

# High concentration of glucose causes impairment of the function of the glutathione redox cycle in human vascular smooth muscle cells

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**Abstract** We demonstrated that high glucose reduced H<sub>2</sub>O<sub>2</sub> scavenge activity in human vascular smooth muscle cells. In the cells exposed to high glucose, the intracellular glutathione content decreased, although the NADPH content was unchanged. The rate of uptake of cystine, which is a rate-limiting precursor of the glutathione synthesis, decreased in the high glucose group compared with the control group. These decreases were shown to be dependent on glucose concentration. It was suggested that high glucose causes impairment of the function of the glutathione redox cycle in human vascular smooth muscle cells, resulting in reduced H<sub>2</sub>O<sub>2</sub> scavenge activity.

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**Key words:** H<sub>2</sub>O<sub>2</sub>; Glutathione; Cystine uptake; High glucose; Human vascular smooth muscle cell

## 1. Introduction

Reactive oxygen species are always generated in the process of energy metabolism and biophylaxis [1,2]. Although there are defense systems against reactive oxygen species inside cells, when the free radicals generated exceed the capability of the defense systems, excessive harmful molecules can locally induce cell or tissue damage [3]. Since reactive oxygen species are emitted by leukocytes, such as macrophages and neutrophils, vascular cells are particularly susceptible to oxidative stress [4]. Increased production and reduced degradation of reactive oxygen species have been reported in diabetes mellitus [5]. It has been proposed that such increased oxidative stress causes cellular injury and results in diabetic complications [6,7]. H<sub>2</sub>O<sub>2</sub> is eliminated by catalase and the glutathione redox cycle. The glutathione redox cycle plays a major role in scavenging H<sub>2</sub>O<sub>2</sub> under physiological conditions. It consists of glutathione, glutathione peroxidase (GPX), glutathione reductase (GR) and NADPH [8,9]. Thus, any decrease or dysfunction in these components may cause impairment of the degradation activity of H<sub>2</sub>O<sub>2</sub>. In the present study, we investigated the glutathione redox cycle in human vascular smooth muscle cells exposed to high concentrations of glucose in culture.

## 2. Materials and methods

### 2.1. Cell culture

Human aortic vascular smooth muscle cells were purchased from Kurabo Co. (Osaka, Japan). Cells were cultured in a HuMedia-SG2

(Kurabo Co., Osaka, Japan) medium with 5% fetal calf serum, human epithelium growth factor (0.5 ng/ml), human fibroblast growth factor (2 ng/ml), insulin (5 µg/ml), gentamycin (50 µg/ml), and amphotericin B (50 ng/ml) in an atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C as previously described [12]. This medium contained L-cysteine HCl·H<sub>2</sub>O (110 µM) and L-glutamic acid (72 µM). Media were changed twice weekly. Cells from the 8th to the 10th passage were used in the present experiment.

### 2.2. Measurement of degradation of H<sub>2</sub>O<sub>2</sub>

Confluent vascular smooth muscle cells in 12-well plates were cultured in media containing 5.5 mM glucose, 11 mM glucose, 27.5 mM glucose, and 5.5 mM glucose plus 22 mM mannitol for 7 days. In order to measure glutathione redox cycle-dependent H<sub>2</sub>O<sub>2</sub> degradation, cells were preincubated for 6 h in media containing 10 mM 3-amino-1,2,4-triazole, a catalase inhibitor. Then culture media were changed to Dulbecco's PBS containing 20 µM H<sub>2</sub>O<sub>2</sub>, and cells were further incubated for 1 h at 37°C. Dulbecco's PBS contains 137 mM NaCl, 3 mM KCl, 0.01% CaCl<sub>2</sub>, 0.01% MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.1% glucose, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, and 8 mM Na<sub>2</sub>HPO<sub>4</sub>. H<sub>2</sub>O<sub>2</sub> was measured by the method by Root et al. [13] with slight modifications. Briefly, 1 ml of the PBS containing H<sub>2</sub>O<sub>2</sub> was incubated in the presence of 2.0 µM scopoletin and 12.0 nM horseradish peroxidase at 37°C for 5 min. The decrease in fluorescence intensity of scopoletin by peroxidase reaction with H<sub>2</sub>O<sub>2</sub> was measured at an excitation wave length of 366 nm and emission wave length of 460 nm.

