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別タイトル	高血糖および酸化LDL 培養下のメサンギウム細胞におけるNADPH oxidase 発現とアポトーシスに対するプロブコールの効果
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# Protective Effect of Probucol Against Oxidative Cell Damage Induced by Oxidized Low-Density Lipoprotein and High Glucose in Human Mesangial Cells

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## ABSTRACT

**Background:** We previously reported that probucol decreases systemic oxidative stress and delays progressive loss of renal function in type 2 diabetes with nephropathy. Apoptosis is an important pathogenic factor for microvascular injury in diabetes. In the present study, we investigated the effect of probucol on oxidative damage induced by oxidized low-density lipoprotein (OxLDL) and high glucose in normal human mesangial cells (NHMCs).

**Methods:** Messenger ribonucleic acid (mRNA) “p22phox” and protein “p47phox” expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits was measured by real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot, respectively. Production of intracellular reactive oxygen species (ROS) was measured by fluorescence-activated cell sorting. Apoptotic signaling was evaluated by measuring caspase-3 activity.

**Results:** Both high glucose (500 mg/dl) and OxLDL enhanced intracellular ROS production. There were increases in the expression of p22phox mRNA and p47phox protein from NADPH oxidase subunits, and caspase-3 activity also increased. Probucol treatment inhibited increased expression of NADPH oxidase subunits and ROS production, as well as activation of caspase-3, induced by OxLDL and high glucose.

**Conclusions:** Probucol appears to suppress oxidative damage and activation of apoptosis signaling induced by OxLDL and high glucose in NHMCs.

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**KEYWORDS:** diabetic nephropathy, oxidative stress, apoptosis

Diabetic nephropathy is a common cause of renal failure. Despite antihypertensive therapy and strict metabolic control, overt diabetic nephropathy leads to progressive loss of renal function. Diabetic nephropathy is character-

ized by glomerular morphologic changes such as thickening of the glomerular basement membrane, mesangial expansion, and glomerulosclerosis. In the renal glomerulus, mesangial cells are vulnerable to hyperglycemia-induced

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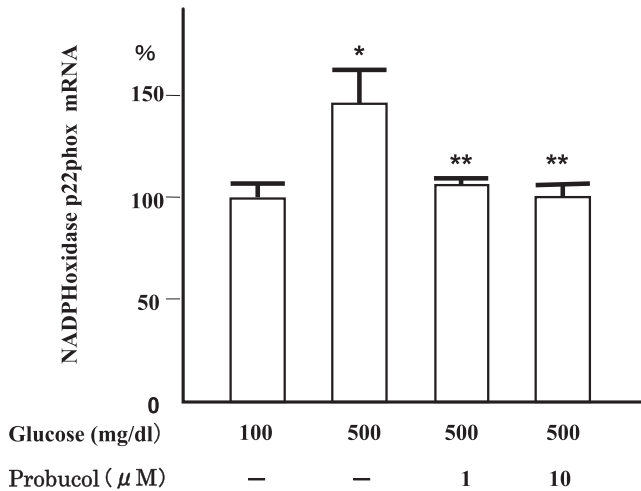


Fig. 1a: NADPH oxidase subunit p22phox mRNA expression in NHMCs cultured in normal and high concentrations of glucose.

NHMCs were incubated with 100 or 500 mg/dl glucose and treated with probucol at a final concentration of 1 or 10 μM. NADPH oxidase subunit p22phox mRNA in NHMCs was detected by RT-PCR. The products were quantified by digital scanning.

Values are expressed as mean ± SD.

