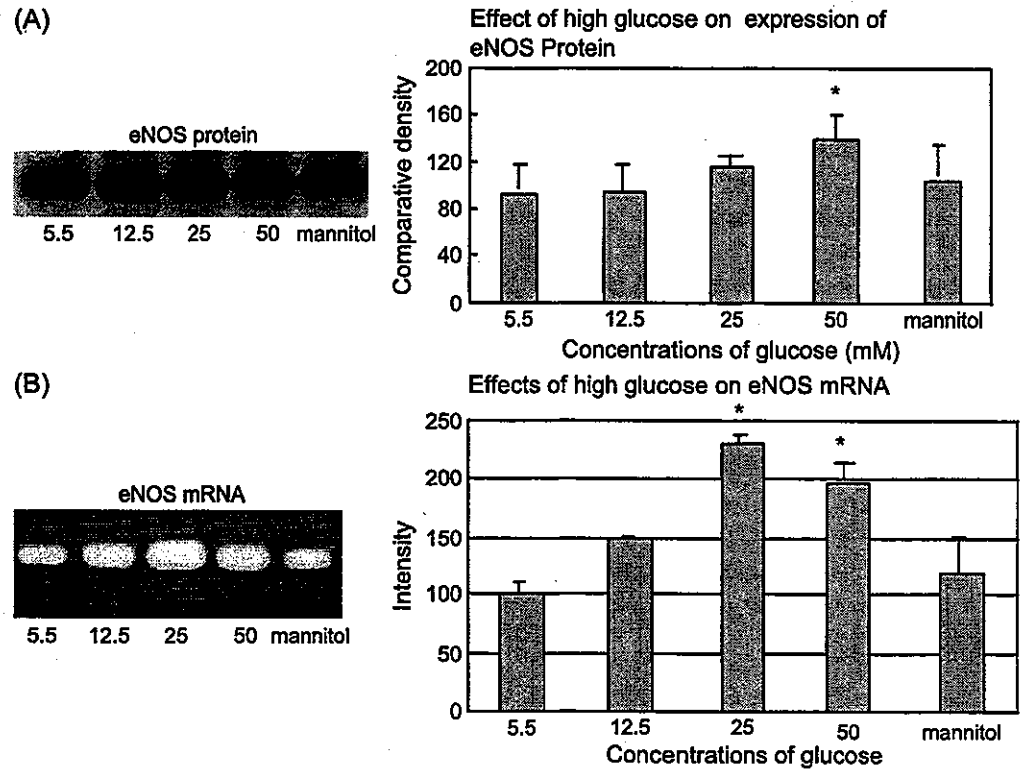

Fig. 1. The effects of high glucose on eNOS expression. Western blot and RT–PCR analysis of eNOS protein **(A)** and mRNA expression **(B)** after 24 hours exposure to different concentrations of glucose. Data represents the mean ± SEM of three separated experiments. The effects of high glucose on NOx production **(A)** *$P < 0.05$ vs control.