### 2.3. Measurement of the NADPH

Confluent vascular smooth muscle cells in a dish 35 mm in diameter were cultured in media containing 5.5 mM glucose (NG) or 27.5 mM glucose (HG) for 7 days and then incubated in the presence or absence of 200 µM H<sub>2</sub>O<sub>2</sub> for 1 h. The cells were disrupted by sonication in 500 µl of extraction buffer (3 mM potassium phosphate buffer containing 70% ethanol at pH 8.5) and then centrifuged at 15000×g for 5 min. A 100 µl aliquot of the supernatant was subjected to high-performance liquid chromatography (HPLC). HPLC was performed using a Toso DEAE-5pw anion exchange column equilibrated with 318 mM potassium phosphate buffer containing 3% ethanol and 7% methanol at pH 7.4 [14]. The cell extract was eluted by the same buffer at a flow rate of 1 ml/min. The fluorescence intensity of the effluent was detected by a fluorescence spectrophotometer (F2000, Hitachi, Japan) with excitation at 340 nm and emission at 450 nm. The retention times of NADH and NADPH were 190 s and 520 s, respectively.

### 2.4. Determination of intracellular GSH

Cells in a dish 35 mm in diameter were rinsed three times in PBS, and glutathione was extracted with 1.2 ml of 5% trichloroacetic acid. The acid extract was treated four times with 2 ml of 0.01 N HCl-saturated diethyl ether to remove trichloroacetic acid. The resulting solution was used for the assay of glutathione. Glutathione was measured enzymatically by the method of Tietze [15]. This method measures total glutathione, the reduced form (GSH) and the oxidized form (GSSG), but in the present experiment almost all glutathione in smooth muscle cells existed as GSH.

### 2.5. Determination of GSH efflux

Cells in a dish 35 mm in diameter were quickly washed twice with PBS, and then incubated with 1.2 ml of PBS at 37°C for 10 min. The

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Table 1  
Effects of glucose concentration on intracellular NADPH content and GSH efflux in cultured human aortic smooth muscle cells

	NADPH content (mol/mg protein)		GSH efflux rate	
	H <sub>2</sub> O <sub>2</sub> (–)	H <sub>2</sub> O <sub>2</sub> (+)	(mol/10 min/mg protein)	% of the initial cellular GSH effluxed in 10 min
NG group	0.266 ± 0.004	0.249 ± 0.011	13.1 ± 1.8	43.6 ± 0.8
HG group	0.243 ± 0.001	0.249 ± 0.011	10.8 ± 1.1	44.5 ± 0.8

Cells were cultured for 7 days in 5.5 mM glucose (NG) or 27.5 mM glucose (HG).

The data are expressed as mean ± S.E.M. (*n*=4).

PBS was then removed and the GSH in cells and in PBS was measured [15].

## 2.6. Determination of cystine uptake

The initial rate of uptake of cystine was measured as described previously [16]. Cells in a dish 35 mm in diameter were rinsed three times in PBS. The cells were then incubated in 0.5 ml of warm uptake medium for 2 min at 37°C. The uptake medium contained 0.05 mM L-cystine and 0.4 µCi/ml <sup>14</sup>C-labeled cystine (New England Nuclear, Boston, MA, USA) in the PBS used in rinsing the cells. Uptake was terminated by rapidly rinsing the dish three times in 1.5 ml of ice-cold PBS which did not contain CaCl<sub>2</sub>, MgCl<sub>2</sub>, or glucose. Then, 0.5 ml of 0.5 N NaOH was added to each dish to dissolve the cells. Of the solution, 0.1 ml was used for the assay of protein [17], and 0.2 ml was used for determining radioactivity.

## 2.7. Statistical analysis

The values obtained from the experiments were indicated using mean ± S.E.M. Student's *t*-test was used for the determination of the significance of the difference between the two groups, and Fisher's Protected Least Significant Difference method was used to compare differences among more than four groups. Differences with a probability of 5% (*P* < 0.05) or less were considered to be statistically significant.

