

down-regulated day3. However, only hsp70 still kept a significantly higher expression level at 42°C compared to at 37°C.

eNOS and phosphorylated eNOS showed a similar alteration, they were up-regulated at 42°C on day1, but down-regulated at 42°C on day3 to below the level at 37°C on day3.

DISCUSSION

Biological stress has been reported to induce negative effects on the maintenance of telomere length in various cells^[1,13]. However, heat stress-associated telomeric changes have not been well investigated, so far. In the present study, HUVECs were used to analyze the telomere-associated alterations induced at 42°C. Cell growth was suppressed at 42°C especially on day3. The proportion of senescent cells elevated indeed on day1 at 42°C but returned to a same level as at 37°C on day3. This observation indicated that a heat-sensitive cell population diminished during 3-day-exposure to 42°C, and a heat-resistant population survived until day3. In the telomere length distribution, apparently long telomere decreased and telomere of medium size (4.4-9.4kb) increased at 42°C on day1. This indicated that growth rate was suppressed more in cells with long telomere than those with short telomere under exposure to heat-stress in an early stage of heat exposure. The shortest range of telomere (<4.4kb) showed an increasing trend of increase at 42°C on day3, although it was not significant. A part of cell population bearing short telomeres might be damaged and diminished at 42°C, resulting in non-clear increase of them on day3. The grown subpopulation at 42°C by day1 seemed to diminish by day3, because the TL distribution of the survived cell population at 42°C on day3 returned to a similar pattern at 37°C on day3.

This suggested that a subpopulation of cells with short telomere grew initially in heat-exposure but lost the heat-endurance during a prolonged heat exposure. The mechanism of the apparent heat-associated growth suppression of cells with long telomere is not clear, so far. Cells containing long telomeres, i.e., young cells, may be equipped with an unknown mechanism suppressing cell growth under a heat-stressed condition, possibly being a favor for the cell survival.

All the analyzed proteins up-regulated on day1 at 42°C. The survived cells for 3 days at 42°C revealed an elevated expression of Hsp70, and maintained the expression of TERC, TRF2, and Hsp90. All the others, TERT, TRF1, Hsp60, and NOS were suppressed in expression at 42°C. The expression of Hsp70 might contribute to survival advantage under a prolonged heat-exposure. We here show that an up-regulation after 3-day-culture at 42°C is maintained only in Hsp70 among analyzed heat shock proteins. Heat shock protein70 (Hsp70) protects cellular elements from injury by reducing oxidation, inflammation and apoptosis and by refolding damaged proteins. Hsp70 improves viability of stressed vascular smooth muscle cells, possibly via its chaperone functions^[14]. The beneficial effects on cell viability are here shown to contribute not only to vascular smooth muscle cells but also possibly to vascular endothelial cells.

The expression of NOS was up-regulated in an early stage of heat-exposure and was down-regulated later. This suggested that heat-stress had an inducible potential for NOS expression. NOS has been reported to contribute to vasodilatation, and so heat exposure would result in vasodilatation^[6]. However the vasodilatation by NOS would diminish after prolonged heat exposure. The heat-associated alterations of NOS expression seemed rather to result in changing vasodilatation potential than contribute to survival advantage for heat stress.

Longer telomeres seemed transiently affected under exposure to heat stress. However, prolonged heat exposure did not leave a persistent altered pattern of telomere length distribution. In contrast to the growth suppression and the down-regulation of various proteins including telomere-associated proteins, other heat shock proteins, and NOS, only Hsp70 up-regulated persistently under heat stress. Hsp70 may be a strong survival factor for heat-damaged cells. Further study is necessary to confirm the vascular cellular heat-resistance associated with Hsp70.

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Altered expression of genes associated with telomere maintenance and cell function of human vascular endothelial cell at elevated temperature

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Abstract The pathophysiological alterations of vascular endothelial cells induced by heat were studied. Human umbilical venous endothelial cells were cultured for 1 day at three different temperatures (37, 39, and 42 °C). The telomere lengths, the expressions of proteins associated with telomere length maintenance, apoptosis, heat shock, and vascular function were analyzed. The cell growth was not suppressed at 39 °C but suppressed at 42 °C. The mean telomere length did not change, whereas the telomere length distribution altered at 42 °C. Long telomere decreased and middle-sized telomere increased in the telomere length distribution at 42 °C. The telomerase activity did not show any heat-associated alterations. However, of the components of telomerase, telomerase reverse transcriptase was up-regulated along temperature elevation. In contrast, the expression level of RNA component TERC did not alter. Among the analyzed apoptosis-associated proteins, p21 was down-regulated and phosphorylated p53 was up-regulated. Heat shock proteins and NO synthase were up-regulated at 42 °C. These results suggested that induced growth suppression or cell senescence was induced by strong heat stress rather than mild one predominantly in cells bearing long telomeres with p53

activation, and simultaneously activated some telomere-associated factors, heat shock proteins, and NO synthesis probably for heat-resistant cell survival.

Keywords Heat stress · Vascular endothelial cell · Telomere · Telomerase · Apoptosis

Abbreviations

HUVECs	Human umbilical venous endothelial cells
TERT	Telomerase reverse transcriptase
TERC	Telomerase RNA component
Hsp	Heat shock proteins
eNOS	Endothelial nitric oxide synthase
PD	Population doubling
SA-β-Gal	Senescence-associated β-galactosidase
TL	Telomere length
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
TRAP	Telomerase repeat amplification protocol
ANOVA	Analysis of variance

Background

Telomere forms a structure capping the chromosomal ends, which consists of repetitive DNA sequence and accessory proteins [1]. Telomere becomes shorter due to DNA duplication starting not from the very end but from an internal site close to the end. It is also known that many kinds of stress facilitate the telomere attrition [2]. Mental or physical stress promotes the telomere attrition [2]. Telomere shortening occurs in somatic cells with aging through many rounds of cell cycle and pathological stress [3]. On the other hand, there is a telomere-maintaining

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mechanism, where telomerase plays a central role. Telomerase consists of an enzymatic protein, reverse transcriptase (TERT), and an RNA molecule containing a complementary sequence of a repetitive unit DNA sequence of telomere (TERC) and mediates the telomere elongation in cancer cells, stem cells, and reproductive cells. The telomerase activity is maintained usually in a very low level in somatic cells and it cannot fully prevent the telomere shortening of somatic cells. Stress factors have been regarded to affect not only telomere shortening but also telomerase function. However, the effects of heat stress on somatic telomere length or telomerase activity have not been satisfactorily investigated yet.