\*p < 0.01 vs 100 mg/dl glucose

\*\*p < 0.01 vs 500 mg/dl glucose, probucol (—)

NADPH: nicotinamide adenine dinucleotide phosphate, mRNA: messenger ribonucleic acid, NHMCs: normal human mesangial cells, RT-PCR: reverse transcription polymerase chain reaction

stress and injury.<sup>1-3)</sup> Cell death may occur by necrosis or apoptosis. Hyperglycemia-induced apoptosis may be an important pathogenic factor for microvascular injury in diabetes.<sup>4)</sup> It has been suggested that chronic hyperglycemia injures mesangial cells by evoking pathways that lead to apoptotic cell death.<sup>5,6)</sup> Kang et al.<sup>7)</sup> reported that high glucose stimulated caspase-3 cleavage and deoxyribonucleic acid (DNA) fragmentation in cultured human mesangial cells. Several mechanisms have been proposed to explain mesangial injury by hyperglycemia, including production of inflammatory cytokines and growth factors and formation of advanced glycation end products.<sup>8-10)</sup> Furthermore, the involvement of oxidative stress is reported to be an important mechanism by which complications develop in diabetes mellitus.<sup>4)</sup>

Probucol was developed as a cholesterol-lowering agent and was found to have antioxidant effects.<sup>11)</sup> We previously reported that probucol decreased urinary 8-hydroxy-2'-deoxyguanosine—a biomarker of overall systemic oxidative stress *in vivo*—in type 2 diabetes<sup>12)</sup> and that it delayed progressive loss of renal function and prolonged initiation

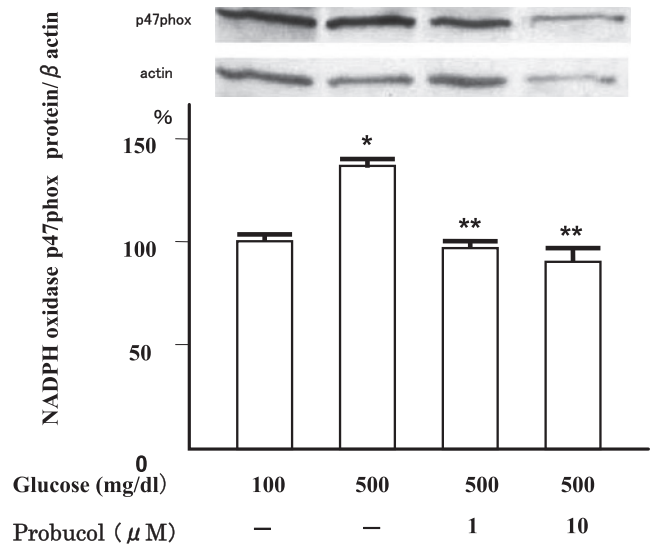


Fig. 1b: NADPH oxidase subunit p47phox protein expression in high glucose-induced NHMCs incubated with probucol.

NHMCs were incubated with 100 or 500 mg/dl glucose and treated with probucol at a final concentration of 1 or 10 μM. NADPH oxidase subunit p47phox protein in NHMCs was detected by Western blot analysis. The products were quantified by digital scanning.

Values are expressed as mean ± SD.

\*p < 0.01 vs 100 mg/dl glucose

\*\*p < 0.01 vs 500 mg/dl glucose, probucol (—)

NADPH: nicotinamide adenine dinucleotide phosphate, NHMCs: normal human mesangial cells

of hemodialysis in type 2 diabetes with overt albuminuria.<sup>13)</sup> We hypothesized that the antioxidant effect of probucol against oxidized products such as oxidized low-density lipoprotein (OxLDL), lipid peroxide, and oxysterols contributes to its renoprotective effect. However, there has been no report of an association between probucol, mesangial oxidative stress, and cell injury.

By examining the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase—an important source of reactive oxygen species (ROS)—and the apoptotic signaling pathway in cultured mesangial cells, we investigated whether probucol protects against oxidative cell damage induced by OxLDL and high glucose.

