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Abnormal glutathione metabolism and increased cytotoxicity caused by H_2O_2 in human umbilical vein endothelial cells cultured in high glucose medium

A. Kashiwagi, T. Asahina, M. Ikebuchi, Y. Tanaka, Y. Takagi, Y. Nishio, R. Kikkawa, Y. Shigeta

Third Department of Medicine, Shiga University of Medical Science, Shiga, Japan

Summary To determine whether increased oxidative stress in diabetes mellitus is due to an impaired free-radical scavenger function in endothelial cells, GSH-dependent H_2O_2 degradation in human umbilical vein endothelial cells was studied. The GSH-dependent, NaN_3-uninhibitable H_2O_2-degradation in endothelial cells was reduced by 48% ($p < 0.001$) when the cells were exposed to 33 mmol/l D-glucose vs 5.5 mmol/l D-glucose. This impairment was dependent not only on the D-glucose concentration in the medium but also on D-glucose specific metabolism, since neither 27.5 mmol/l L-glucose nor 27.5 mmol/l D-raffinose had any effect on the peroxide degradation activity. Activation of the glutathione redox cycle by H_2O_2 in cells exposed to high glucose concentrations was attenuated as compared with 5.5 mmol/l D-glucose because of: 1) a 42% decrease ($p < 0.001$) in intracellular NADPH content, and 2) a 34% reduction ($p < 0.01$) in glutathione release into the media. This results in an accumulation of GSSG in the cells following exposure to H_2O_2. Both H_2O_2-evoked ^{51}Cr-release and H_2O_2-induced endothelial cell damage were significantly ($p < 0.01$) greater in the 33 mmol/l D-glucose group than in the 5.5 mmol/l D-glucose group. These results indicate that the abnormal glutathione redox cycle observed in endothelial cells is induced by high glucose concentrations in the medium, resulting in an impairment of reduced GSH-dependent H_2O_2-degradation. These abnormalities may associate with the increased cellular damage following an exogenous exposure to H_2O_2. [Diabetologia (1994) 37: 264–269]

Key words Human umbilical vein endothelial cells, high glucose, oxygen radicals, radical scavenger, glutathione redox cycle.

Oxygen free radicals such as the superoxide anion (O^-_2), and the hydroxyl radical ($OH\cdot$) have been reported to be generated from endothelial cells various pathophysiological conditions including diabetes mellitus [1–5]. Although endothelial cells are capable of scavenging these radicals, in excess they can cause profound endothelial cell damage [6–8]. Such endothelial cell dysfunction may be associated with microvascular complications as well as large vessel diseases commonly observed in diabetes.

H_2O_2 is a potentially harmful molecule produced by the process of oxygen reduction from superoxide dismutase and many oxidase reactions in vivo [5]. Interestingly, a previous report [9] suggests that the glutathione redox cycle is the key step to scavenging H_2O_2 in cytosol as well as in the mitochondria which may contribute to the integrity of the endothelial cells [10]. The glutathione redox cycle is regulated by the intracellular contents of GSH and GSSG, and by glutathione peroxidase and glutathione reductase activities as well as the NADPH level [9, 11]. Therefore, any decreases in these enzyme and substrate levels may profoundly impair H_2O_2-scavenging activity, resulting in exacerbated cell damage following exposure to H_2O_2. Although abnormal glutathione redox cycle in erythrocytes is re-

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Corresponding author: Dr. A. Kashiwagi, Third Department of Medicine, Shiga University of Medical Science, Seta, Otsu, Shiga 520-21, Japan

Abbreviations: GSH, Reduced glutathione; GSSG, oxidized glutathione; BSO, L-buthionine-[S,R]-sulfoximine

Table 1. Effects of NaN_3 and BSO on H_2O_2-degradation in human endothelial cells cultured in high glucose media

D-Glucose (mmol/l)	H_2O_2-degrading activity (nmol · μg $DNA^{-1} \cdot h^{-1}$)			
	Total	BSO-inhibitable	NaN_3-inhibitable	Residual
5.5	6.00 ± 0.04	4.03 ± 0.03	1.92 ± 0.06	0.05 ± 0.05
33	4.10 ± 0.03[a]	2.10 ± 0.12[a]	2.03 ± 0.18	0

[a] $p < 0.001$ vs 5.5 mmol/l glucose. The data are expressed as mean ± SEM ($n = 3$). The cells were incubated in the presence or absence of either 200 μmol/l BSO for 18 h before and H_2O_2-treatment for 1 h to the cells or 4 mmol/l NaN_3 for 1 h during the H_2O_2-treatment

ported in diabetes [12], abnormalities in the glutathione redox cycle in endothelial cells exposed to high concentrations of glucose have never been demonstrated.