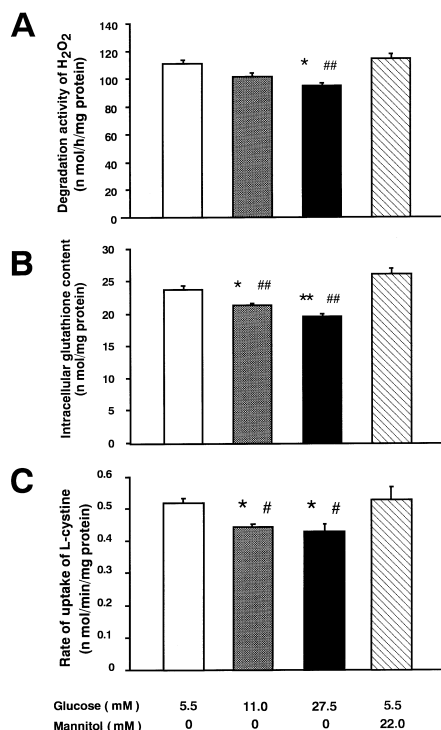


Fig. 1. Effects of glucose concentrations on degradation activity of H<sub>2</sub>O<sub>2</sub> (A), GSH content (B) and cystine uptake (C) in cultured human aortic smooth muscle cells. Cells were cultured for 7 days in the presence of 5.5 mM glucose, 11 mM glucose, 27.5 mM glucose, and 5.5 mM glucose plus 22 mM mannitol. GSH content (B) was determined two days after the last exchange of the media. The data are expressed as mean ± S.E.M. (A: *n* = 11, B: *n* = 8, C: *n* = 5). \**P* < 0.05 and \*\**P* < 0.01 vs. 5.5 mM glucose, #*P* < 0.05 and ##*P* < 0.01 vs. 5.5 mM glucose plus 22 mM mannitol.

## 3. Results

### 3.1. Degradation activity of H<sub>2</sub>O<sub>2</sub>

As shown in Fig. 1A, glutathione redox cycle-dependent degradation activity of H<sub>2</sub>O<sub>2</sub> in human aortic smooth muscle cells was reduced by 25% (*P* < 0.05) in cells exposed to a high glucose medium (HG; 27.5 mM) for 7 days compared with control group cells (NG; 5.5 mM glucose). This reduction was shown to be dependent on glucose concentration in the medium. Although the degradation activity of H<sub>2</sub>O<sub>2</sub> in the high glucose group decreased compared to that in the control group, the activity in the hyperosmolar control group (5.5 mM glucose plus 22 mM mannitol) did not change compared to that in the control group.

### 3.2. Intracellular NADPH content

To investigate whether the reduction of degradation activity of H<sub>2</sub>O<sub>2</sub> was due to the decrease of NADPH content in the glutathione redox cycle, we measured the intracellular NADPH content by HPLC. As shown in Table 1, NADPH content did not differ significantly between the NG group and HG group in the absence or presence of H<sub>2</sub>O<sub>2</sub>.

### 3.3. Intracellular GSH content

As shown in Fig. 1B, intracellular GSH content decreased by 13% (*P* < 0.05) with 11 mM glucose and by 18% (*P* < 0.01) with 27.5 mM glucose compared with values with 5.5 mM glucose. In the hyperosmolar control group, intracellular GSH content was not reduced. This high glucose effect was manifested as early as 2 days after exposure of cells to the high glucose condition and was maintained to day 7 in the high glucose medium (Fig. 2).

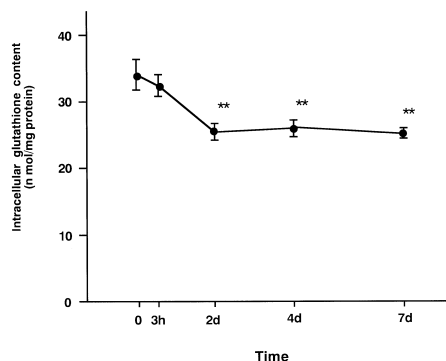


Fig. 2. Time course of GSH content in cultured human aortic smooth muscle cells. Cells were cultured in media containing 27.5 mM glucose for the indicated periods of time. GSH content was determined one day after the last exchange of the media. The data are expressed as mean ± S.E.M. (*n* = 3 dishes). \*\**P* < 0.01 vs. the starting point.

### 3.4. GSH efflux

To study the mechanism of the decrease of intracellular GSH content, GSH efflux was examined. As shown in Table 1, there was no significant difference in the rate of GSH efflux between the NG group and HG group.

### 3.5. Cystine uptake

The rate of uptake of cystine decreased by 15% ( $P < 0.05$ ) with 11 mM glucose and by 18% ( $P < 0.05$ ) with 27.5 mM glucose compared with values for 5.5 mM glucose (Fig. 1C). In the hyperosmolar control group, rate of uptake of cystine was not decreased.

## 4. Discussion

The present study demonstrated the following: (1) Degradation activity of  $H_2O_2$  was reduced significantly in vascular smooth muscle cells exposed to a high glucose medium compared with the control group. (2) In the cells exposed to high glucose, intracellular GSH content decreased, although the NADPH content was unchanged. (3) The rate of uptake of cystine was decreased in the high glucose group compared with the control group.