Stress stimuli including heat stimulus can cause various cellular effects including induction of cell senescence, and activation of cellular protective reactions. Heat shock proteins are one of stress-associated proteins and are ubiquitously synthesized in virtually all species and it is hypothesized that they might have beneficial health effects [4]. In response to stress stimuli, mammalian cells activate a signaling pathway leading to the transient expression of heat shock proteins (hsp). Hsp's are a family of proteins serving as molecular chaperones that prevent the formation of nonspecific protein aggregates and assist proteins in the acquisition of their native structures. Physiologically, hsp's play a protective role in the homeostasis of the vessel wall consisting of endothelial cells and smooth muscle cells [5]. Heat stress induces various alterations of cell functions. One of vascular functions is vasodilatation in reaction to relaxation stimuli. Vasodilatation occurs in association with heat stress with production of NO [6]. This production is mediated by endothelial nitric oxide synthase (eNOS) expressed in vascular endothelial cells, resulting in relaxation of the surrounding smooth muscle cells of vascular wall. NO production can be an indicator of vascular endothelial function. Thus, pathological stress including heat stress induces various cellular responses in human somatic cells. In order to evaluate heat effects on vascular endothelial cell, we analyzed telomere DNA, and the expressions of telomere-associated components, apoptosis-associated proteins, heat shock proteins, and nitric oxide synthase of HUVECs at high temperature.

Methods

Cell culture

Human umbilical venous endothelial cells (HUVECs) were purchased from Clonetics Corp. (San Diego, CA, USA). The cells were cultured according to the manufacturer's instructions in endothelial cell growth medium (Clonetics Corp; San Diego, CA, USA) supplemented with 10 % fetal

bovine serum (Gibco, Grand Island, NY, USA) in a humidified incubator maintained at 37, 39, or 42 °C and supplied with 5 % CO₂ and 95 % air at 37, 39, or 42 °C and 5 % CO₂ in a gelatin-coated flask (Iwaki Glass, Chiba, Japan). Three passages were used in all experiments. Culture media were refreshed every 24 h. On day 1, the cells were collected and were counted using a hemocytometer. Population doublings (PDs) were calculated using the formula: $PD = [\log(\text{expansion})/\log 2]$, where expansion was the number of cells harvested divided by the initial number of cells seeded.

Senescence-associated β -galactosidase (SA- β -Gal) expression

The cells were washed in PBS, fixed for 10 min at room temperature in 2 % formaldehyde/0.2 % glutaraldehyde, and incubated at 37 °C (no CO₂) with fresh SA- β -Gal staining solution containing 1 mg/mL of X-gal, pH 6.0 for 12 h. One hundred cells were scored from each well.

Telomere detection

Telomere detection was performed as previously described [7]. Cellular DNA was extracted from samples and the DNA (0.1 μ g) was digested with *MspI*. The digests (10 μ l) were subjected to Southern blot hybridization with a telomere DNA probe. The autoradiogram was captured on an Image Master, and the telomere length was then assessed quantitatively. Every sample was measured in triplicate.

Terminal length (TL) analysis

TL distribution was analyzed by comparing the telomere length using a telomere percentage analysis with three intervals of length (>9.4, \geq 9.4, \geq 4.4, and <4.4 kb) as defined by a molecular weight standard as previously described [7]. The percent of the stratified intensity in each molecular weight range was measured. The mean TL was estimated using the formula $S(\text{ODi} - \text{background}) / S(\text{ODi} - \text{background}/Li)$, where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i.

Semiquantitative RT-PCR for TERC RNA

Total RNA samples were extracted using RNazol B (Teltest). mRNA for human telomerase RNA component (TERC) was determined by RT-PCR using a DIG detection system (Roche Applied Science). Each human cDNA was produced by RT-PCR according to each human-derived sequence. For the amplification of β -actin cDNA, the forward primer (205 bp) 5'-CCTTCCTGGGCATGGAGTCCT-3' and the reverse

primer 5'-GGAGCAATGATCTTGATCTTC-3' were used according to the published human β -actin cDNA sequence [8]. And the TERC forward primer 5'-TCTAACCTAACTGA-GAAGGGCGTAG-3' and the TERC reverse primer 5'-GTTTGCTCTAG AATGAACGGTGGAAG-3' were used [9]. The values for TERC mRNA levels were normalized to the β -actin mRNA level in the same sample. The PCR products were directly synthesized from 2 μ g of total RNA isolated from each sample using the Superscript one-step RT-PCR system with Platinum *Taq* (Invitrogen) and gene-specific primers according to the recommendations provided by the supplier. The PCR products were analyzed by agarose gel electrophoresis (1.3 %) followed by staining with ethidium bromide and scanning with Gel-Doc (Bio-Rad). For semi-quantitative PCR, β -actin was used as an internal control to evaluate total RNA input, as described by our group [10].

Western blot analysis

Cells from a dish were homogenized with 100 μ l lysis buffer (100 mM Tris pH 6.8, 4 % Sodium Dodecyl Sulfate, 20 % glycerol containing the protease inhibitor, M phenylmethanesulfonyl fluoride, 0.1 mM, leupeptin, 0.1 μ l, and aprotinin, 0.1 μ l). Gel electrophoresis was used to separate 10 μ g protein on a 10 % SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (162-0112, Bio-Rad Laboratories, Hercules, CA, USA) blocked with 5 % dry milk or blocking solution for Western blot (Roche). Membranes were blocked and incubated with antibodies against telomerase reverse transcriptase (TERT) (Rockland immunochemicals, PA, USA), TRF1 (Imgenex, CA, USA), TRF2 (Cell Signaling Technology, MA, USA), hsp60 (Assay Designs, GA, USA), hsp70 (Assay Designs, GA, USA), hsp90 (Enzo, PA, USA), eNOS (BD Biosciences, NJ, USA), phospho-eNOS (Cell Signaling Technology, MA, USA), p21 (EXBIO), p53 (Santa Cruz Biotechnology), phospho-p53 (Cell Signaling Technology, MA, USA), Akt (Cell Signaling Technology, MA, USA), phospho-Akt (Cell Signaling Technology, MA, USA), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, CA, USA). Detection was performed with secondary horseradish peroxidase-conjugated antibodies (EMD Millipore, MA, USA) and the ECL detection system as previously described [11]. The relative expression levels were determined compared to that of GAPDH.

Telomerase activity

Telomerase activity was examined by means of a modified telomerase repeat amplification protocol (TRAP) method with TeloChaser (Toyobo, Osaka, Japan), as previously described [12].

Statistical Analysis

Assays were repeated three times and analyzed statistically. The normality of the data was examined with the Kolmogorov-Smirnov test and the homogeneity of variance with the Levene Median test. If both the normal distribution and equal variance tests were passed, intergroup comparisons were performed using a two-way analysis of variance (ANOVA) test followed by all pairwise multiple comparison procedures using Tukey's post hoc test. The data are expressed as the mean \pm standard deviation. The criterion for the significance is $p < 0.05$.