Therefore, we examined the effects of high external glucose on the capacity of endothelial cells to scavenge H_2O_2, and the possible potentiation of oxygen radicals-mediated cell injury using an human umbilical vein endothelial cell model.

Subjects, materials and methods

Chemicals. Class 1 collagenase was purchased from Worthington Biochemical Co. (Freehold, NJ, USA) and fetal calf serum was obtained from Gibco Laboratories (Grand Island, NY, USA). Endothelial cell growth factor was purchased from Boehringer Mannheim (Mannheim, Germany), and heparin from Novo Nordisk A/S (Bagsvaerd, Denmark). Scopoletin and BSO were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). DEAE-5PW was purchased from Toyosoda Co. Ltd. (Tokyo, Japan). ^{51}Cr-Sodium chromate (14.8–44.4 TBq/g chromium) was obtained from DuPont/NEN Research Products (Boston, Mass., USA). All other chemicals were of reagent grade and were obtained from commercial sources.

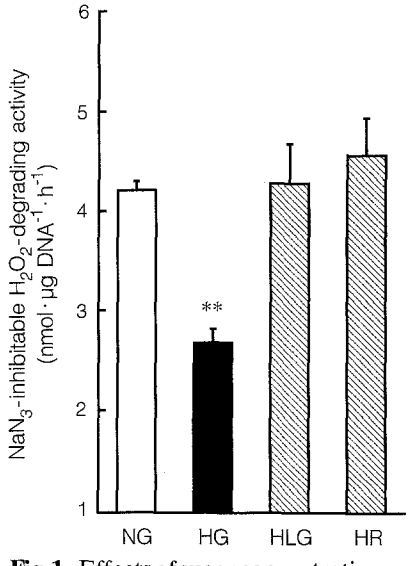

Fig. 1. Effects of sugar concentrations on H_2O_2-scavenging activity in cultured endothelial cells. Cells were cultured for 5–7 days in the presence of 5.5 mmol/l D-glucose (NG, $n = 17$), 33 mmol/l D-glucose (HG, $n = 13$), 5.5 mmol/l D-glucose plus 27.5 mmol/l L-glucose (HLG, $n = 9$), and 5.5 mmol/l D-glucose plus 27.5 mmol/l D-raffinose (HR, $n = 6$). The NaN_3-uninhibitable, GSH-dependent degradation of H_2O_2 was measured by incubating the cells in the presence or absence of 4 mmol/l sodium azide and 20 μmol/l H_2O_2, as described in the Materials and methods. The data are expressed as mean ± SEM (n = number of experiments). ** $p < 0.01$ vs NG, HLG, and HR groups

Isolation and culture of human umbilical vein endothelial cells. Human umbilical vein endothelial cells were isolated from umbilical cord tissue obtained from healthy, pregnant women according to methods previously described [13, 14]. Isolated endothelial cells were cultured in minimal essential medium containing 10 % fetal calf serum, 50 μg/ml endothelial cell growth factor and 90 μg/ml heparin. The medium was changed every 2–3 days, and only 5–14th passage cells were used in the present experiment. Endothelial cells were cultured for 5–7 days in culture media containing 5.5 mmol/l D-glucose without (NG) or with 27.5 mmol/l D-raffinose (HR), or 33 mmol/l D-glucose (HG).