## Methods

### 1. Mesangial cell culture

Normal human mesangial cells (NHMCs) were obtained from Lonza Group Ltd. (Basel, Switzerland) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% delipidated calf serum, 2 mmol/l L-glutamine, and 40 μg/ml gentamicin. The cells ( $5.0 \times 10^4$ )

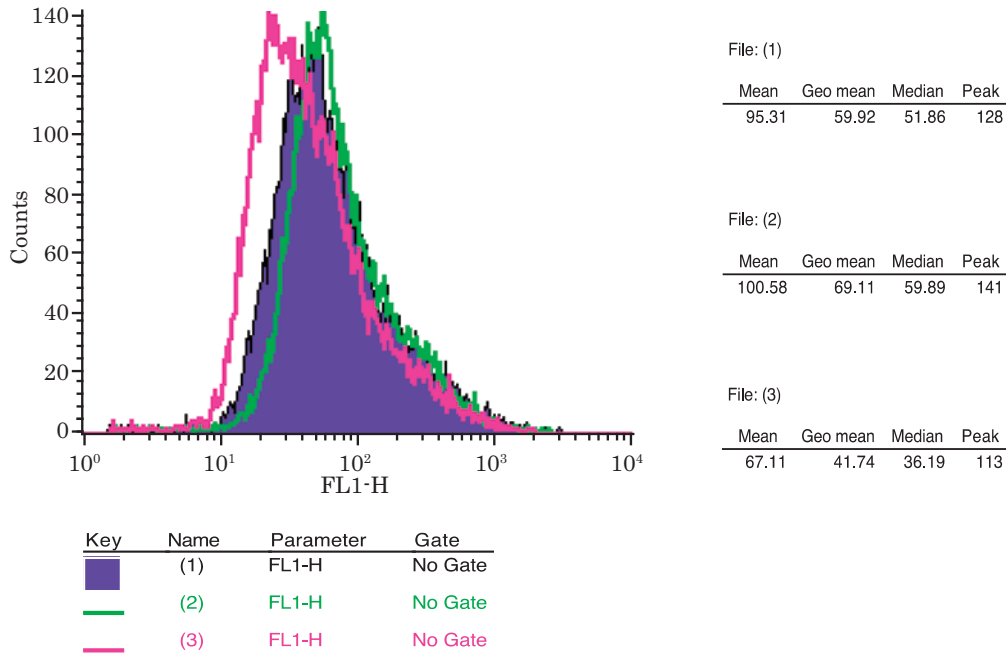


Fig. 1c: ROS in high glucose-exposed NHMCs treated with probucol

NHMCs were incubated with 100 or 500 mg/dl glucose and treated with probucol at a final concentration of 10 μM; 20000 cells were counted and ROS was measured by fluorescence-activated cell sorting (excitation λ = 488 nm, emission λ = 650 nm)

- (1) 100 mg/dl glucose, probucol (-)
- (2) 500 mg/dl glucose, probucol (-)
- (3) 500 mg/dl glucose, probucol 10 μM

ROS: reactive oxygen species, NHMCs: normal human mesangial cells, Geo mean: geometric mean

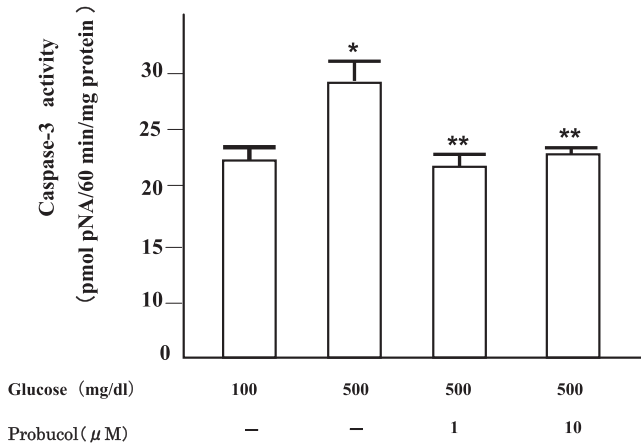


Fig. 1d: Caspase-3 activity in high glucose-exposed NHMCs incubated with probucol.

NHMCs were incubated with 100 or 500 mg/dl glucose and treated with probucol at a final concentration of 1 or 10 μM. The fluorescence of cleaved pNA was measured as caspase-3 activity by spectrofluorometry at an excitation wavelength of 405 nm.