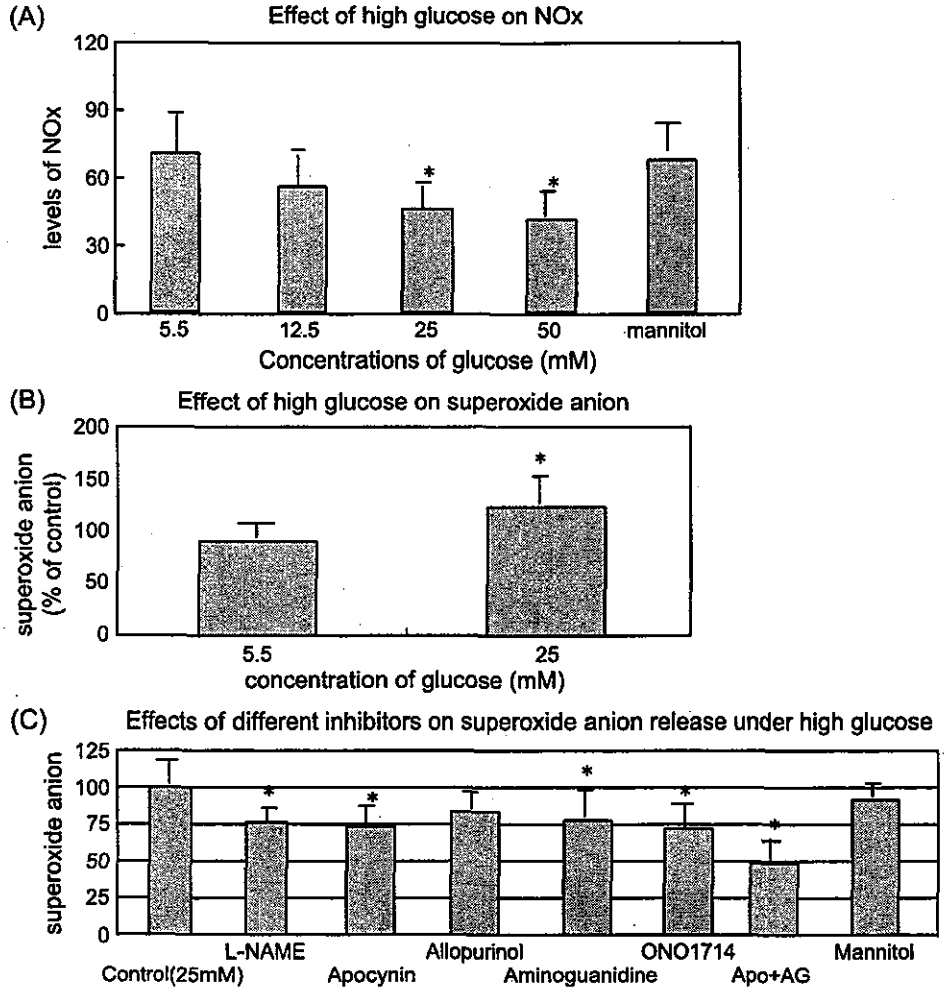

Fig. 2. The effects of high glucose on NOx production **(A)**, superoxide anion **(B)**. Effects of different inhibitors on superoxide anion under the stimulation of high glucose **(C)**. Data represents the mean ± SEM. *P < 0.05 vs control.

Effects of high glucose on intracellular superoxide anion and possible route of superoxide production

The intracellular superoxide anion was largely increased by the stimulation of high glucose (25 mM), compared with control (5.5 mM), after 24 hours exposure (Fig. 2B). And the stimulatory effects of high glucose could be abolished by L-NAME(100 μM) and apocynin(10 μM), respectively (Fig. 2C). However, the effect of allopurinol (10 μM), aminoguanidine(10 μM), or ONO 1714 was relatively limited, and mannitol did not affected superoxide production (Fig. 2C).

Effects of high glucose on intracellular BH4 levels and GTPCH1 activities

As showed in Fig. 3A and B, both of intracellular BH_4 levels and GTPCH1 activities were decreased significantly by high glucose exposure.