Measurement of H_2O_2 degradation by endothelial cells. Cells were incubated in media containing 5.5, 11, 22, 33 or 66 mmol/l D-glucose or either 27.5 mmol/l L-glucose or 27.5 mmol/l D-raffinose in the presence of 5.5 mmol/l D-glucose for a further 5–7 days. H_2O_2-degrading activity was measured by a method previously described [13]. In order to verify GSH-dependent H_2O_2-degradation, H_2O_2-degrading activity was also measured after the cells were preincubated with 200 μmol/l BSO (a gamma-glutamyl-cysteine synthetase inhibitor) [9] for 18 h under both NG and HG conditions. During these conditions, GSH content in the cells fell by 98 %. The residual H_2O_2 in the media was measured by methods previously described [13, 15]. The data are expressed as nmol H_2O_2 degraded per μg DNA per hour.

Measurement of GSH and GSSG in cells and media. The cells were incubated in the absence or presence of 200 μmol/l H_2O_2 at 37 °C for 1 h with the above concentrations of carbohydrates. The intracellular and medium contents of GSH and GSSG were measured by the cyclic enzymic method as previously described [11, 13]. The data are expressed as pmol GSH equivalent per μg DNA.

Measurement of glutathione peroxidase and glutathione reductase activities. At 0, 20, and 60 min after addition of 200 μmol/l H_2O_2 to the incubation medium, the cells were homogenized in 450 μl of cold phosphate-buffered saline (pH 7.4) in three 15-s bursts using an ultrasonic homogenizer and were then centrifuged at 10,000 × g for 30 min. The supernatant was used for the determination of glutathione peroxidase [13, 16] and glutathione reductase activities [13, 17]. Both enzyme activities were expressed as nmol NADPH · μg $DNA^{-1} \cdot min^{-1}$.

Measurement of the reduced form of pyridine nucleotides. The cells were incubated in the presence or absence of various concentrations of H_2O_2 for 1 h in NG and HG conditions. The cells were disrupted by microwave and sonication in a 0.5 ml aliquot of extraction buffer (3 volumes 10 mmol/l phosphate buffer

pH 8.5 to 7 volumes of ethanol) and then were centrifuged at 15,000 × *g* for 1 min. A 100 µl aliquot of the supernatant was subjected to HPLC [18]. HPLC was performed using a Toso DEAE-5pw anion exchanger column, in a Shimadzu RCA-6 (HPLC) equipped with a fluorescence detector. The column was equilibrated in 30% buffer A (10 mmol/l potassium phosphate buffer containing 10% ethanol at pH 7.4) and 70% buffer B (450 mmol/l potassium phosphate buffer containing 10% methanol at pH 7.4). The fluorescence intensity of the effluent was monitored and the retention times of NADH and NADPH were 5.3 min and 16.5 min, respectively.

Measurement of H_2O_2-induced ^{51}Cr-release and endothelial-cell loss. Two KBq ^{51}Cr-sodium chromate was added to the incubation medium and the cells were incubated for a further 18 h at 37 °C. Spontaneous and H_2O_2-evoked ^{51}Cr-release from the cells were measured by incubating the cells at 37 °C for 2 h in the absence or presence of 200 µmol/l H_2O_2. The H_2O_2-evoked ^{51}Cr-release rate was calculated by the following formula: H_2O_2-evoked increase in ^{51}Cr release rate (%) = [(^{51}Cr-release in the presence of H_2O_2 – spontaneous ^{51}Cr-release)/total ^{51}Cr incorporation] × 100 [19].

For the measurement of endothelial-cell loss, cells were incubated for a further 48 h in the presence or absence of 200 µmol/l H_2O_2 in NG, HR, and HG conditions. The DNA content in the remaining cells attached to the culture dish was then measured by a previously described method [20].

Statistical analysis

The data are expressed as mean ± SEM unless otherwise stated. Student's *t*-test was used for the determination of the significance of differences between the two groups, and Scheffe's multiple comparison test was used to compare differences among more than three groups. A *p* value of less than 0.05 was considered significant.

Results

The effect of high external glucose concentration on H_2O_2 degradation. As shown in Table 1, the total H_2O_2-degrading capacity of the endothelial cells was reduced by 32% ($p < 0.001$) in the cells exposed to a high glucose medium for 5–7 days. This reduction in H_2O_2-degradation under high glucose conditions was only observed for BSO-inhibitable (i.e. GSH-dependent) H_2O_2-degradation (48% decrease vs NG group), and not for NaN_3-inhibitable (catalase-dependent) degradation. Although the NaN_3-uninhibitable (GSH-dependent) activity in the HG group decreased by 50% ($p < 0.01$) compared to the NG group, the activities in both hyperosmolar control L-glucose and D-raffinose groups were not reduced compared to the NG group (Fig. 1). Furthermore, this reduction in H_2O_2-scavenging activity became more marked with increasing concentrations of D-glucose in the medium (Fig. 2).