It has been thought that  $H_2O_2$  produced in the vascular system is degraded first in erythrocytes and endothelial cells which are in direct contact with blood [4]. The function of the glutathione redox cycle as a scavenger of  $H_2O_2$  has been studied with regard to mechanisms of diabetic complications only in erythrocytes and endothelial cells [10,11,14,18,19]. However, because the cell membrane is permeable to  $H_2O_2$ , if a large amount of  $H_2O_2$  is produced in the blood vessels it probably reaches the vascular smooth muscle cells through thin stratified endothelial cells. Since the total anatomical volume taken up by smooth muscle cells in blood vessels is much greater than that of endothelial cells, smooth muscle cells are considered to play a more important role in the degradation of  $H_2O_2$  than endothelial cells. Therefore, we investigated the effects of high glucose concentrations on the function of the glutathione redox cycle in human vascular smooth muscle cells. In the present experiment, we measured the glutathione redox cycle-dependent  $H_2O_2$  degradation activity by inhibiting catalase activity with amino-triazole. We showed that degradation activity of  $H_2O_2$  was reduced significantly in the cells exposed to a high glucose medium compared with the control group. This reduced degradation activity was shown to be dependent on the glucose concentration of the medium. This suggests that glutathione redox cycle-dependent degradation activity of  $H_2O_2$  may be decreased in smooth muscle cells exposed to hyperglycemia in diabetes mellitus.

Kashiwagi et al. [14] reported that degradation activity of  $H_2O_2$  in endothelial cells was reduced significantly when the cells were exposed to high glucose levels and that a decrease of such activity was accompanied by a decrease in NADPH content. The decrease in GSH content was reported in erythrocytes exposed to a high glucose culture medium [11] and in erythrocytes from diabetic patients [10], but not in endothelial cells [14]. We found that although the NADPH content did not differ significantly between the NG and HG groups, intracellular GSH in cells exposed to high glucose decreased significantly. The effect of exposure to a high glucose concentration was relatively rapid because intracellular GSH reached a plateau by 2 days after exposure of cells to high glucose con-

ditions. The concentrations of GSH in vascular smooth muscle cells in the present study are close to the reported  $K_m$  value of GPX for GSH [20]. Thus, the degradation activity of  $H_2O_2$  by GPX decreases with decreases in GSH content. The impaired degradation activity of  $H_2O_2$  demonstrated in the smooth muscle cells exposed to high glucose seems to be caused by the decrease of GSH content in these cells.

GSH is not degraded in the cell, and the intracellular GSH content is regulated by GSH synthesis within the cell and GSH efflux from the cell [21]. GSH is synthesized intracellularly by two processes [22]: (1) the formation of  $\gamma$ -glutamylcysteine from glutamate and cysteine, catalyzed by glutamylcysteine synthetase, and (2) the synthesis of GSH from  $\gamma$ -glutamylcysteine and glycine catalyzed by GSH synthetase. Among the amino acids used in GSH synthesis, glutamate and glycine are synthesized intracellularly and found abundantly in the intracellular or extracellular fluid space. On the other hand, cysteine is very sparse in most cells because of lack of synthetic activity. Therefore, it is necessary for most cells to take up cysteine from the medium, and it has been shown that the intracellular cysteine content is a rate-limiting factor in GSH synthesis [23,24]. Cysteine is easily autoxidized to cystine in extracellular fluid, whereas cystine is rapidly reduced to cysteine once it enters the cells [16]. Thus, cystine in the medium is a precursor of the intracellular GSH and its uptake is a rate-limiting factor in maintaining GSH levels in most cells, including vascular smooth muscle cells [21]. In the present study, to investigate the mechanism of the decrease of intracellular GSH content, we measured the uptake of cystine by the cells and the efflux of GSH from the cells. The uptake rate of cystine decreased in the HG group compared with that of the control group. The synthesis of GSH seems to slow down through an impaired cystine uptake capability in the HG group. On the other hand, there was no difference in the rate of efflux of GSH between the two groups.

There is a correlation between GSH efflux and GSH catabolism in cells [25]. GSH efflux has been investigated in kidney cells [26], liver cells [26], fibroblasts [27], and endothelial cells [28], and half lives of GSH are about 20 min, 1.5 h, 1.5 h, and 3 h, respectively. The half life of GSH observed in smooth muscle cells in this study was about 10 min. This is the shortest among the previous studies, meaning that the GSH turnover in vascular smooth muscle cells is very rapid compared with that in other cells. Our study suggests that a large amount of GSH in the vascular smooth muscle cells is released into the extracellular space, forming a defense system against oxidative stress in the whole vascular system. GSH plays an important role in the defense system against oxidative stress as a reducing agent not only in the cells but also in the plasma [29]. The possibility exists that if GSH synthesis is decreased in cells exposed to high glucose concentrations for long periods, such as in diabetes, the impaired defense system against oxidative stress causes dysfunction not only in the vascular smooth muscle cells but also in other vascular systems. Further investigations on GSH metabolism in vascular smooth muscle cells will be necessary for understanding the defense system against oxidative stress throughout the vascular system.

