

Original Article

Enhancement of reactive oxygen species and induction of apoptosis in streptozotocin-induced diabetic rats under hyperbaric oxygen exposure

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Received January 31, 2011; accepted February 17, 2011; Epub February 20, 2011; published March 31, 2011

Abstract: An important source of reactive oxygen species (ROS) production is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which on activation induces superoxide production via oxidation in the mitochondria, inflammation and stress; such ROS are implicated in the pathogenesis of diabetic complications, including neuropathy. Hyperbaric oxygen (HBO) treatments are applied various diseases including diabetic patients with unhealing foot ulcers, however, and also increases the formation of ROS. In a previous study, we showed that a clinically recommended HBO treatment significantly enhanced oxidative stress of pancreatic tissue in the diabetic rats. However, no study has been undertaken with regard to the effects of HBO on the activity and gene expression of the NADPH oxidase complex and on apoptosis in the pancreas of diabetic animals. The purpose of this study was to investigate the effect of HBO exposure on gene expression of the NADPH complex, and pancreatic expression of genes related to apoptosis via the mitochondria, using the NADPH oxidase inhibitor apocynin. The mRNA expression of genes related to NADPH oxidase complex and apoptosis increased significantly ($P < 0.05$) in the pancreas of diabetic rats under HBO exposure. Similarly, activities of NADPH oxidase and caspase-3 changed in parallel with mRNA levels. These results suggest that oxidative stress caused by HBO exposure in diabetic animals induces further ROS production and apoptosis, potentially through the up-regulation of NADPH oxidase complex. Thus, this study can contribute to development of a better understanding of the molecular mechanisms of apoptosis via the mitochondria in diabetes, under HBO exposure.

Keywords: Diabetes mellitus, hyperbaric oxygen, reactive oxygen species, nicotinamide adenine dinucleotide phosphate, apoptosis, apocynin

Introduction

Reactive oxygen species (ROS), which cause cellular damage via oxidation, have been implicated in the pathogenesis of diabetes mellitus [1, 2]. Persistent hyperglycemia in diabetes induces ROS production by glucose autooxidation [3, 4], activation of protein kinase C (PKC), and increased flux through the hexosamine pathway [1]. An important source of ROS production is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which has been implicated in the pathogenesis of diabetic complications, including neuropathy [5, 6].

Hyperbaric oxygen (HBO) is applied in treatment of various diseases including diabetic patients with non-healing foot ulcers [7]; however, this

treatment also increases the formation of ROS, which are known to result in cellular damage through the oxidation of lipid, protein, and DNA [8]. The oxidative effects of HBO have been investigated in animals and humans [9-11]; however, the side effects of HBO treatment in diabetic patients and animals have been little investigated. In a previous study, we showed that a clinically recommended HBO treatment significantly decreased both mRNA expression and activities of antioxidant enzymes in the streptozotocin (STZ)-induced diabetic rat, suggesting that HBO causes oxidative stress [12].

STZ-induced β -cell death in the pancreas is associated with oxidative stress caused by the production of excess intracellular ROS [13, 14]; furthermore, STZ may damage pancreatic tissue

via imposition of oxidative as well as nitrosative stresses, which in turn can induce apoptosis in pancreatic cells [15]. In addition, the oxidative stress caused by HBO exposure induces apoptosis via the mitochondrial pathway [16]. However, to our knowledge, no study has been undertaken on the effects of HBO on the activity and gene expression of the NADPH oxidase complex in the pancreas of diabetic animals. In the present study, to evaluate the effects of HBO in the pancreas of STZ-induced diabetic rats, we examined the activity and gene expression of the NADPH complex; we also examined pancreatic expression of genes related to apoptosis via the mitochondria, and evaluated the effect of apocynin, an NADPH oxidase inhibitor [17-19].

Materials and methods

Animals and experimental design

Male Wistar rats (weight range, 250–270 g) were purchased from Japan SLC Inc (Shizuoka, Japan) at 7 weeks of age. They were housed at 23–25 °C with light from 7:00 AM to 7:00 PM and free access to water at all times. All rats were fed a commercial diet during the experiment. All study procedures were implemented in accordance with the Institutional Guidelines for Animal Experiments at the College of Biore-source Sciences, Nihon University under the permission of the Committee for Experimental Animals in our College. Rats were allowed to acclimatize for 1 week prior to treatment, and at the start of the experiment they were randomly divided into four groups, 6 rats per group. The groups were as follows: non-diabetic induction and non-HBO group (Control), non-diabetic induction and HBO group (HBO), diabetic induction and non-HBO group (DM), and diabetic induction and HBO group (DM + HBO). In this study, diabetes developed on the third day after diabetic induction, and rats in the DM + HBO group were then exposed to HBO once daily for 7 days. Rats in the HBO group were also exposed to HBO once daily for 7 days. Similarly, rats in the four groups were injected with apocynin daily for 7 days, and these groups were designated “treated with apocynin”. The mean body weight of animals in all groups was measured at the start and end of the study period.