Effect of high glucose on H_2O_2-induced changes in intracellular glutathione metabolism. As shown in Table 2, there was no difference in the basal GSH and

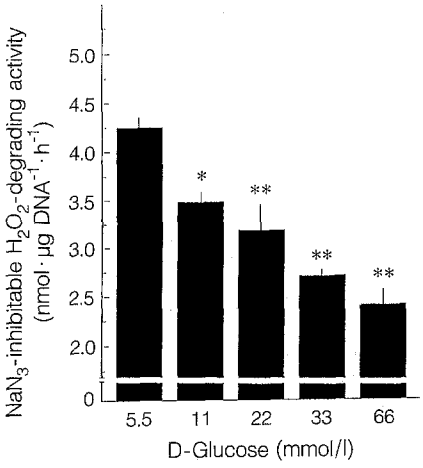

Fig. 2. Effects of D-glucose concentrations on NaN_3-uninhibitable, GSH-dependent H_2O_2-degradation activity in cultured endothelial cells. Endothelial cells were cultured for 5–7 days in the presence of 5.5–66 mmol/l D-glucose. H_2O_2-degradation was then measured as described in Figure 1. The data are expressed as mean ± SEM ($n = 8$). * $p < 0.05$, ** $p < 0.01$ vs the 5.5 mmol/l D-glucose group

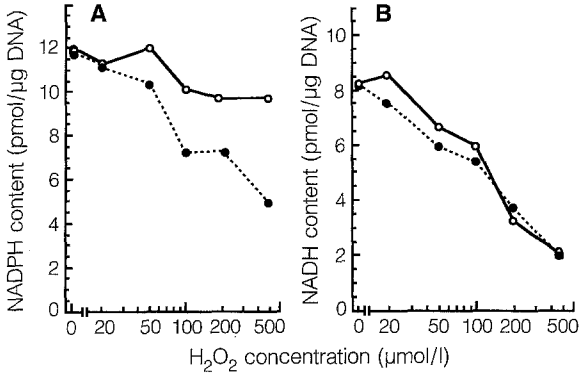

Fig. 3 A, B. Effect of H_2O_2 concentration on (A) intracellular NADPH content and (B) intracellular NADH content in cells cultured in media containing 5.5 mmol/l D-glucose (○) and 33 mmol/l D-glucose (●) for 5–7 days. The cells were then incubated with 0, 20, 50, 100, 200, and 500 µmol/l H_2O_2 at 37 °C for 1 h, and the NADPH and NADH contents measured as described in the Materials and methods. The data are expressed as the mean of two independent experiments

GSSG contents between the NG and HG groups. By exposing the cells to H_2O_2, the GSH content in the cells decreased significantly by 41% (NG group) and by 30% (HG group). The H_2O_2-evoked decrease in GSH content in the HG group was 73% ($p < 0.05$) of the one shown in the NG group. In contrast, the intracellular GSSG content increased significantly by 13.5-fold (NG group), and by 19-fold (HG group) of the respective basal value in the presence of H_2O_2. The increase in GSSG content was 44% greater ($p < 0.01$) in the HG group than in the NG group. The total glutathione (GSSG + GSH) release into the medium for 1 h after exposure to H_2O_2 was decreased by 34% ($p < 0.01$) in the HG group compared to the NG group.