Results

Population doubling (PD) and Cell senescence

The PD of the HUVECs was assessed on day 1 of culture. The PD's were 1.69 ± 0.10 , 1.67 ± 0.19 , and 1.21 ± 0.14 , at 37, 39, and 42 $^{\circ}$ C, respectively. The PD was significantly suppressed at 42 $^{\circ}$ C ($p = 0.024$) (Fig. 1).

SA- β -Gal expression was observed in 1.9 % of cells at 37 $^{\circ}$ C, and 3.8 % at 39 $^{\circ}$ C, and 4.0 % at 42 $^{\circ}$ C (Fig. 1). Senescent cells significantly increased at 39 and at 42 $^{\circ}$ C.

Growth suppression was not observed but senescent cells increased already at 39 $^{\circ}$ C. An apparent suppress of cell growth was observed at 42 $^{\circ}$ C. Cell population having survived through the heat condition of 42 $^{\circ}$ C for 1 day bore a similar proportion of senescent cells as that at 39 $^{\circ}$ C. This indicated that cell population retained a constant ratio of heat-induced senescent cells in independent of growth suppression.

The mean TL and TL distribution

The mean TL's of HUVECs were measured to assess how much high temperature condition affected telomere DNA (Fig. 2). There was no significant heat-associated change in mean TL. However, in TL distribution, long telomere (>9.4 kb) decreased and middle-sized telomere (9.4–4.4 kb) increased at 42 $^{\circ}$ C. Yet, the proportional amount of short telomere (<4.4 kb) was not significantly affected. Cells bearing longer telomere tended to be affected more by heat stress, compared to those with shorter telomere.

Telomerase activity

The telomerase activity of HUVECs at three temperatures was evaluated by TRAP assay (Fig. 3). Average relative TPG at 37 $^{\circ}$ C was put as $1(1 \pm 0.66)$. The relative TPG's at 39 and 42 $^{\circ}$ C were 0.74 ± 0.37 and 1.33 ± 0.84 , respectively. Thus, the relative telomerase activity of HUVECs was

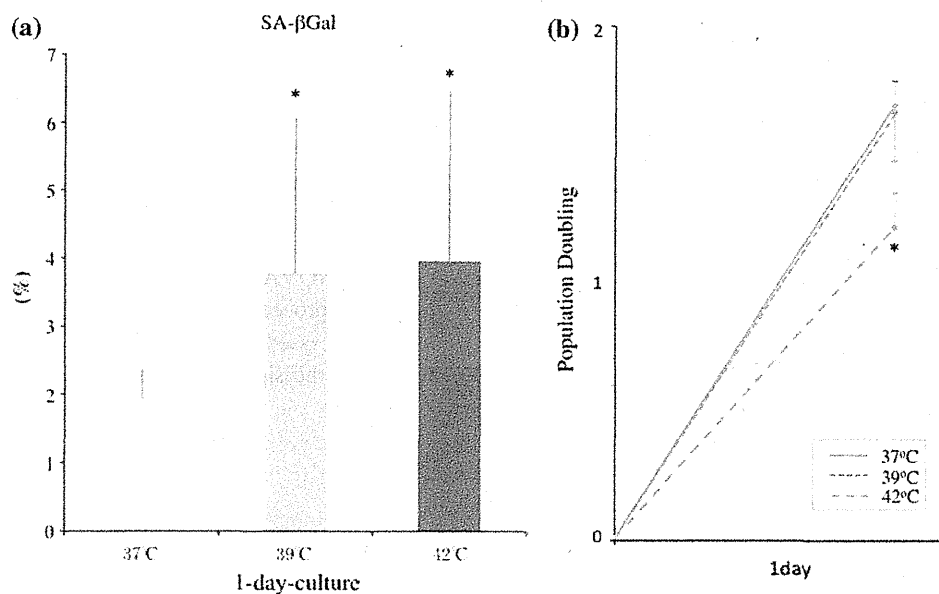
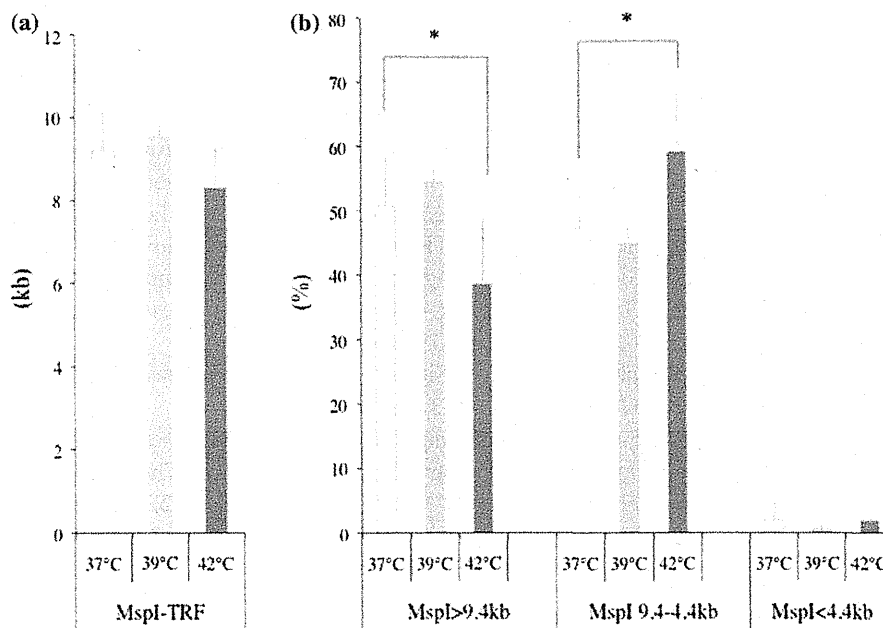


Fig. 1 The population doubling and the ratio of senescence-associated β -galactosidase (SA- β -Gal) staining of HUVECs cultured at different temperatures. The upper panel (a) shows the PD on day 1 in

culture. The horizontal bars are standard deviations. The lower panel (b) shows the percentages of the cells that were SA- β -Gal stained. * $p < 0.05$ versus at 37 °C

Fig. 2 The mean telomere length and the telomere length distribution of HUVECs cultured at different temperatures. The mean telomere lengths and the telomere length distributions are shown. The MspI-terminal restriction fragment lengths are presented as the mean values \pm standard deviation. The horizontal bars represent the standard deviation. A significant difference was observed between the control cells at 37 °C and those at higher temperatures. The horizontal bars represent the standard deviation. * $p < 0.05$ versus at 37 °C



not affected by heat. The telomerase activity of HUVEC did not change at different temperatures.