Diabetes induction and apocynin treatment

In four groups (DM and DM + HBO groups

treated or untreated with apocynin), diabetes was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ: 40 mg/kg body weight) dissolved in 0.05 M citrate buffer (pH 4.5), as described previously [12]. Control and HBO groups treated or untreated with apocynin were treated with an equal volume and concentration of STZ injection vehicle, citrate buffer. Diabetes was confirmed in the STZ-treated rats by measuring the fasting plasma glucose concentration 48 h post-injection. After an overnight fast, blood was obtained from the tail vein, centrifuged at 3,000 × g for 5 min, and the plasma collected. Fasting plasma glucose and insulin concentrations were measured every day using commercially available enzyme-linked colorimetric diagnostic kits (DRI-CHEM4000, FUJIFILM, Tokyo, Japan) and Rat Insulin ELISA KIT; AKRIN-130 (Shibayagi, Gunma, Japan), respectively. Diabetes was considered to have been induced when the blood glucose level reached at least 14 mmol/l [20]. Two days after the induction of diabetes, rats injected with citrate buffer were orally administered saline (Control and HBO groups) or 15 mg/kg per day apocynin [21]; rats injected with STZ were orally administered saline (DM and DM + HBO groups) or 15 mg/kg per day apocynin. The treatment period was 1 week.

HBO exposure

For all experiments, HBO was applied at a clinically used pressure of 2.4 ATA for 90 min, once daily for 7 days, in a hyperbaric chamber for small animals (Nakamura Tekkosho, Tokyo, Japan). The ventilation rate was 4–5 L/min. Each exposure was started at the same hour in the morning (10 AM) to exclude any confounding issues associated with changes in biological rhythm.

Tissue preparation and blood sampling

After the final HBO exposure, rats were fasted overnight before blood sampling was performed. Blood samples were collected from the inferior vena cava just before rats were sacrificed. Blood samples were centrifuged at 3000 rpm for 5 min, and the plasma was then collected and stored at -80 °C until analysis. Animals in all groups were sacrificed the following day under ether anesthesia, and pancreatic samples were then collected in liquid nitrogen for analysis of antioxidant enzymes, lipid peroxidation, and NADP⁺/NADPH concentrations, and

stored at -80 °C until analysis. Additionally, pancreatic samples were collected in RNAlater solution (Qiagen, Hilden, Germany) for molecular biologic studies, and stored at -80 °C until analysis.

Biochemical analysis

Fasting plasma glucose was measured using commercially available enzyme-linked colorimetric diagnostic kits (DRI-CHEM4000, Fujifilm, Tokyo, Japan), and the plasma free fatty acid (FFA) concentration was measured by an enzyme method using a JCA-BM2250 (JEOL Ltd., Tokyo, Japan). Fasting plasma insulin concentrations were determined using a rat insulin ELISA kit (AKRIN-010H, Shibayagi, Gunma, Japan).

Analysis of antioxidant defense enzymes, lipid peroxidation, and NADP⁺/NADPH concentrations

Pancreatic homogenates were prepared as a 1:10 (w:v) dilution in 10 mM potassium phosphate buffer, pH 7.4, using an Ultra-Turrax® homogenizer (IKA® Japan, Nara, Japan). Samples were centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatants were collected and immediately assayed for enzyme activities. For total glutathione (GSH), ~50 µg of liver was homogenized in 5% trichloroacetic acid at a ratio of 1:10 (w:v) and centrifuged for 5 min at 8000 rpm and 4 °C. Total GSH was measured in the tissues as previously described [22]. Total superoxide dismutase (SOD), catalase, and total glutathione peroxidase (Gpx) activities were measured according to Sun *et al.* [23], Aebi [24], and Paglia and Valentine [25], respectively. Lipid peroxidation levels were measured by the thiobarbituric acid (TBA) reaction using the method of Ohkawa *et al.* [26]. Quantitation of NADP⁺/NADPH concentrations in pancreatic samples was undertaken using the Enzy-Chrom™ NADP⁺/NADPH assay kit (BioAssay Systems, CA, U.S.A). This kit is based on a glucose dehydrogenase cycling reaction, in which a tetrazolium dye (MTT) is reduced by NADPH in the presence of phenazine methosulfate. The intensity of the reduced product color is proportionate to the NADP⁺/NADPH concentration in the sample as described previously [27].

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

An in situ cell death detection kit (Roche) was

used to visualize apoptotic pancreatic cells. After being dewaxed in xylene and absolute ethanol, slides were rehydrated with decreasing concentrations of ethanol and rinsed in PBS buffer. Rehydrated sections were incubated at room temperature for 30 min with proteinase K (20 µg/ml in 10 mM Tris-HCl, pH 8). After rinsing with PBS, sections were permeabilized with 0.1% Triton X-100 in PBS for 2 min at 4 °C. Positive control sections were treated for 10 min at 37 °C with DNase. Slides were rinsed with PBS and incubated for 1 h at 37 °C in the TUNEL reaction mixture (terminal deoxynucleotidyl transferase enzyme (TdT) with nucleotide-fluorescein-conjugated mixture in reaction buffer). In negative controls, TdT enzyme was omitted from the reaction mixture. The slides were rinsed in 0.01 M PBS buffer, pH 7.4, and mounted in fluorescent mounting medium (Dako, Carpinteria, CA, USA).

Apoptotic nuclei analysis

The counts of apoptotic nuclei in each group of rats were compared using images obtained with the Image J software version 1.8 systems (Wayne Rasband National Institutes of Health, USA). One hundred islets of Langerhans were chosen at random from each group of individual rats. The percentage of apoptotic nuclei was calculated for each group.