Table 2. Effects of D-glucose concentration on H_2O_2-evoked changes in intracellular glutathione content in cells and glutathione release into media in cultured endothelial cells

D-Glucose (mmol/l)	H_2O_2	Cell GSH content (pmol GSH · μg DNA^{-1})	Cell GSSG content (pmol GSH · μg DNA^{-1})	GSH + GSSG release into the media (pmol GSH · μg DNA^{-1} · h^{-1})
5	−	417 ± 22	2 ± 1	23 ± 3
	+	248 ± 25[e]	27 ± 2[e]	154 ± 10[e]
	(+) − (−)	− 169 ± 16	+ 25 ± 2	+ 137 ± 8
33	−	420 ± 21	2 ± 1	25 ± 4
	+	296 ± 18[d]	+ 38 ± 2[c,e]	115 ± 8[b,e]
	(+) − (−)	− 124 ± 7[a]	+ 36 ± 2[b]	+ 90 ± 12[b]

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs the 5 mmol/l D-glucose group; [d] $p < 0.01$, [e] $p < 0.001$ vs the value in the absence of H_2O_2.
The data are expressed as mean ± SEM ($n = 8$). The cells were incubated in the presence or absence of 200 μmol/l H_2O_2 at 37 °C for 1 h. The GSH and GSSG content in the cells as well as the total glutathione released into the medium were measured as described in the Materials and methods

Table 3. Increases in H_2O_2-evoked ^{51}Cr-release from endothelial cells cultured in high glucose media

D-Glucose (mmol/l)	H_2O_2-evoked ^{51}Cr-release rate (%)
5.5	5.6 ± 0.3
33.0	8.5 ± 0.7[a]

[a] $p < 0.01$ vs 5.5 mmol/l D-glucose group. The data are expressed as mean ± SEM ($n = 4$).
The cells were prelabelled with 2 KBq ^{51}Cr at 37 °C for 18 h. ^{51}Cr-release from the cells was then measured at 37 °C for 2 h in the presence or absence of 200 μmol/l H_2O_2. The H_2O_2-evoked ^{51}Cr-release rate (%) calculations are described in the Materials and methods

Glutathione peroxidase and glutathione reductase activities. Glutathione peroxidase activity (pmol NADPH · min^{-1} · μg DNA^{-1}) in the HG group was 356 ± 17, which was 19 % ($p < 0.01$) less than the value recorded for the NG group (438 ± 21). After exposure to H_2O_2 for 20 min, the glutathione peroxidase activities of both groups decreased to a similar level (NG; 231 ± 29, HG; 210 ± 20) and the activities then returned to 338 ± 58 (NG) and 329 ± 13 (HG) 1 h after exposure to H_2O_2. The basal glutathione reductase activity was not significantly different between the two groups (567 ± 67 vs 592 ± 27 pmol NADPH · min^{-1} · μg DNA^{-1}).

Effects of high glucose concentrations on H_2O_2-evoked decreases in intracellular NADPH and NADH concentrations. As shown in Figure 3, there was a marked difference in the H_2O_2-evoked decrease in NADPH concentration between NG and HG groups. By increasing the H_2O_2 concentration in the medium, a greater decrease in the intracellular NADPH concentration was seen in the HG group than in the NG group. To confirm the D-glucose effect, we studied another set of experiments. In the presence of 200 μmol/l H_2O_2, NADPH content in the HG group (4.5 ± 0.4 pmol/μg DNA, $n = 8$) was less ($p < 0.001$) than the NG group (7.8 ± 0.6 pmol/μg DNA, $n = 9$). By contrast, there were no differences in the H_2O_2-evoked decrease in NADH concentration between the NG and HG groups.

H_2O_2-induced endothelial-cell loss and ^{51}Cr-release. In the absence of H_2O_2, the DNA content per dish was not significantly different among the NG (2.89 ± 0.08, $n = 8$), HG (2.45 ± 0.15, $n = 8$), and HR (2.68 ± 0.14, $n = 6$) groups (μg DNA/dish). However, the DNA content in the presence of 200 μmol/l H_2O_2 in the HG group was 1.70 ± 0.17, which was 25 % ($p < 0.05$) less than the respective values in the NG (2.27 ± 0.17) and HR (2.26 ± 0.12) groups (μg DNA/dish). Similarly, ^{51}Cr-release from the cells in the HG group during a 2-h incubation in the presence of 200 μmol/l H_2O_2 was 52 % ($p < 0.01$) greater than that from cells in the NG group (Table 3).