Values are expressed as mean ± SD.

\*p < 0.01 vs 100 mg/dl glucose

\*\*p < 0.01 vs 500 mg/dl glucose, probucol (-)

NHMCs: normal human mesangial cells, pNA: p-nitroaniline

were plated in 12-well plates (or 6-well plates when measuring NADPH oxidase subunits) and incubated at 37°C in 5% CO<sub>2</sub> atmosphere for 72 h. Then the medium was replaced with DMEM containing a normal (100 mg/dl) or high (500 mg/dl) glucose concentration. After 24 h incubation, 1 or 10 μM of probucol (Daiichi Sankyo Co. Ltd., Tokyo, Japan) was added, and the cells were incubated for an additional 24 h. Probucol was dissolved in ethanol at a final concentration of 10 mM. After probucol treatment for 24 h, we investigated NADPH oxidase expression and the apoptotic signaling pathway in the NHMCs.

### 2. Preparation of OxLDL

Native low-density lipoprotein (LDL) was separated from human plasma of a healthy donor by sodium bromide stepwise density gradient ultracentrifugation at 4°C. Fractions with densities ranging from 1.019 to 1.063 g/ml were used as LDL. Native LDL was oxidized by 500 μM CuCl<sub>2</sub> at 4°C for 72 h. The reaction was stopped by adding ethylenediaminetetraacetic acid (EDTA). The OxLDL prepared under these conditions showed increased relative mobility on agarose gel electrophoresis and a higher level of thio-

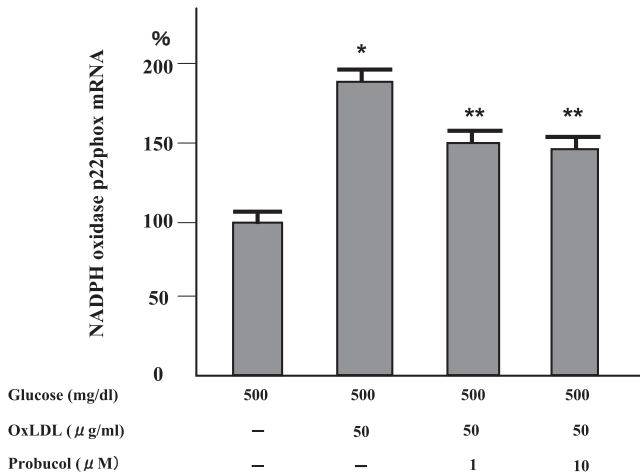


Fig. 2a: NADPH oxidase subunit p22phox mRNA expression in OxLDL-exposed NHMCs incubated with probucol.

NHMCs were incubated with 500 mg/dl glucose with or without OxLDL (50 μg/ml) and treated with probucol at a final concentration of 1 or 10 μM. NADPH oxidase subunit p22phox mRNA in NHMCs was detected by RT-PCR. The products were quantified by digital scanning. The glucose concentration in control cultures was 500 mg/dl.

Values are expressed as mean ± SD.

\*p < 0.01 vs 500 mg/dl glucose

\*\*p < 0.01 vs 500 mg/dl glucose, OxLDL (+), probucol (-)

NADPH: nicotinamide adenine dinucleotide phosphate, mRNA: messenger ribonucleic acid, OxLDL: oxidized low-density lipoprotein, NHMCs: normal human mesangial cells, RT-PCR: reverse transcription polymerase chain reaction