RNA extraction and Quantitative Real-time PCR

RNA was isolated by homogenization with a Micro Smash-100R™ (Tomy Seiko, Tokyo, Japan) using Isogen (Nippon Gene, Tokyo, Japan) for pancreatic tissue. RNA was purified using RNeasy Mini kits (Qiagen, Hilden, Germany) for all tissues studied. All samples were treated with DNase I (RNase Free DNase set, Qiagen). The concentrations of total RNA were measured by absorbance at 260 nm using a NanoDrop® ND-1000 (NanoDrop, USA). Purity was estimated on the basis of the 260/280 nm absorbance ratio. Total RNA (1 µg) was subjected to reverse transcription using oligo(dT)₁₂₋₁₈ primer and M-MuLV reverse transcriptase (SuperScript™ III First-Strand Synthesis, Invitrogen Life Science, USA) according to the manufacturer's instructions. The transcript levels of specific primers (**Table 1**) were quantified by real-time PCR (7500 Real Time PCR System, Applied Biosystems, CA, USA). The cDNA was amplified under the following conditions: 95 °C for 10 min, followed by 45 cycles

Table 1. Primers sequences used for real-time PCR reactions

Gene		5' - 3'
gp91 ^{phox}	Forward	CGGAATCTCCTCTCCTTCCT
	Reverse	GCATTACACACCACTCCAC
p22 ^{phox}	Forward	TGTTGCAGGAGTGCTCATCTGTCT
	Reverse	AGGACAGCCCGGACGTAGTAATTT
p47 ^{phox}	Forward	AGGTTGGGTCCCTGCATCCTATTT
	Reverse	TGGTTACATACGGTTCACCTGCGT
Bcl-2	Forward	GGGAGCGTCAACAGGGAGA
	Reverse	CAGCCAGGAGAAATCAAACAGA
Bax	Forward	CCAAGAAGCTGAGCGAGTGT
	Reverse	GCAAAGTAGAAGAGGGCAACC
Caspase-3	Forward	CAGAGCTGGACTGCGGTATTGA
	Reverse	AGCATGGCGCAAAGTGACTG
18s rRNA	Forward	GTAACCCGTTGAACCCATT
	Reverse	CCATCCAATCGGTAGTAGCG

of 15 s at 95°C and 1 min at 59°C, using Power SYBR® Green PCR Master Mix (Applied Biosystems) with each primer at a concentration of 400 nM/L. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, which was displayed as a single peak (data not shown). All samples were analyzed in triplicate. The relative expression ratio (*R*) of a target gene was expressed for the sample versus the control in comparison to 18S rRNA [28]. *R* was calculated based on the following equation [29]: $R = 2^{-\Delta\Delta C_t}$, where C_t represents the cycle at which the fluorescence signal was significantly from background and $\Delta\Delta C_t$ was $(C_{t, \text{target}} - C_{t, \text{18s rRNA}})_{\text{treatment}} - (C_{t, \text{target}} - C_{t, \text{18s rRNA}})_{\text{control}}$.

Determination of caspase-3 activity

Caspase-3 activity was determined using the Caspase-3/CPP32 Fluorometric Assay Kit (Biovision, Inc., Mountain View, Calif., USA). For each assay, 50 µg of pancreatic tissue was used. Samples were read in a microplate spectrofluorometer (Gemini EM, Molecular Devices, CA, and USA) with a 400-nm excitation and a 505-nm emission filter. Fold increase in caspase-3 activity was determined by comparison with fluorescence of 7-amino-4-trifluoromethyl coumarin in controls and with data reported from other groups [30].

Data analysis

Results are expressed as mean ± standard error

of the mean (SEM). Statistical significance was determined by unpaired *t*-test with a *P* value of < 0.05.

Results

The baseline body weight of rats at the beginning of the study was similar in all groups. At the end of the treatment, there was no difference in body weight between control and HBO rats treated or untreated with apocynin (mean group weights ranged from 253 to 282 g). However diabetic rats presented with weight loss, and the body weight of DM + HBO rats (238 ± 8 g) in particular, decreased significantly, when compared with the rats in the other 3 groups (control, 275 ± 7 g; HBO, 260 ± 7 g; DM, 251 ± 5 g). In addition, there was no difference in body weight between DM and DM + HBO rats treated or untreated with apocynin (data not shown).

Biochemical findings

Hyperglycemia occurred within 3 days after STZ administration. Fasting plasma glucose concentrations in the DM + HBO group were significantly higher than those in the DM group (**Table 2**). Moreover, the fasting plasma insulin concentrations of the DM + HBO group were significantly decreased compared with the DM group (**Table 2**). The fasting plasma glucose and insulin concentrations in the DM and DM + HBO groups, and the fasting plasma glucose concentrations in the DM and DM + HBO groups treated with apocynin were decreased com-

Table 2. Plasma glucose and insulin concentrations in experimental groups treated with or without apocynin.

Group	Apocynin (-/+)	Plasma glucose (mmol/l)	Plasma insulin (pmol/l)
Control	(-)	5.5 ± 0.2	126 ± 9
	(+)	5.3 ± 0.6	131 ± 6
HBO	(-)	5.7 ± 0.4	122 ± 7
	(+)	5.4 ± 1	121 ± 7
DM	(-)	23.8 ± 2.5	57 ± 11
	(+)	22.2 ± 3.1 †	84 ± 7 †
DM + HBO	(-)	26.5 ± 4 *	26 ± 5 *
	(+)	23.9 ± 2.5 †	49 ± 6 †

Values are expressed as mean ± SEM (*n* = 6).