Discussion

In the present study, the total H_2O_2-degradation capacity of human umbilical vein endothelial cells cultured in the HG group fell by 32 % compared to that of the NG group. In endothelial cells, H_2O_2 is primarily metabolized by two enzymes, catalase and glutathione peroxidase [9, 12]. In our in vitro system, only the GSH-dependent degradation which was not inhibited by a catalase inhibitor (NaN_3), but inhibited by decreasing intracellular GSH content in the presence of BSO was specifically affected. These abnormalities were D-glucose-specific and dependent on D-glucose concentrations. Furthermore, the BSO-inhibitable (GSH-dependent) portion of the activity plus the NaN_3-inhibitable (catalase-dependent) activity approximates the total H_2O_2 degrading activity by endothelial cells. Thus the other enzymatic or non-enzymatic pathway of H_2O_2-degradation may not be significant during the present experimental conditions [21].

The GSH-dependent degradation of H_2O_2 is primarily regulated by the glutathione redox cycle [9, 11]. In the present study, basal content of each component of glutathione redox cycle was not significantly different between the NG and HG groups, although basal glutathione peroxidase activity in the HG group was only 19 % less than that of the NG group. However, fol-

lowing H_2O_2 treatment, cells in the HG group showed a smaller decrease in GSH content which may be associated with the impairment of H_2O_2-induced increase in glutathione release into media and a greater decrease in NADPH content with a greater increase in GSSG content compared to cells in the NG group. Therefore, abnormal glutathione level in the cells exposed to high glucose media can be explained by both decreases in glutathione transport and NADPH level. In terms of glutathione transport, it has been reported that GSSG can be actively transported out of erythrocytes by a Mg^{2+}-ATPase [30] and a similar impairment of GSSG transport and GSSG accumulation has been reported in erythrocytes obtained from patients with non-insulin-dependent diabetes mellitus [31].

By stoichiometric analysis, the major factor contributing to the decrease in H_2O_2-degrading activity in cells exposed to a high glucose medium may be the NADPH supply to the glutathione redox cycle, since the NADPH content in these cells (8–10 pmol·μg DNA^{-1}) was only 2–2.5% of the GSH content (400 pmol·μg DNA^{-1}). This was despite the high H_2O_2-degrading activity of the cells (more than 4 nmol·μg $DNA^{-1}\cdot h^{-1}$) and even higher glutathione peroxidase and glutathione reductase activities (about 24–36 nmol·μg $DNA^{-1}\cdot h^{-1}$). In the present study, we could not determine the exact mechanism whereby the NADPH content in cells in the HG group dropped upon exposure to H_2O_2. However, based on data from previous studies, one may speculate that this decrease may be due to an insufficient activation of the pentose phosphate shunt pathway [22], and to over-utilization of NADPH by activation of the polyol pathway [23, 24]. Thus, the NADPH supply to the glutathione redox cycle may be crucial for H_2O_2-degrading activity in the endothelial cells. In contrast, intracellular NADH content decreased equally during the treatment of cells with H_2O_2 in both NG and HG groups. These decreases in intracellular NAD and NADH content are explained by their consumption during the process of DNA repair after the DNA damage by H_2O_2 [25, 26].

An impairment of the GSH-dependent H_2O_2-degradation based on abnormal glutathione redox cycle may induce an increase in the cytotoxic effect of H_2O_2 on endothelial cells [9, 13]. Our results clearly show that endothelial cells exposed to high external glucose concentrations were more susceptible to an exogenous exposure of 200 μmol/l H_2O_2 than cells cultured under the normal glucose condition. Our data suggest that a biologically relevant H_2O_2-concentration which can be easily achieved in the proximity of stimulated neutrophils (50 μmol/l H_2O_2) and other biological systems [11, 28, 29] can induce endothelial cell dysfunction and that these injuries can be exacerbated by high external glucose. However, intra- and extracellular concentrations of free radicals have never been adequately measured in the in vivo diabetic state, although evidence demonstrating elevated levels of lipid peroxidation and autoxidation products suggests an increased oxidative stress in diabetes [3, 30–34].

Altogether, one can speculate that not only excess free-radical production but also any impairment of radical scavenging activity induced by a high glucose concentration, as found in diabetes, may profoundly affect endothelial cell integrity. The clinical relevance of such perturbations of the glutathione redox cycle in diabetes with respect to the pathogenesis of diabetic vascular complications awaits future study.

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