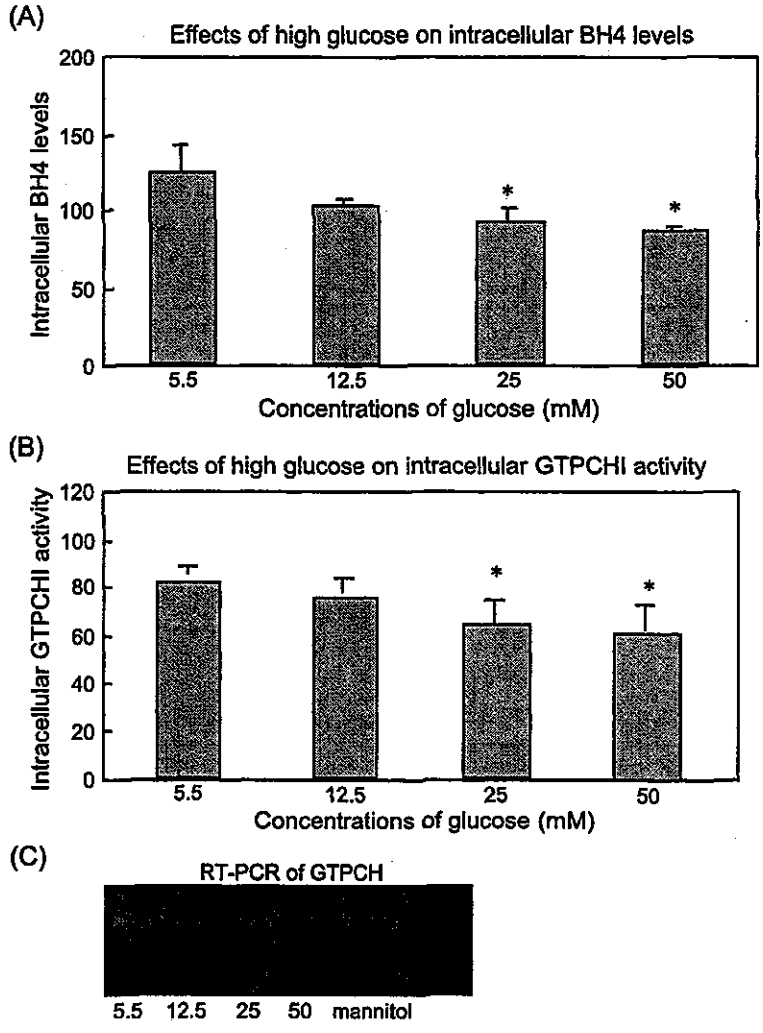

Fig. 3. The effects of high glucose on intracellular BH_4 levels (A), GTPCH1 activities (B) and GTPCH1 mRNA expression (C).

Effects of high glucose on expression of GTPCH1 mRNA

As revealed in Fig. 3C, in accordance with the inhibition of intracellular GTPCH1 activities, the expression of GTPCH1 mRNA abundance was also decreased by exposure to high glucose. It tended to correlate with GTPCH1 protein and activities (data not shown).

Effect of HMG-CoA reductase inhibitor on intracellular GTPCH1 activity and BH4 level

Atrovastatin exhibited a stimulatory effect on intracellular BH_4 accumulation (Fig. 4A) and GTPCH1 activities (Fig. 4C) and it was shown in a time- and concentration–dependent manner (part of data not shown).