Non-diabetic rats in the non-HBO (control), non-diabetic rats in the HBO (HBO), diabetic rats in the non-HBO (DM), and diabetic rats in the HBO (DM + HBO) groups.

* Represents significance at *P* < 0.05 compared with the DM group untreated with apocynin.

† Represents significance at *P* < 0.05 compared with the same group treated with or without apocynin.

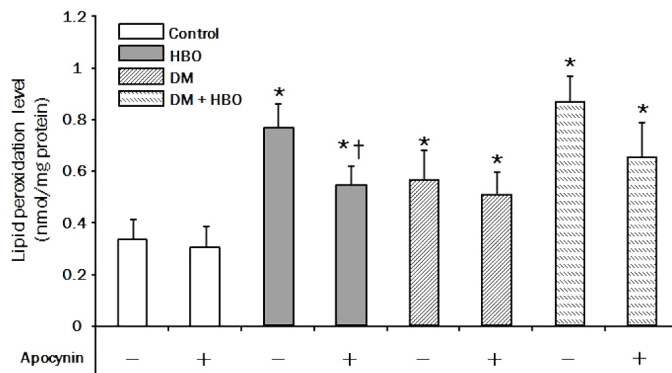


Figure 1. Comparison of lipid peroxidation levels (TBARS) in the pancreas of non-diabetic rats in the non-HBO (control), non-diabetic rats in the HBO (HBO), diabetic rats in the non-HBO (DM), and diabetic rats in the HBO (DM + HBO) groups. TBARS values are indicated as nmol/mg protein in pancreatic tissue. Values are expressed as mean ± SEM (*n* = 6). * Represents significance at *P* < 0.05 compared with the control group untreated with apocynin. † Represents significance at *P* < 0.05 compared with the same group treated with or without apocynin.

pared with the corresponding groups untreated with apocynin (Table 2); in addition, the insulin concentrations in the DM and DM + HBO groups treated with apocynin were increased (Table 2). The fasting FFA concentrations in the DM and DM + HBO groups were significantly higher than in the control and HBO groups (Table 2). Similarly, no significant differences were observed between the DM and DM + HBO groups treated with or without apocynin.

Antioxidant defense activities

TBARS levels in the pancreatic tissues examined are presented in Figure 1. TBARS levels in the HBO, DM, and DM + HBO groups treated with or without apocynin were significantly increased compared with those of the control group. In addition, TBARS levels were higher in the HBO and DM + HBO groups than in the DM

group (Figure 1). In addition, TBARS levels in the HBO, DM, and DM + HBO groups treated with apocynin were decreased compared with those groups untreated with apocynin (Figure 1). The GSH, total SOD and CAT activities in the pancreatic tissues examined in the HBO, DM, and DM + HBO groups treated with or without apocynin were significantly decreased compared with those of the control group, whereas the Gpx activities in the DM + HBO groups treated with or without apocynin were increased compared with those of the control group (Table 3). Moreover, the GSH, total SOD and CAT activities in the DM + HBO group treated with apocynin were increased compared with those of the DM + HBO group untreated with apocynin (Table 3).

NADP⁺ and NADPH concentrations

NADP (including NADP⁺ and NADPH) is likely to

Table 3. Antioxidant enzyme activities in experimental groups treated with or without apocynin.

Group	Apocynin (-/+)	GSH	Cu-Zn SOD	Catalase	Gpx
Control	(-)	76 ± 8	13.8 ± 2	282 ± 18	0.6 ± 0.15
	(+)	72 ± 5	13.9 ± 3	290 ± 16	0.5 ± 0.07
HBO	(-)	55 ± 7 *	6.3 ± 0.9 *	166 ± 9 *	0.7 ± 0.1
	(+)	58 ± 6 *	11.5 ± 2.5 †	205 ± 22 *†	0.6 ± 0.14
DM	(-)	67 ± 4 *	8.2 ± 1.2 *	233 ± 26 *	0.6 ± 0.14
	(+)	70 ± 3 *	10.7 ± 2.9	266 ± 37	0.5 ± 0.1
DM + HBO	(-)	49 ± 5 *	5.8 ± 0.9 *	94 ± 17 *	0.9 ± 0.12 *
	(+)	60 ± 3 *†	7.4 ± 0.7 *†	134 ± 36 *†	0.8 ± 0.1 *

Values are expressed as mean ± SEM ($n = 6$). Non-diabetic rats in the non-HBO (control), non-diabetic rats in the HBO (HBO), diabetic rats in the non-HBO (DM), and diabetic rats in the HBO (DM + HBO) groups. Levels of glutathione (GSH) were measured and expressed as nmol/mg protein. The activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx) are expressed as U/mg of protein. * Represents significance at $P < 0.05$ compared with the control group untreated with apocynin. † Represents significance at $P < 0.05$ compared with the same group treated with or without apocynin.