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## References

- [1] Freeman, B.A. and Crapo, J.D. (1982) *Lab. Invest.* 47, 412–426.
- [2] Zweier, J.L., Kuppusamy, P. and Luty, G.A. (1988) *Proc. Natl. Acad. Sci. USA* 85, 4046–4050.
- [3] Hennig, B. and Chow, C.K. (1988) *Free Radic. Biol. Med.* 4, 99–106.
- [4] Sacks, T., Moldow, C.F., Craddock, P.R., Bowers, T.K. and Jacob, H.S. (1978) *J. Clin. Invest.* 61, 1161–1167.
- [5] Lyons, T.J., Silvestri, G., Dunn, J.A., Dyer, D.G. and Baynes, J.W. (1991) *Diabetes* 40, 1010–1015.
- [6] Baynes, J.W. (1991) *Diabetes* 40, 405–412.
- [7] Hunt, J.V., Smith, C.C. and Wolff, S.P. (1990) *Diabetes* 39, 1420–1424.
- [8] Andreoli, S.P., Mallett, C.P. and Bergstein, J.M. (1986) *J. Lab. Clin. Med.* 108, 190–198.
- [9] Harlan, J.M., Levine, J.D., Callahan, K.S., Schwartz, B.R. and Harker, L.A. (1984) *J. Clin. Invest.* 73, 706–713.
- [10] Murakami, K., Kondo, T., Ohtsuka, Y., Fujiwara, Y., Shimada, M. and Kawakami, Y. (1989) *Metabolism* 38, 753–758.
- [11] Yoshida, K., Hirokawa, J., Tagami, S., Kawakami, Y., Urata, Y. and Kondo, T. (1995) *Diabetologia* 38, 201–210.
- [12] Okuda, Y., Ezure, M., Sawada, T., Mizutani, M., Tsukahara, K., Soma, M. and Yamashita, K. (1994) *Life Sci.* 55, P115–118.
- [13] Root, R.K., Metcalf, J., Oshino, N. and Chance, B. (1975) *J. Clin. Invest.* 55, 945–955.
- [14] Kashiwagi, A., Asahina, T., Ikebuchi, M., Tanaka, Y., Takagi, Y., Nishio, Y., Kikkawa, R. and Shigeta, Y. (1994) *Diabetologia* 37, 264–269.
- [15] Tietze, F. (1969) *Anal. Biochem.* 27, 502–522.
- [16] Bannai, S. and Kitamura, E. (1980) *J. Biol. Chem.* 255, 2372–2376.
- [17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275.
- [18] Asahina, T., Kashiwagi, A., Nishio, Y., Ikebuchi, M., Harada, N., Tanaka, Y., Takagi, Y., Saeki, Y., Kikkawa, R. and Shigeta, Y. (1995) *Diabetes* 44, 520–526.
- [19] Urata, Y., Yamamoto, H., Goto, S., Tsushima, H., Akazawa, S., Yamashita, S., Nagataki, S. and Kondo, T. (1996) *J. Biol. Chem.* 271, 15146–15152.
- [20] Flohe, L., Loschen, G., Gunzler, W.A. and Eichele, E. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* 353, 978–999.
- [21] Bannai, S. and Tateishi, N. (1986) *J. Membr. Biol.* 89, 1–8.
- [22] Kosower, N.S. and Kosower, E.M. (1978) *Int. Rev. Cytol.* 54, 109–160.
- [23] Bannai, S., Sato, H., Ishii, T. and Sugita, Y. (1989) *J. Biol. Chem.* 264, 18480–18484.
- [24] Bannai, S. (1984) *J. Biol. Chem.* 259, 2435–2440.
- [25] Orrenius, S., Ormstad, K., Thor, H. and Jewell, S.A. (1983) *Fed. Proc.* 42, 3177–3188.
- [26] Griffith, O.W. and Meister, A. (1979) *Proc. Natl. Acad. Sci. USA* 76, 5606–5610.
- [27] Bannai, S. (1979) *J. Biol. Chem.* 254, 3444–3450.
- [28] Miura, K., Ishii, T., Sugita, Y. and Bannai, S. (1992) *Am. J. Physiol.* 262, C50–58.
- [29] Anderson, M.E. and Meister, A. (1980) *J. Biol. Chem.* 255, 9530–9533.