Gene expression

Among the analyzed telomere-associated components, TERT and TRF2 were affected by hyperthermia. TERT was up-regulated at 39 and 42 °C. TRF2 was up-regulated at 42 °C. TRF1 or TERC was not affected by heat in

expression (Fig. 4). The expression of apoptosis-associated components, p21, p53, pp53, Akt, and p-Akt, was appraised. p21 was down-regulated and pp53 was up-regulated at 42 °C, whereas the expression of p53 was not affected (Fig. 4).

All the analyzed heat shock proteins (hsp60, hsp70, and hsp90) were up-regulated at 42 °C. However, the up-regulation of heat shock proteins was not observed at 39 °C (Fig. 4).

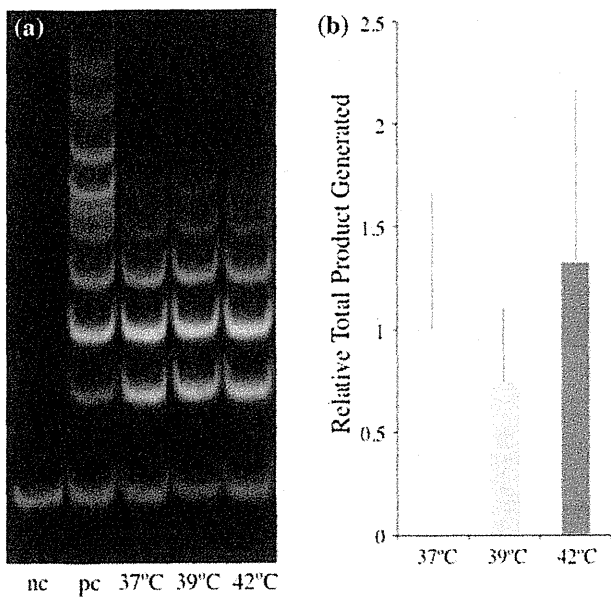


Fig. 3 The telomerase activity of endothelial cells at different temperatures. The *left panel (a)* shows a photograph of representative TRAP assay results for HUVECs. The *right panel (b)* shows the relative telomerase activity at different temperatures. The relative telomerase activity is presented as a proportional ratio of the density the ladder of a sample to that of the mean value at 37 °C. The materials used for the positive control (pc) and negative control (nc) were provided with the kit

eNOS and phosphorylated eNOS showed a similar alteration. They tended to be down-regulated at 39 °C, but it was not significant. They were up-regulated at 42 °C (Fig. 4).

Discussion

Biological stress affects telomere attrition [1, 13]. Heat can be a stress stimulus affecting telomere-shortening rate of somatic cells. However, the relationship between heat stress and telomere shortening has not been well studied, so far. In the present study, human vascular endothelial cell was used to analyze the telomere-associated alterations induced by heat stress. Population doubling was not lowered at 39 °C but at 42 °C. On the other hand, senescent cells increased at 39 °C to a similar extent observed at 42 °C. These indicated that heat-induced cell senescence occurred even with a mild heat stimulation which did not yet influence the cell growth rate. Significant change of gene expression at 39 °C was observed only in TERT. Cell senescence induction and the up-regulation of TERT could be regarded as the most sensitive biological changes associated with heat stress.

In telomere length distribution, apparently long telomere decreased and telomere of medium size (4.4–9.4 kb)

increased at 42 °C. The growth rate seemed to be suppressed more in cells bearing longer telomere (>9.4 kb) than those bearing shorter telomere (\leq 9.4 kb) at 42 °C. The amount of the shortest range of telomere (<4.4 kb) showed no significant change. A cell population bearing short telomeres might be damaged and diminished at 42 °C, and so consequently not increased.

The mechanism of the heat-dependent growth suppression of cells bearing long telomeres needs to be further studied. Cells containing long telomeres might bear a protective mechanism to heat stress for cell survival, which accompanies growth suppression.

This growth suppression did not depend on the telomerase activity, the level of which was not altered at three temperatures. Of the telomerase components, TERC expression did not change, yet TERT expression was elevated under hyperthermia. The elevated TERT expression did not contribute to promote telomerase activity but might be associated with cell survival under heat stress. TERT expression has been reported to relate to cell survival independently of the TERC expression level.

In addition, TRF2 was also up-regulated at 42 °C. TRF2 is a ubiquitously expressed protein binding directly to the tandem array of duplex telomeric repeats and involved in telomere structure and chromosome end protection. TRF2 has been reported to prevent end-to-end chromosome fusions resulting in growth arrest or apoptosis [14].

TRF2 plays key roles in telomere maintenance [15, 16]. Moreover, a recent report suggests roles for TRF2 protein in DNA repair in addition to chromatin reorganization and telomere maintenance [17]. TRF2 has also been suggested to protect young neurons against death induced by DNA-damaging agents. [18]. Thus, TRF2 affects cell survival and differentiation by modulating DNA damage pathways and gene expression, and the elevated expression of TRF2 could be beneficial for cell survival.

Among the analyzed apoptotic signal-associated factors, the expression of p21 decreased and that of pp53 increased. Anti-apoptotic factor, Akt and p-Akt, tended to be down-regulated, but it was not significant. These results suggested that heat stress by 42 °C partially induces apoptosis pathway. It seems from our observation that the function of p53 at high temperature is controlled not by elevation in its expression but by activation with its phosphorylation at Ser15.

Cell cycle suppressing genes p53/p21 showed cross results in expression in cells cultured at 42 °C. The expression of p21 was suppressed in a temperature-dependent manner. On the other hand, p53 did not show a clear tendency with temperature, whereas phosphorylated p53 elevated prominently at 42 °C. Akt is an aging-promoting gene and control p53/p21 expression. However, no significant temperature-dependent alteration in Akt