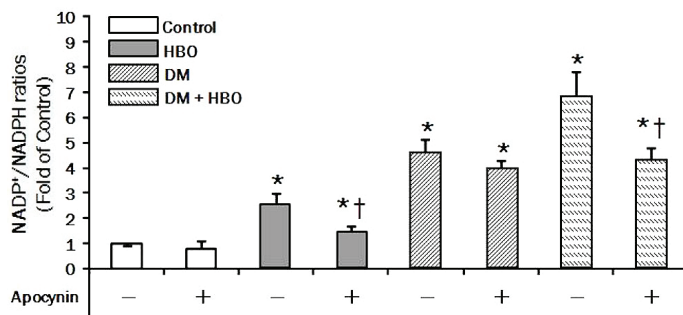


Figure 2. NADPH oxidase activity (NADP⁺/NADPH ratios) in the pancreas of non-diabetic rats in the non-HBO (control), non-diabetic rats in the HBO (HBO), diabetic rats in the non-HBO (DM), and diabetic rats in the HBO (DM + HBO) groups. NADPH oxidase activity is indicated as fold over control in pancreatic tissue. Values are expressed as mean ± SEM ($n = 6$). * Represents significance at $P < 0.05$ compared with the control group untreated with apocynin. † Represents significance at $P < 0.05$ compared with the same group treated with or without apocynin.

belong to the class of fundamental common mediators of diverse biological processes, including energy metabolism, mitochondrial functions, prevention/generation of oxidative stress, and cell death. In particular, NADP modulates multiple key factors in cell death, such as apoptosis-inducing factor [27] (Vilcheze et al., 2005). In the present study, we determined the levels of NADP⁺ and NADPH in pancreas of all groups, then calculated the NADP⁺/NADPH ratio. The NADP⁺/NADPH ratio was significantly higher in the HBO, DM, and DM + HBO groups treated

with or without apocynin than in the control group (Figure 2). The NADP⁺/NADPH ratios in the HBO and DM + HBO groups treated with apocynin were decreased compared with the corresponding groups untreated with apocynin (Figure 2).

Immunodetection of apoptotic nuclei in pancreatic tissue

In the present study, apoptosis of islets of Langerhans in pancreas was detected by the TUNEL method and the nuclei were counted and indicated as a percentage of total nuclei (see Materials and Methods). As shown in Figure 3a and 3b, STZ treatment induced a higher percentage of apoptosis of islets of Langerhans in the DM and DM + HBO groups treated without apocynin (DM; 33.5 ± 6.5%, DM + HBO; 55.3 ± 9.2%). The percentage of apoptosis in the DM and DM + HBO groups treated with apocynin decreased compared with the corresponding groups untreated with apocynin (Figure 3b).

Expression of genes of NADPH oxidase complex (gp91^{phox}, p22^{phox}, p47^{phox})

Gene expression of NADPH complex components studied is summarized in Figure 4. We measured the expression of NADPH oxidase complex, gp91^{phox}, p22^{phox}, and p47^{phox}. In the