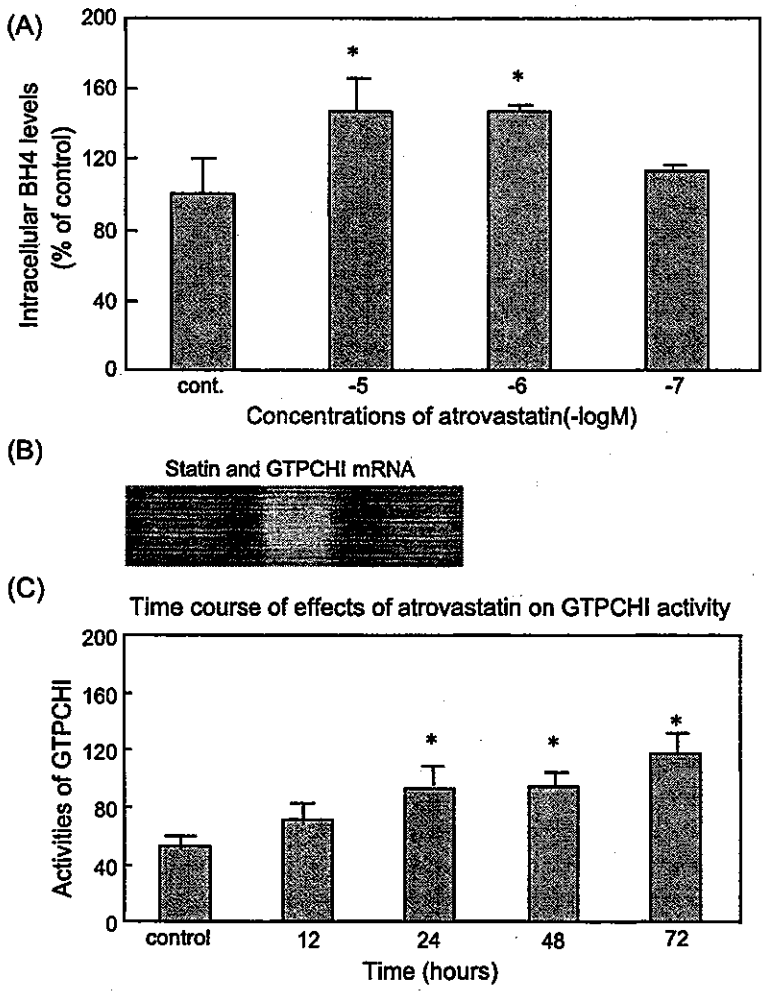

Fig. 4. Effects of different concentrations on atrovastatin on intracellular BH_4 levels (**A**) and GTPCH1 mRNA (**B**). Time-course of effects of atrovastatin on intracellular GTPCH1 activities (**C**). Data represents the mean ± SEM of three independent experiments. *P < 0.05 vs control.

HMG-CoA reductase inhibiters and expression of GTPCH1 mRNA

In accordance with the stimulatory increase of intracellular GTPCH1 activities and BH_4 accumulations, expression of GTPCH1 mRNA was also augmented by atrovastatin (Fig. 4B).

Discussions

This study demonstrated that in bovine aortic endothelial cells, the expression of eNOS was increased by exposure to high glucose (Fig. 1A). This may be the result of augmentation of eNOS mRNA expression by high glucose (Fig. 1B). Although, the effect of high glucose on eNOS protein

was dose dependent, the maximum level of eNOS mRNA expression was maximum between 25 and 50 mM glucose. We cannot elucidate the mechanism in the difference of effective concentration between protein and mRNA levels. In the preliminary experiment of 72 hours exposure to high glucose, eNOS expression was maximum between 25 and 50 mM glucose. We speculate that the difference of effective glucose concentration between eNOS protein and mRNA is due to the difference of time course. It exhibited conflictions to the reasonable hypothesis that, the eNOS abundance should be decreased by high glucose, which is based upon the clues of impaired endothelium-dependent relaxation in diabetic vessels of both human and animal experiments (Consrtino et al., 1997; Ding et al., 2000; Johnstone et al., 1993; Makimattila et al., 1996; Noyman et al., 2001; Steinberg et al., 1996). Further, measurement of NOx revealed a marked decrease when cells were allow to grow in high glucose (Fig. 2). Two possible explanations could be applied: one comes from the rapid reaction between NO and superoxide, while another one means the possibility if virtually decreased capacity of NO production by eNOS. One possible clue for eNOS dysfunction caused by high glucose gives rise to the hypothesis that increased abundance of eNOS caused by high glucose could not produce NO in proportion to that under normal glucose.

It is now generally agreed that oxidative stress plays a crucial role in the formation and deterioration of atherosclerosis (Tomas et al., 2002; Chen et al., 1995). In order to clarify the dysfunction of eNOS, we also studied the production of superoxide anion after exposure of high glucose by FACS. As shown in Fig. 2, high glucose increased intracellular superoxide anion significantly. In order to identify the sources of superoxide, different kinds of inhibitors which is related to possible pathways of superoxide were applied. It is amazingly to find that L–NAME, which is the specific inhibitor of NOS, exhibited a strong inhibitory effect on superoxide production and restored superoxide anion to almost the same level as control. It means that eNOS becomes an important source of superoxide anion in high glucose. From this point of view, high glucose could lead to dysfunction of eNOS.