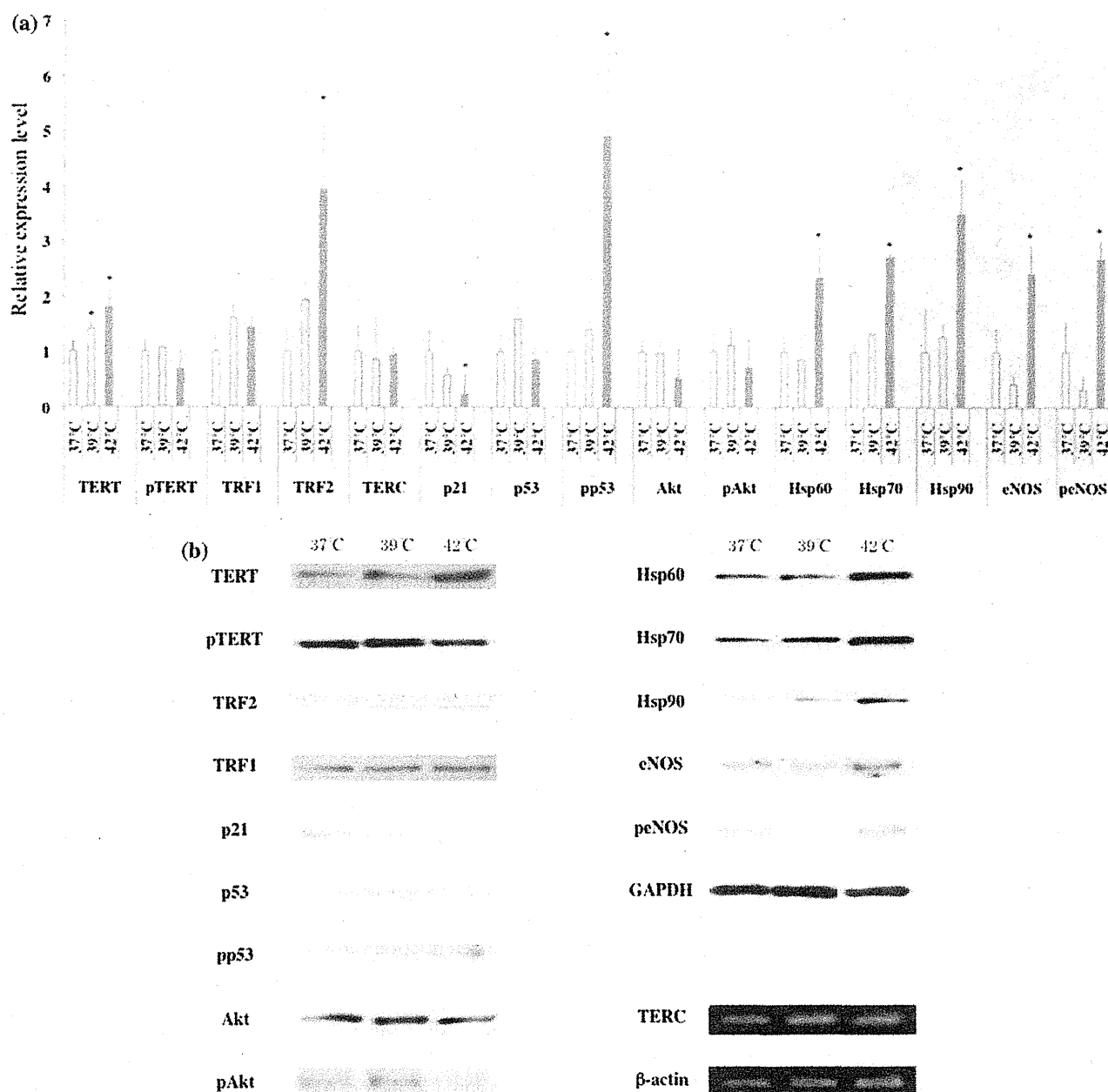


Fig. 4 The expression levels of telomere-associated proteins, TERC RNA, cell cycle-associated proteins, heat shock proteins, and NOS of HUVECs cultured on day 1 at different temperatures. The representative western blot results and a TERC RNA RT-PCR result at 37, 39 and 42 °C are shown in the *upper panel* (a). The *lower panel* (b) shows the relative expression levels determined by the proportion of

each band density to that of β -actin or GAPDH (set at 1 each), β -actin (RNA) for TERC and GAPDH (protein) for western results. 'p' at the beginning depicts phosphorylated form of each protein in cases of TERT, p53, Akt, and NOS. The horizontal bars represent the standard deviation. * $p < 0.05$ versus at 37 °C

expression but only mild and an insignificant tendency of lowered expression of Akt and phosphorylated Akt at 42 °C were observed. These results imply heterogeneity in the cell population in which the genes are expressed in different levels of expression among cells. Some cells bear low expression of Akt and they might be heat-resistant cells and others are not.

Phosphorylated p53 at Ser15 enhances NER/NHEJ repair, which causes anti-apoptotic effects on cancer cells [19].

p21 is one of the cyclin-dependent kinase inhibitors to govern the cell cycle checkpoint and to be essential for determining whether a cell enters into an arrested state [20]. Thus, lowered expression of p21 might contribute to partially prevent cell growth suppression.

Induction of apoptosis is a function of external stimuli and cellular gene expression. Many cells respond to DNA damage by the induction of apoptosis, which depends on a functional p53 protein and is signaled by elevation of p53 levels. A prior exposure to mild heat stress (42 °C) can induce anti-apoptotic effects for cells. The anti-apoptotic effect of thermal stress is mediated by increasing HSP70 and p53 [21]. p53 proteins are accumulated after heat stress in association with HSP72/HSC73 [22]. Whole-body thermal preconditioning has been shown to elevate carotid arterial Hsp70, p-Akt, and p-eNOS expression, resulting from the activation of phosphatidylinositol 3-kinase (PI3 K)/Akt-dependent heat shock protein (Hsp)/endothelial nitric oxide synthase (eNOS) pathways [23].

Several groups have shown that thermotolerant cells are less sensitive to cytotoxicity induced by hyperthermia, growth factor withdrawal, heavy metals, radiation, and anti-cancer drugs. Thermotolerance is associated with the synthesis and accumulation of a family of highly conserved proteins referred to as Hsps. The expression of Hsps in response to mild stress induces thermotolerance and protection from apoptosis [24].

Heat shock protein 90 (HSP90) is induced in response to cellular stress and stabilizes client proteins involved in cell cycle control and proliferative/anti-apoptotic signaling. Tanespimycin, an HSP90 inhibitor, reduces tumor cell survival in vitro. In multiple myeloma, HSP90 inhibition affects multiple client proteins that contribute to tumor cell survival, including elements of the PI3/Akt, STAT3, and MAPK signaling pathways. HSP90 inhibition also abrogates the protective effect of bone marrow stromal cells and inhibits angiogenesis and osteoclastogenesis [25].

A recent study has shown that the inhibition of endothelial senescence involves phosphorylation of Akt at Ser473 and increased expression eNOS [26].

In the present study, Hsp and eNOS were up-regulated in protein expression without significant increase of Akt expression at 42 °C. This implies that Hsp and eNOS might be regulated in expression by other pathway not involving Akt expression in HUVEC at high temperature condition.