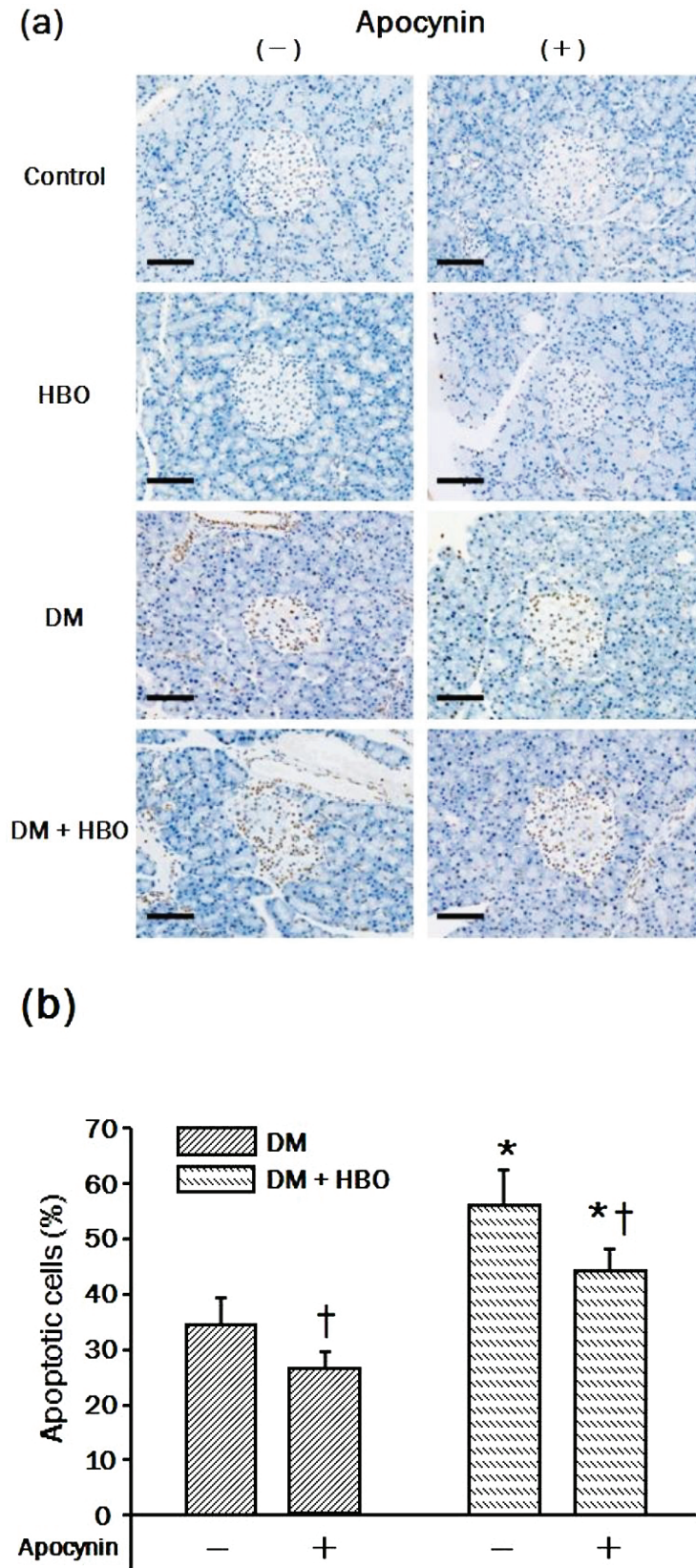


Figure 3. Apoptotic cells detected by Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique (see Materials and Methods) in slides prepared from pancreatic tissue in the non-HBO (control), non-diabetic rats in the HBO (HBO), diabetic rats in the non-HBO (DM), and diabetic rats in the HBO (DM + HBO) groups. Apoptotic nuclei in islets of Langerhans from the control, HBO, DM, and DM + HBO groups treated with or without apocynin (a). Scale Bar = 50 μ m. Apoptotic index in pancreatic islets in the DM and DM + HBO groups treated with or without apocynin. Positive nuclei were counted in 100 islets per group and expressed as percentage of apoptotic nuclei with respect to the total nuclei in pancreatic islets (b). * Represents significance at $P < 0.05$ compared with the DM group untreated with apocynin (vs. DM + HBO group treated with or without apocynin). † Represents significance at $P < 0.05$ compared with the same group treated with or without apocynin.

DM and DM + HBO groups untreated with apocynin, gp91^{phox} (also called Nox2) and p22^{phox} were significantly up-regulated (gp91^{phox}; 2.6-, and 2.4-fold, p22^{phox}; 2.5-, and 2.7-fold, respectively) compared with the control group (Figure 4a). In addition, there was no difference in the expression of gp91^{phox} and p22^{phox} between DM and DM + HBO rats treated with or without apocynin (data not shown). The expression of p47^{phox} in the HBO, DM, and DM + HBO groups untreated with apocynin was significantly up-regulated (3.3-, 2.0-, and 5.2-fold, respectively) compared with the control group (Figure 4a). Moreover, the expression of p47^{phox} in the HBO and DM + HBO groups treated with apocynin was down-regulated compared with the corresponding groups untreated with apocynin (Figure 4a).

Expression of genes of apoptosis (Bcl-2, Bax, and Caspase-3)

As shown in Figure 4b, the expression of Bcl-2 in the HBO, DM, and

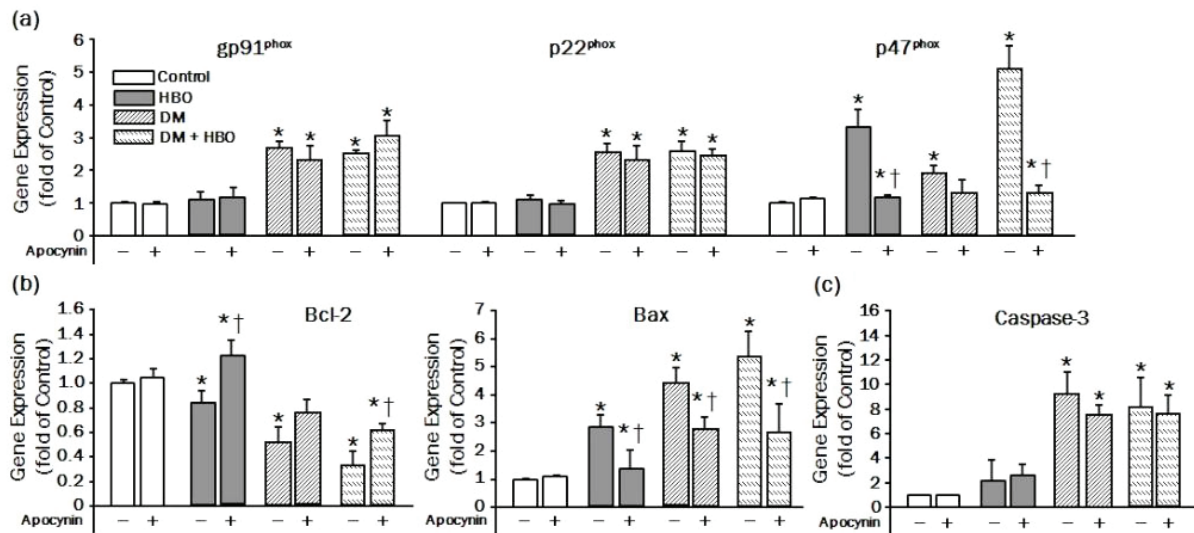


Figure 4. Real-time PCR analysis of NADPH oxidase complex (a), apoptosis (b), and caspase-3 gene expression in the pancreas of non-diabetic rats in the non-HBO (control), non-diabetic rats in the HBO (HBO), diabetic rats in the non-HBO (DM), and diabetic rats in the HBO (DM + HBO) groups. Values are expressed as mean $\pm$ SEM ($n = 6$). * Represents significance at $P < 0.05$ compared with the control group untreated with apocynin. † Represents significance at $P < 0.05$ compared with the same group treated with or without apocynin. gp91^{phox}, gp91^{phox} protein; p22^{phox}, p22^{phox} protein; p47^{phox}, p47^{phox} protein; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