Since increased superoxide anion could also be inhibited partially by apocynin, but not by allopurinol and aminogunidine independently, it confirmed that in case of high glucose, NADPH oxidase, but neither xanthine oxidase nor iNOS is not the possible source of superoxide production as well as eNOS. The mechanisms underline these phenomena are still unknown. Evidences from diabetic animal models and human studies showed cofactor of eNOS, tetrahydrobiopterin (BH_4), may be the redox of NO or superoxide production of eNOS (Heitzer et al., 2000; Pieper, 1997). We further focused on the effects of high glucose on intracellular BH_4 level and activity of GTPCH1-the rate-limiting enzyme in the de novo biosynthesis of BH_4, which is the most important pathway under physiological conditions. BH_4 is absolutely required for eNOS activity (Chen et al., 1995; Hattori et al., 2003). By acting as a cofactor of eNOS, evidences showed that it is involved in: 1) stabilization eNOS in its dimeric form, which is pivotal for eNOS to function normally; 2) electron transfer from the reductase domain to oxidase domain; 3) active site integrity. And in some pathological situations, it could even help overcome 'paradoxical deficiency' of L-arginine. As it is showed in Fig. 3, both intracellular BH_4 levels and activities of GTPCH1 were markedly decreased by the exposure to high glucose comparing with control. It has been revealed that BH_4 react with superoxide rapidly, thus decreases BH_4 accumulation in cells. Results of the present study show that in case of high glucose, the deceased activity of GTPCH1 could also be an important reason for the decreased BH_4 levels. So it is reasonable to think that it is the combination of the two possibilities lead to an absolute shortage of intracellular BH_4, and accordingly, the dysfunction of eNOS arises. But it is still difficult to identify which one plays a more important role.

We speculated that the transcriptional regulation of GTPCH1 mRNA is responsible for the decreased GTPCH1 mRNA by high glucose treatment. Preliminary experiment showed that the decreased activity of GTPCH1 associated decreased protein level. Gesierich et al. reported the importance of the complex formation of GTPCH1 with GTPCH1 feedback regulatory protein (GERP) in negative feedback regulation by end product BH_4, and phenylalanine upregulated GTPCH1 mRNA without changing GERP (Gesierich et al., 2003; Hattori et al., 2003). They speculated that the substrate level and transcription of the interacting protein regulation of BH_4 biosynthesis. In the present study, the amount of BH_4 was decreased by high glucose treatment, and the protein also decreased.

HMG-CoA reductase inhibitors are now generally convinced to be a potent antiatherosclerotic agent. Its pleiotropic effects include a direct stimulatory effect on eNOS or iNOS as reported (Gorren et al., 2002; Hayashi et al., 1995a; List et al., 1997). And we have for the first time revealed that HMA-CoA reductase inhibitors could upregulate GTPCH1 mRNA expression, thus stimulate the activity of *GTPCH1* as well as intracellular BH_4 levels in cultured endothelial cells, directly. Statin was reported to enhance cytokine-mediated inducible nitric oxide synthesis in smooth muscle cells (Hattori et al., 2002). It has been reported that the effect of statin was abolished by exogenous mevalonate or GTPCH1 inhibitor, GGTI-298. Our data further, give a richer meaning to the pleiority of antiatherosclerotic effects of HMG–OA reductase inhibitor (Laufs et al., 1998; Tsunekawa et al., 2001). Finally, mannitol concentration was adjusted to the osmotic pressure in 50 mM high glucose. Preliminarily, we examined the effect of mannitol on high glucose treatment, and we made sure that it did not significant effect on eNOS protein and mRNA and GTPCH1 mRNA, and BH_4 concentration.

Conclusively, high glucose could lead to the dysfunction of eNOS by inhibiting the synthesis of BH_4 and activating NADPH oxidase. Statin could enhance eNOS activity through stimulating GTPCH1, thus increases BH_4 levels, directly.