In summary, high temperature induces cell senescence, growth suppression, lowered proportional amount of long telomere, up-regulation of TERT, TRF2, pp53, hsp's, and eNOS, suppression of p21, and no clear alterations of p53 and Akt in expression. These cross results may reflect that HUVEC population exposed to heat for one day contains at least two types of subpopulation, one of which is committed to enter cell senescence stage and the other of which gains heat resistance with expression of anti-apoptotic genes. One of hypothetical explanations for the heat-associated telomere length change and altered gene expression related to cell survival is that cell-protective genes such as TERT, TRF2, Hsp's, and eNOS are

commonly regulated in HUVECs at 42 °C regardless of their telomere size, and growth of HUVECs with longer telomere would be more suppressed. In addition, HUVECs with short telomere, i.e., old cells would not grow but undergo cell death. This hypothesis can explain the relative decrease of long telomere, the increase of middle-sized telomere, but no increase of short telomere. The cell-protective mechanism in heat condition seems to have higher effect on cells bearing longer telomere. Further study is necessary to elucidate mechanism of heat resistance of vascular endothelial cells.

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Conflict of interest The authors have no financial competing interest to declare in relation to this manuscript.

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Effects of Immersion in Artificial Carbon Dioxide on Endothelial Function Assessed with Flow-Mediated Dilatation in Patients with Type 2 Diabetes

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Abstract

Purpose: The aim of present study was to investigate the endothelial function of immersion of patients with diabetes in carbon dioxide (CO₂)-enriched water

Methods: Sixteen diabetic patients with minor complications were immersed in CO₂-enriched water for 4 weeks, and 8 patients were immersed in normal spa water for the same duration. To assess endothelial function, forearm flow-mediated dilatation (FMD) was measured in those patients, and %FMD at pre-immersion was compared to that at post-immersion in CO₂-enriched water. The pulse wave velocity (PWV) was also measured to determine whether vascular stiffness was affected in those patients. The percent coefficient of variation of R-R intervals was examined as CVR-R (%). All patients were medicated with antidiabetic drugs, which were not changed during the study.

Results: %FMD showed no significant difference in any patients between pre- and post-CO₂-enriched water bathing. However, %FMD was significantly increased in patients under 8.0% of HbA1c after CO₂-enriched water bathing ($p < 0.05$), but it was not significantly increased in patients over 8.0 of HbA1c. PWV and CVR-R (%) were significantly reduced in all patients after CO₂-enriched water bathing.

Conclusion: CO₂-enriched water immersion had a positive effect on endothelial function, and reduced arterial wall stiffness in patients with diabetes. These findings suggest that CO₂-enriched water bathing may improve microcirculation, as well as subjective symptoms, in patients with controlled diabetes.

Keywords: diabetes, flow-mediated dilatation, CO₂ water, endothelial function, PWV

I INTRODUCTION

Type 2 diabetes is associated with a high prevalence of atherosclerosis and with several-fold increases in cardiovascular events. This prevalence increases with male gender, aging, longer duration of diabetes, and the presence of additional cardiovascular risk factors, nephropathy, retinopathy, and peripheral or carotid occlusive arterial disease^{1), 2)}. Endothelial dysfunction is an early phenomenon during diabetic atherogenesis³⁾ and has been associated with a poor

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cardiovascular prognosis in the diabetic population^{4), 5)}. Therefore, it is now known that peripheral endothelial dysfunction is considered an integrator of cardiovascular risk. The association between endothelial and smooth muscle dysfunction was evaluated previously by flow-mediated dilation (FMD) in patients with type 2 diabetes^{4), 6)}. Some of those authors reported a higher prevalence of angiopathy in patients with abnormal FMD⁶⁾.

Immersion in water enriched with carbon dioxide (CO₂) reportedly had positive microcirculatory effects^{7), 8)} and increased blood flow to a much higher extent than did immersion in plain water. Toriyama et al.⁹⁾ demonstrated that CO₂ immersion improved the limb salvage rate in patients with critical limb ischemia without a revascularization option. CO₂ immersion was recently shown to induce local plasma vascular endothelial growth factor (VGEF) production, resulting in NO-dependent new capillary formation associated with mobilization of endothelial progenitor cells¹⁰⁾. These results suggest that CO₂ immersion could be effective in atherosclerosis as an adjunctive treatment in diabetic patients. The aims of the present study were to investigate type 2 diabetic patients with minor complications who underwent CO₂ immersion for 4 weeks, and to assess whether this therapy was associated with endothelial dysfunction as evaluated by FMD measurements.

II METHODS

Study populations

We conducted a prospective study in patients with type 2 diabetes who had not achieved their treatment goal with diet, exercise, and antidiabetic drugs (sulfonyl-urea, metformin, or DDP-4 inhibitors or insulin). We recruited 24 patients (men and women) ranging from 50 to 75 years of age (Table 1). All patients had had diabetes for more than 5 years and also had at least two cardiovascular risk factors. Diabetes mellitus was defined as fasting glucose >126 mg/dL or the use of hypoglycemic medications. Hypertension was defined as systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg, or the use of medication prescribed for hypertension. Body mass index was calculated as weight (kg)/height (m²). Total and high-density lipoprotein (HDL) cholesterol levels were measured from blood samples obtained after a 12-hour fast. Low-density lipoprotein (LDL) cholesterol was estimated by the Friedewald equation¹¹⁾. The exclusion criteria were as follows: treatment with type 1 diabetes, HbA1c ≥ 9.0%, systolic blood pressure ≥ 160 mmHg, and serum creatinine ≥ 1.5 mg/dL at baseline. Patients were also excluded from the study if they had clinically infected skin, severe heart failure, malnutrition, or a history of disease affecting the vascular system. Patients were excluded if they had ever enrolled in a clinical evaluation of another wound-care device or drug.

Study protocol

Sixteen enrolled patients bathed in 40°C water in a bathtub (200 L), which was solved with 50 g of CO₂ bathing additive (Kao, Tokyo, Japan). The concentration of CO₂ in the bathtub was 145 ± 11 ppm at 10 minutes after mixing. They soaked for 10 min at a time at least four times a week for 4 weeks. As control subjects, 10 diabetic patients bathed in water without the

Table 1 Characteristics in control and diabetic patients in present study

	Controls (n=8)	CO ₂ bath (n=16)
Age (y.o.)	61±3.6	64.7±4.8
Sex(M/F)	5/3	12/4
BMI	25.1±2.1	24.7±2.1
Hypertension	6	10
Dyslipidemia	4	6
Diabetes		
Duration (y)	6.4±0.50	8.6±0.81
Diabetes Complications		
CVD	2	3
Stroke	0	2
Nephropathy	1	3
Retinopathy	2	4
Neuropathy	1	1

DM; diabetes mellitus, BMI; body mass index, CVD; cardiovascular disease. Data are means ± SD or number patients.

addition of artificial CO₂. All patients were medicated with antidiabetic, antihypertensive, and other medications, which were not changed during the study. Before and after CO₂ bathing, each patient's, blood pressure, %FMD, brachia intima-media thickness (bIMT), pulse wave velocity (PWV), % coefficient of variation of R-R intervals (%CVR-R), serum lipid profiles, and HbA1c were measured. The study protocol was approved by the Ethics Committee of Kyushu University Graduate School of Medicine. Written informed consent was obtained from all patients before any study procedure was undertaken.