DM + HBO groups untreated with apocynin was significantly down-regulated (0.8-, 0.43-, and 0.3-fold, respectively) compared with the control group. In addition, the expression of Bcl-2 in the HBO and DM + HBO groups treated with apocynin was up-regulated compared with the corresponding groups untreated with apocynin (Figure 4b). Expression of Bax in the HBO, DM, and DM + HBO groups untreated with apocynin was significantly down-regulated (0.8-, 0.43-, and 0.3-fold, respectively) compared with the control group. Moreover, the expression of Bax in the HBO, DM, and DM + HBO groups treated with apocynin was down-regulated compared with the corresponding groups untreated with apocynin (Figure 4b). The expression of caspase-3 in the DM and DM + HBO groups untreated with apocynin was up-regulated (8.8-, and 7.8-fold, respectively) compared with the control group (Figure 4c). No difference in the mRNA expression of caspase-3 was found between DM and DM + HBO rats treated with or without apocynin (data not shown).

Caspase-3 activity in pancreatic tissue

Caspase-3 activity was significantly higher in the DM and DM + HBO groups untreated with apocynin compared with the control group (Figure

5). No difference in caspase-3 activity was found between DM and DM + HBO rats treated with or without apocynin (data not shown).

Discussion

In the present study, we showed that development of hyperglycemia and hypoinsulinemia was facilitated in diabetic rats receiving HBO treatment, and that activity of the NADPH complex was also significantly increased, suggesting the production of ROS and apoptosis-related caspase-3 activity. Moreover, we have also shown, for the first time, the dynamic panel of mRNA expression of genes related to ROS and apoptotic factors in diabetic rat pancreas exposed to HBO.

Hyperglycemia subsequent to diabetes causes oxidative stress, mainly leading to enhanced production of mitochondrial ROS [1]. STZ has been proposed to act as a diabetogenic agent due to its ability to destroy pancreatic β -islet cells, possibly via the formation of excess free radicals [15, 31]. Furthermore, STZ-induced β -cell death is associated with oxidative stress caused by the production of excess intracellular ROS [13, 14]. Moreover, the oxidative stress caused by HBO exposure induces apoptosis via

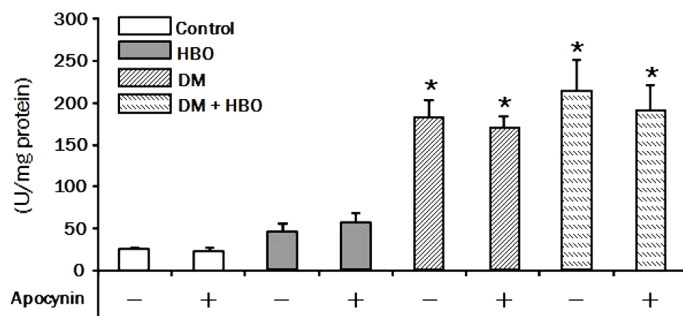


Figure 5. Caspase-3 activity in the pancreas of non-diabetic rats in the non-HBO (control), non-diabetic rats in the HBO (HBO), diabetic rats in the non-HBO (DM), and diabetic rats in the HBO (DM + HBO) groups. Caspase-3 activity is indicated as U/mg protein in pancreatic tissue. Values are expressed as mean $\pm$ SEM ($n = 6$). * Represents significance at $P < 0.05$ compared with the control group untreated with apocynin. † Represents significance at $P < 0.05$ compared with the same group treated with or without apocynin.

the mitochondria [16]. Thus, our demonstration of changes in activity and mRNA expression related to ROS and apoptosis may correlate with oxidative stress induced and enhanced by both diabetes induction and HBO exposure.

An important source of ROS production is NADPH oxidase [5, 6]. Increased activation of NADPH oxidase-dependent superoxide production has a role in hypertension, hypercholesterolemia, diabetes and increased superoxide bioavailability [32-34]. However, to our knowledge, no study has been undertaken of the effects of HBO on the activity and gene expression of the NADPH oxidase complex of pancreatic tissue in animals with or without diabetes. In the present study, we showed that the activity of NADPH oxidase in the HBO, DM, and DM + HBO groups was significantly increased, while such activity was significantly decreased in the HBO and DM + HBO groups treated with the NADPH oxidase inhibitor apocynin, compared to the corresponding activity in the absence of apocynin. These results suggest that even clinically used HBO may induce NADPH oxidase activity in pancreatic tissue of diabetic rats.

Activation of NADPH oxidase induces superoxide production via processes such as oxidation in the mitochondria, inflammation, and stress [35]. NADPH oxidase is a multi-component enzyme complex consisting of the subunits gp91^{phox} and p22^{phox}, as well as the cytosolic factors p47^{phox} and p67^{phox}, and the small GTP-

binding protein Rac-1 [19]. Recent reports have provided evidence for increased mRNA and/or protein expression of these subunits in pancreatic islets of diabetic animals and human patients [36, 37]. Apocynin, an NADPH oxidase inhibitor, prevents the translocation of p47^{phox} to gp91^{phox}, and impedes p47^{phox} subunit assembly within the membrane complex in human and animal endothelial cells, thereby inhibiting the activity of NADPH oxidase and production of superoxide [38, 39]. In the present study, there was no difference in the expression of gp91^{phox} and p22^{phox} between HBO and DM + HBO groups treated with or without apocynin, whereas the expression of p47^{phox} was significantly down-regulated. In addition, the activity of NADPH oxidase in HBO and DM + HBO groups treated with apocynin was significantly decreased compared with the corresponding groups without apocynin. These results suggest that apocynin may have inhibited the production of ROS by specifically preventing the translocation of p47^{phox} to gp91^{phox} [39]. Furthermore, the expression of p47^{phox} in the HBO group untreated with apocynin was significantly up-regulated compared to that in the DM group, suggesting that HBO exposure may have specifically promoted p47^{phox} mRNA expression. Further analysis would be necessary of the detailed mechanism of translocation of p47^{phox} to gp91^{phox} under HBO exposure.