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The treadmill exercise-tolerance test is useful for the prediction and prevention of ischemic coronary events in elderly diabetics

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Abstract

Background: Approximately 80% of cases of ischemic heart disease (IHD) occur in patients with nonstenotic coronary arteries, and few studies have systematically assessed exercise testing (TMT) as a predictor of risk in the elderly. **Methods:** TMT was carried out using a protocol for the independent and active elderly (n=176). After 4.1±0.5 years follow-up, logistic regression analysis was performed for each coronary risk factor such as diabetes mellitus (DM) and hypercholesterolemia (HC). According to the results, patients were divided into Gp HC, hypercholesterolemic patients; Gp DM, diabetics; Gp HC+DM, hypercholesterolemic diabetics; and Gp C, nonhyperlipidemic and nondiabetics. Sensitivity and specificity of TMT for IHD (significant stenosis or acute coronary syndrome) were analyzed. **Results:** Odds ratios for each risk factors are as follows: DM, 4.167; HC, 4.485; and DM+HC, 8.652. Notably, TMT was 17.59. Age was a significant risk, but hypertension was not. Positive ischemic signs in TMT were observed in 52.7%, 28.6%, 33.3%, and 16.3% in the Gp HC+DM, HC, DM, and C groups, respectively. Only three participants complained of chest pain during the TMT. Significant stenosis was observed in 75.0%, 71.4%, 69.2%, and 60.0% of coronary angiography (CAG)-receiving patients of Gp HC, DM, HC+DM, and C. During the observation term, acute coronary syndromes occurred in 4.7%, 3.3%, 5.5%, and 0% of patients in the Gp HC, DM, HC+DM, and C groups, respectively. The sensitivity of TMT for IHD was higher than 66.7% and specificity was higher than 94.1% in each group. **Conclusion:** An exercise tolerance test in the elderly, especially for diabetics and hypercholesterolemic patients, is useful for the diagnosis of IHD.

1. Introduction

Recent mega-trials have revealed that strict control of complicated coronary risk factors such as hyperlipidemia is important for the prevention of diabetic vascular lesions (Jonsson, Cook, & Pedersen, 1999). Exercise stress testing is an accepted means of estimating and diagnosing cardiovascular disease, as well as of predicting cardiovascular and all-cause mortality (Gianrossi, Detrano, Mulvihill, et al., 1989). However, approximately 80% of cases of ischemic heart disease (IHD) occur in patients with nonstenotic coronary arteries, and these cases cannot be predicted by an exercise-tolerance test (Bezerra, Higuchi, Libby, Ramires, et al., 2001). Furthermore, few studies have systematically assessed exercise testing as a predictor of risk in the elderly. Diabetic coronary lesions are known to have long segmental narrowing, and the incidence of IHD seems to be especially increased in patients who have had diabetes for more than 10 years (Al-Attar, Mahussain, & Sadanandan, 2002; Stein, Weintraub, Gebhart, et al., 1995). We have speculated that an exercise-tolerance test would be useful for the evaluation and prevention of IHD in elderly diabetics, if it could be carried out in a safe manner. We therefore modified the protocol of the exercise burden for the treadmill exercise-tolerance test (TMT) to make it more suitable for elderly patients.

The present study focused on the relationship between the frequency of cardiovascular ischemia, the exercise-tolerance test, and coronary risk factors in the elderly.

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2. Research design and method

2.1. Patient selection

Between April 1997 and March 2000, 342 patients were enrolled in this study. All patients were ambulatory and were either referred to our geriatric clinic (Nagoya University Hospital) or enrolled in our hospital to receive educational hospitalization for diabetes. Among them, 176 patients who were older than 65 years and who underwent an exercise-tolerance treadmill test were prospectively enrolled and followed for 4.1±0.5 years (Table 1). All patients gave their informed consent to participate in this study. None of the patients had experienced a myocardial infarction in the 3 months prior to enrollment, and they were independently active in daily life, as determined by their Lawton and Berthal scores (Collin, Wade, Davies, & Horne, 1988; Lawton & Brody, 1969).