Flow-mediated dilation (FMD)

Endothelium-dependent dilation was assessed as a parameter of vasodilation according to the guidelines for ultrasound assessment of FMD of the brachial artery in the fasting state⁶¹. Using a 10-MHz linear-array transducer probe (Unex, Nagoya, Japan), longitudinal images of the brachial artery at baseline were recorded with a stereotactic arm, and the artery diameter was measured after supine rest for ≥5 min, as previously described by us¹². Artery diameter was measured from clear anterior (media-adventitia) and posterior (intima-media) interfaces, which were manually determined. Then, suprasystolic compression (50 mmHg higher than systolic blood pressure) was performed at the right forearm for 5 min, and the artery diameter was measured continuously from 30 sec before to ≥2 min after cuff release. Maximum vasodilation was then evaluated from the change in artery diameter after the release of occlusion (%FMD). FMD is known to be affected by a wide range of biological, environmental, and methodological factors¹³. Simultaneously, intima-media thickness (IMT) was defined as the distance from the leading edge of the first acrogenic line to the leading edge of the second echogenic line on the scans¹⁴. The highest value among three averaged IMTs was used as a representative value for each individual. To quantify inter- and intra-observer reproducibility, baseline brachial diameter

and FMD were measured by three individuals. Inter- and intra-observer coefficients were high ($r > 0.90$).

Measurement of aortic PWV

baPWV was measured using a volume-plethynographic apparatus (form PWV/ABI version-112; Colin, Komaki, Japan). This instrument records PWV, ankle brachial index (ABI), blood pressure, electrocardiogram, and heart sounds simultaneously. Details of the method, the validity, and the reproducibility of this approach were described previously¹⁵. The interobserver and intraobserver variation coefficients were 8.4 and 10.0%, respectively.

Statistical analysis

The results are expressed as mean \pm SD. Differences in efficacy measures between baseline and 4 weeks after CO₂ bathing were compared using the paired Student's t-test. Values of $P < 0.05$ were considered statistically significant.

III RESULTS

Characteristics of the enrolled patients are summarized in Table 1. No significant differences were seen in age, BMI, or duration of diabetes in all patients, although complications for diabetes in both groups were not different. Table 2 shows the data for diabetic patients before and after CO₂ bathing. Blood pressure, %FMD, b-IMT, PWV, CVR-R (%), and HbA1c were not significantly different between control and pre-CO₂ bathing subjects. Compared to pre-CO₂ bathing, at post-CO₂ bathing, both sides of PWV were significantly decreased, and CVR-R (%) was increased. We did not find any difference in %FMD between pre- and post-CO₂ bathing in

Table 2 Comparison of physical and biochemical parameters between before and after CO₂ bathing

	Controls (n=8)	CO ₂ bathing (n=16)	
		Pre-CO ₂	post-CO ₂
s-BP(mmHg)	141 \pm 14	134 \pm 11	134 \pm 14
d-BP(mmHg)	76 \pm 5.8	80 \pm 5.1	82 \pm 6.2
%FMD	5.4 \pm .51	5.9 \pm 0.53	5.2 \pm 0.48
b-IMT(mm)	0.31 \pm 0.03	0.32 \pm 0.03	0.30 \pm 0.02
ABI(Rt)	1.16 \pm 0.06	1.23 \pm 0.08	1.17 \pm 0.06
ABI(Lt)	1.12 \pm 0.04	1.25 \pm 0.06	1.16 \pm 0.85
rt-PWV(m/sec)	1792 \pm 128	1840 \pm 125	1749 \pm 143 [†]
lt-PWV(m/sec)	1821 \pm 170	1879 \pm 132	1726 \pm 122 [†]
CVR-R(%)	2.33 \pm 0.17	2.68 \pm 0.12	3.65 \pm 0.25 ^{*†}
BS(2hr;mg/dl)	148 \pm 14	162 \pm 12	168 \pm 18
HbA1c(%)	7.42 \pm 0.51	7.71 \pm 0.41	7.74 \pm 0.38

S-BP; systolic blood pressure, d-BP; diastolic pressure, % FMD: %flow mediated dilation, b-IMT; brachial intima-media thickness, ABI; ankle brachial index, PWV; pulse wave velocity, CVR-R (%): % coefficient of variation of R-R intervals, BS; blood sugar level. Data are means \pm SD. *: $p < 0.05$ vs control subjects; † : $p < 0.05$ vs values at before.

those patients (Figure 1). From these data on %FMD, we furthermore separated subjects into those with under vs over 8.0% in HbA1c. Figure 2 shows that %FMD was significantly increased in patients under 8.0 in HbA1 (p<0.05), but was not significantly increased in patients over 8.0% in HbA1 (p<0.05).

At the end of the study, we asked the subjects to complete several questionnaires regarding their subjective feelings about whether CO₂-enriched bathing was beneficial, neutral, or aggravating. Figure 3 summarizes the results: 14 patients felt the CO₂ bath kept them warm, 13 patients sweated during the bath, 12 experienced reduced fatigability, 10 patients experienced release from lower back pain or muscle pain, and 9 patients reported that they had a good night's sleep after bathing.

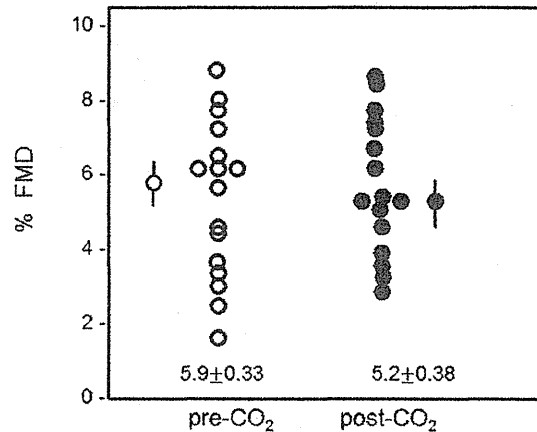


Fig. 1 Results of %FMD are shown in control diabetic patients (n=8) and in patients (n=16) before and after carbon dioxide bathing. Average value indicates the mean ± SD.