In the present study, activities of the antioxidant enzymes GSH, Cu-Zn SOD, catalase, and Gpx in the DM + HBO group untreated with apocynin were decreased compared with the other groups. On the other hand, in the DM + HBO group treated with apocynin, GSH, Cu-Zn SOD, and catalase activities were increased compared with activities in the same group untreated with apocynin. These results suggest that an increase in the activity of the antioxidant enzyme is attributable to apocynin, which decreases the expression of p47^{phox} and activity of the NADPH oxidase.

The mechanisms by which hyperglycemia exerts its deleterious effects on β -cells include generation of ROS; the latter cause cellular apoptosis via oxidation and have been implicated in the pathogenesis of diabetes mellitus [1]. More-

over, the oxidative stress caused by HBO exposure is linked to the mitochondrial pathway of apoptosis [16]. However, there is no study with regard to the effects of HBO on the activity and expression of genes related to apoptosis via mitochondria in the pancreas of diabetic animals. In the present study, we demonstrated down-regulation of the expression of the anti-apoptotic factor Bcl-2, in contrast to up-regulation of mRNA levels of the apoptotic factor, Bax in the DM + HBO group untreated with apocynin. In addition, we found that the decrease of apoptotic nuclei in islets of Langerhans in the DM + HBO group treated with apocynin was more than that in the same group untreated with apocynin. These results suggested that exposure of pancreas to apoptosis in diabetic animals is attributable to HBO. The present study, however, showed that there was no difference in caspase-3 activity and mRNA expression between the DM + HBO group treated with or without apocynin. This suggests that the production of ROS caused by HBO exposure may involve enhanced apoptosis in diabetic rat pancreas via some other route, such as the main death receptor Fas [16]. However, Weber et al reported that the death receptor Fas is down-regulated by HBO, suggesting that such down-regulation may be a protective action of the cell in response to stress [16]. Recently, the apoptotic signal of death receptors has been shown to be transduced to caspase-8 via FADD [40]; inhibition of caspase-8 by HBO blocks the signal to caspase-3 and inhibits execution of apoptosis [16]. Thus, these reports suggest that HBO has some other route by which it induces apoptosis of pancreatic tissue. Further studies are necessary to clarify the mechanism of apoptosis due to HBO exposure in diabetic animals.

An important pathogenetic mechanism of pancreatic β -cell damage during experimental STZ-induced diabetic animals is increased expression of pro-inflammatory cytokines and increased ROS production in pancreatic islets [13, 14, 41]. Recently, Manna et al reported that TNF α stimulated by ROS production, which was induced in pancreatic tissue of STZ-intoxicated animals, up-regulated the expression of phospho-extracellular signal-regulated kinase (ERK) 1/2 and phospho-p38; furthermore, activation of phosphorylated kinases was suggested to be a critical component in the oxidative stress-induced apoptotic process [15]. In the present study, we showed that the percentage of apop-

tosis of islets of Langerhans in the DM and DM + HBO groups treated with apocynin decreased compared with the corresponding groups untreated with apocynin; however, there was no difference in caspase-3 activity and mRNA expression between DM and DM + HBO rats treated with or without apocynin. Thus, apocynin may inhibit apoptosis via pancreatic ERK1/2 and p38 in STZ-induced diabetic animals due to HBO exposure; however, no further information is available to date on this. The present results have the potential to be applied to further studies of the mechanisms of apoptosis via ERK1/2 and p38.

In conclusion, this study demonstrates various gene expression dynamics of the NADPH oxidase complex and apoptosis in the pancreatic tissue of STZ-induced diabetic rats exposed to HBO. This study further suggests that oxidative stress caused by HBO exposure in diabetic animals induces further ROS production and apoptosis, potentially through the up-regulated expression of genes related to NADPH oxidase complex, and down-regulated expression of anti-apoptotic factors in the mitochondria. Thus, the present study advances understanding of the molecular mechanisms of apoptosis in the diabetic pancreas under HBO exposure; however, the role of other molecules involved in the apoptotic signal cascade such as Fas, ERK1/2 and p38 remains unclear. Further studies are also needed to address the detailed mechanisms operating in the apoptotic pathway under HBO exposure and diabetes induction. These observations suggest that identification of a protective mechanism against ROS production caused by HBO exposure may be beneficial in humans with essential diabetes mellitus. Furthermore, our study model could serve as a useful model for toxicological evaluation of the side effects of HBO treatment.

Acknowledgements

This study was partially supported by the Academic Frontier Project "Surveillance and control for zoonoses" and Strategic Research Base Development Program "International research on epidemiology of zoonoses and training for young researchers" from Ministry of Education, Culture, Sports, Science and Technology, Japan.

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