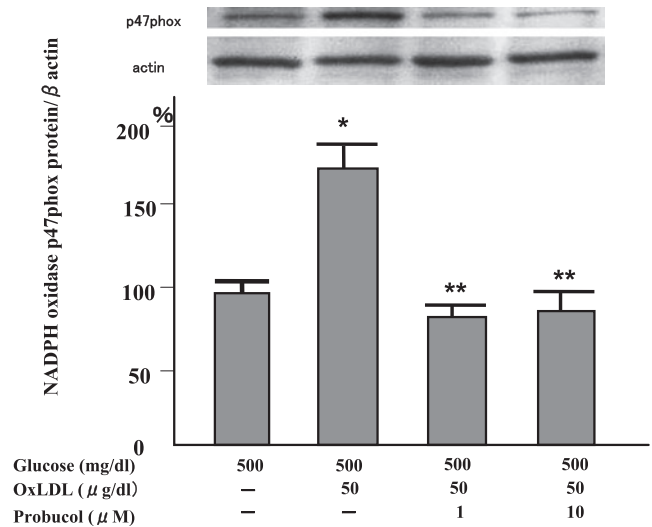


Fig. 2b: NADPH oxidase subunit p47phox protein expression in OxLDL-exposed NHMCs incubated with probucol.

NHMCs were incubated with 500 mg/dl glucose with or without OxLDL (50 μg/ml) and treated with probucol at a final concentration of 1 or 10 μM. NADPH oxidase subunit p47phox protein in NHMCs was detected by Western blot analysis. The products were quantified by digital scanning. The glucose concentration in control cultures was 500 mg/dl. Values are expressed as mean ± SD.

\*p < 0.01 vs 500 mg/dl glucose

\*\*p < 0.01 vs 500 mg/dl glucose, OxLDL (+), probucol (-)

NADPH: nicotinamide adenine dinucleotide phosphate, OxLDL: oxidized low-density lipoprotein, NHMCs: normal human mesangial cells

barbituric acid-reactive substances (TBARS). The final amount of OxLDL protein was 3.6 mg/ml. OxLDL (50 μg/ml) and probucol were added at the same time.

### 3. Western blot analysis of NADPH oxidase p47phox protein expression in mesangial cells

Western blot analysis was used to determine the relative quantities of NADH/NADPH oxidase subunit p47phox in NHMCs. Cells were suspended in a lysis buffer containing 10 mmol/l tris (hydroxymethyl) aminomethane hydrochloride [Tris-HCl (pH 7.5)], 150 mmol/l NaCl, 0.5% Triton® X-100 (The Dow Chemical Co., Midland, MI, USA), 0.5 mmol/l phenylmethylsulfonyl fluoride, and 1 mmol/l EDTA at 4°C for 4 h. After centrifugation at 12000 g, protein concentration in the supernatant was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples were diluted in an equal volume of electrophoresis sample buffer containing 100 mM Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue, and 5% b-mercaptoethanol. After boiling for 5 min, the samples were electrophoresed

on 10% SDS-polyacrylamide gel. Proteins were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA). After blotting, the membrane was washed with Tris buffer saline [TBS; 100 mM, Tris-HCl (pH 7.5), 0.9% NaCl], blocked with 5% bovine serum albumin in TBS, and then briefly washed in TBS containing 0.1% Tween-20 (TTBS). The membrane was incubated with goat polyclonal immunoglobulin G (IgG) at a dilution of 1: 1000 for 2 h at room temperature. After washing with TTBS, the blot was incubated with antibody against goat polyclonal IgG at a dilution of 1: 1000 for 1 h at room temperature and then washed with TTBS. Antigen-antibody complex was visualized by photo detection. The products were quantified by digital scanning and Image J software (Scion Corp., Frederick, MD, USA).

### 4. Subcloning mRNA fragment by reverse transcription polymerase chain reaction for NADPH oxidase p22phox in mesangial cells