IV DISCUSSION

The present study demonstrates that CO₂-enriched bathing enhances %FMD in

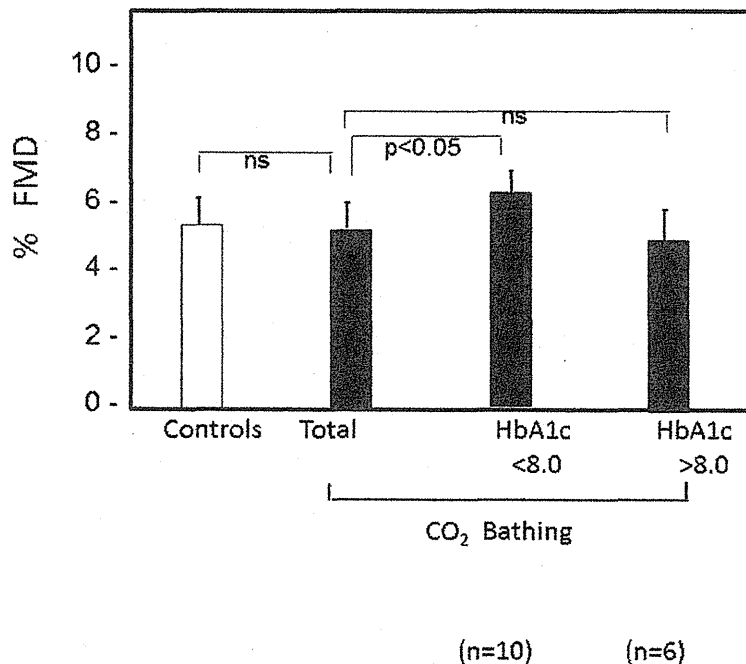


Fig. 2 Results of %FMD in control diabetic patients and in patients before and after (n=6) carbon dioxide bathing. All patients with carbon dioxide bathing were divided into two groups: those with HbA1 levels under (n=10) or over 8.0% (n=6). Open and closed bars indicate values before and after carbon dioxide bathing, respectively. Each value indicates the mean ± SD.

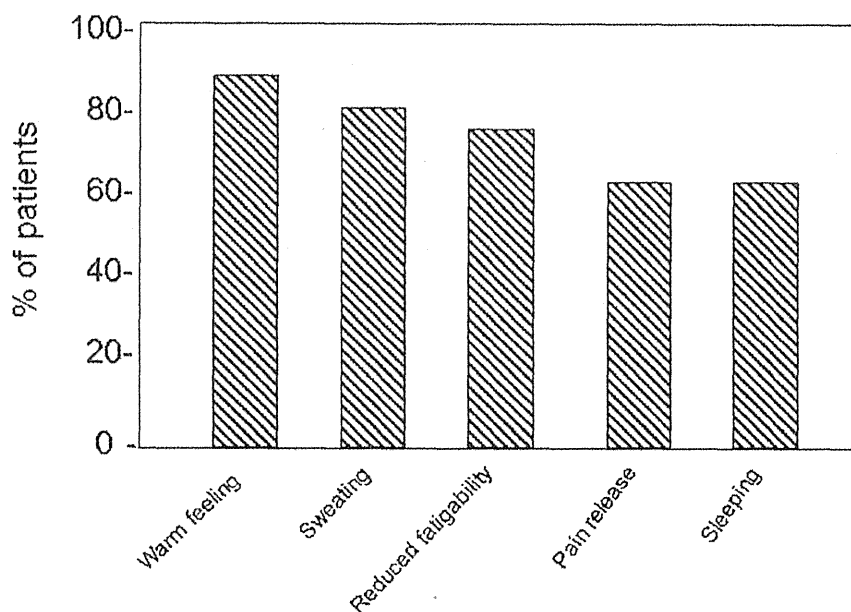


Fig. 3 Percent number of patients who answered a questionnaire about their subjective feelings after carbon dioxide bathing. Each patient answered three questions about improvement, no change, or worsening of symptoms. Data show the number of patients who reported only improvements.

diabetic patients under 8.0% in HbA1c. The results indicate the improvement of endothelial function after CO₂ bathing for one month. In fact, the benefits of CO₂ bathing have been described for the past 50 years, and this therapy is now thought to be effective for the treatment of peripheral vascular disorder¹⁶⁾. However, the mechanism or mechanisms underlying this traditional therapy remain poorly defined. The effect of CO₂-enriched water on cutaneous circulation depends primarily on the vasodilation elicited by the CO₂ that diffuses into the subcutaneous tissue through the skin layers¹⁷⁾. Our results showed that CO₂ bathing significantly reduced arterial stiffness in the peripheral arteries of diabetic patients. This reduction may have contributed to the induction of local VEGF synthesis associated with activation of the NO-cGMP pathway¹⁰⁾, resulting in increased peripheral blood flow. We have also shown that CVR-R (%), a marker of parasympathetic nerve activity, was increased in diabetic patients who were treated with CO₂ bathing. These observations therefore suggest that CO₂ bathing induces peripheral vasodilation and may increase parasympathetic nerve activity or may decrease sympathetic nerve activity as previously described by Toriyama et al.⁹⁾.

Each group in this study constituted a small number of patients who had no serious complications and various levels of HbA1c in diabetes, as well as control patients (Table 1). Our data indicated no difference in %FMD between pre- and post-CO₂ bathing, although CO₂ bathing did reduce arterial stiffness in patients with diabetes. However, CO₂ bathing was beneficial for patients under 8.0 in HbA1c because of the resulting increase in %FMD. On the other hand, this treatment was not beneficial for patients over 8.0 in HbA1c (Table 2). Although the

mechanisms underlying the effects of CO₂ water bathing remain unknown, there is evidence of a link between ROS damage¹⁸⁾ and the development of microvascular or macrovascular complications in diabetes^{19), 20)}. It is also possible that levels of ROS products, including serum concentrations of advanced oxidation protein products, advanced glycation end products, and lipoprotein lipase, are higher in patients over 8.0 in HbA1c than in those under 8.0%²¹⁾. Those producing factors may induce endothelial dysfunction in patients with high levels of HbA1c.

FMD studies have been reported to predict cardiovascular events²²⁾. Diameter changes after an increase in flow depend on the endothelium, mainly through a nitric oxide-dependent mechanism but also through vascular smooth muscle cell contraction and relaxation. Endothelial dysfunction may be considered a cardiovascular risk factor or at least a cardiovascular risk marker^{6), 23)}, but impairment of vascular smooth muscle cell function has also been reported in diabetic patients²⁴⁾ and may be involved in altered FMD. The mechanism underlying impairment of FMD cannot be explained by our results, since we did not test vascular smooth muscle cell function specifically. The important fact is that an impaired FMD response per se, whatever its mechanism, predicted a poor cardiovascular prognosis in several large population studies²⁵⁾. The present study was short-term and consisted of small numbers of subjects in both groups. Our findings therefore need to be confirmed in a large cohort of patients with type 2 diabetes and with a long observation period.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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