2.2. Protocol and method

TMT was performed according to a protocol for the elderly, which we adapted from a protocol used for veterans in the United States (Prakash, Myers, & Froelicher, 2001). We changed the test so that each step lasted 2 min due to the age-related limitation of exercise tolerance (Hagberg, 1994; Tamesis et al., 1993; Table 2). The chronotropic response to exercise was assessed by estimating the proportion of the heart-rate reserve (220-age) used at peak exercise (Lauer, Francis, Okin, et al., 1999). Ischemic changes in the treadmill test were diagnosed using the Minnesota protocol; in brief, 1.0 mm or more ST segment elevation or depression in two or more leads was identified as positive. Exercise tolerance was estimated as METS, which was calculated from the participant's TMT results, body weight, age, and estimated Vo2 at rest. Plasma lipid and glucose levels were also measured. The diagnosis of hypercholesterolemia (HC) and diabetes followed the guidelines of the American Heart Association and Diabetes Association (Krauss, Eckel, Howard, et al., 2000; Resnick, Harris, Brock, et al., 2000). This study was approved by our institutional review board.

2.3. Follow-up data/definition of adverse outcome

All patients were followed until April 2002, with the mean follow-up period being 4.1±0.5 years after the treadmill test. The outcome was determined from patient interviews, hospital chart reviews, and telephone interviews. An adverse outcome was defined as the finding of significant stenosis in coronary angiography (CAG) with or without coronary intervention, such as percutaneous coronary angioplasty or ischemic cardiac events in the follow-up term. Cardiac events were defined as cardiac death, nonfatal MI, and resuscitated ventricular fibrillation or tachycardia after the TMT. Only the most severe outcome was considered an endpoint. Twenty-nine patients were excluded because of patient or physician refusal to follow-up (n=13), an inability to repeat the exercise treadmill test safely due to hearing loss (n=2), or geographic relocation (n=14). A total of 147 elderly individuals could be followed, and data on their histories of ischemic coronary disease, results of CAG, medication, and other parameters were recorded (Table 1). Based on the odds ratios evaluated as described below, patients older than 65 years were divided into four groups: Gp HC, hypercholesterolemic patients (n=42); Gp diabetes mellitus (DM), diabetic patients (n=30); Gp HC+DM, hypercholesterolemic and diabetic patients (n=36); and Gp C, nondiabetic and nonhyperlipidemic patients (n=39).

Table 1
Profile of patients

Patients number	147 (Male 71, Female 76)
Age (years)	71.7+0.4
Hypercholesterolemia	78 (Male 38, Female 40)
Diabetes mellitus (DM)	66 (Male 32, Female 34)
Hypertension	78 (Male 40, Female 38)
[Hypercholesterolemia+DM]	[36 (Male 17, Female 19)]

Table 2
Protocol of treadmill test for elderly

Stage	1	2	3	4	5	6	7	8	9
Period (min)	2	2	2	2	2	2	2	2	2
Speed (miles/h)	1	2	2	2	2	2	2.5	3.3	3.3
Gradient (%)	0	0	5	10	15	20	20	20	25
METs	2.5	3	5	6	8	9	10	11	13

2.4. Statistical analysis

Continuous data were expressed as the means±S.D. Categorical variables were analyzed by the chi-square test or Fisher's Exact Test. Continuous variables within groups were analyzed by repeated measures using analysis of variance (ANOVA). The Student's t test was used to identify significant differences in means. Stepwise multiple logistic regression analyses were used to identify the independent predictors of outcome, as well as the additive prognostic values of the clinical data and the exercise treadmill test. Fisher's Exact Test was used to calculate odds ratios or the probability of detecting any variables included in the logistic regression analysis in patients with adverse outcomes relative to patients with good outcomes.

3. Results

The odds ratios of each risk factor as determined by logistic regression analysis are shown in Table 2. Briefly, the odds ratios were as follows: DM, 4.167; HC, 4.485; and DM+HC, 8.652 (P<.01, respectively). That of age was significantly high (2.953; P<.05), whereas that of hypertension was not significant (2.151; P=.053). Notably, the odds ratio for positive ischemic signs as evaluated by TMT was 17.59.