Total cellular ribonucleic acid (RNA) was isolated from

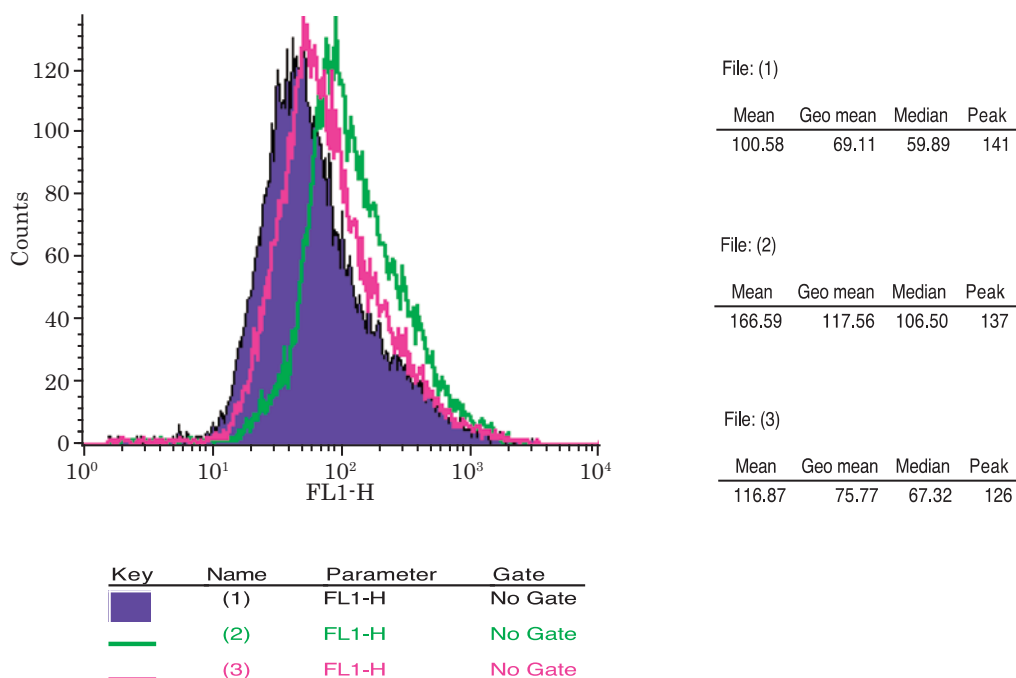


Fig. 2c: ROS in NHMCs exposed to high glucose and OxLDL and treated with probucol  
 NHMCs were incubated with 500 mg/dl glucose with or without OxLDL (50  $\mu$ g/ml) and treated with probucol at a final concentration of 10  $\mu$ M; 20000 cells were counted and ROS was measured by fluorescence-activated cell sorting (excitation  $\lambda$  = 488 nm, emission  $\lambda$  = 650 nm)

(1) glucose 500 mg/dl, OxLDL (-), probucol (-)

(2) glucose 500 mg/dl, OxLDL (+), probucol (-)

(3) glucose 500 mg/dl, OxLDL (+), probucol 10  $\mu$ M

ROS: reactive oxygen species, NHMCs: normal human mesangial cells, OxLDL: oxidized low-density lipoprotein, Geo mean: geometric mean

mesangial cell cultures in a 6-well plate using an RNeasy Kit (Qiagen GmbH, Hilden, Germany). The amount of RNA was measured as absorbance at 260 nm. Synthesis of complementary DNA (cDNA) and polymerase chain reaction (PCR) were performed using the TaKaRa RNA PCR<sup>TM</sup> Kit (Takara Bio Inc., Ohtsu, Japan). Expression of the house-keeping gene NADPH messenger RNA (mRNA) was used as an internal standard. A pair of gene-specific PCR primers was designed for NADPH oxidase (sense: TTGGTGCC TACTCCATTGTG; antisense: GAGAGCAGGAGATGCA GGAC).

Amplification was performed for 33 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 56°C for 20 s, and extension at 72°C for 18 s. PCR products were separated by electrophoresis on a 3% agarose gel and then visualized by staining with 1 mg/ml ethidium.

### 5. Detection of ROS production

ROS production was detected with 2',7'-dichlorodihydrofluorescein diacetate (Invitrogen Corp. Carlsbad, CA,

USA). After culture in 6-well plates under varying experimental conditions, the cells were washed, trypsinized, and suspended in phosphate buffered saline (PBS).

After these treatments, mesangial cells were incubated for 30 min at 37°C with 3  $\mu$ M/l 2',7'-dichlorodihydrofluorescein diacetate, then washed and scraped. Furthermore, 20000 cells were counted and were measured by fluorescence-activated cell sorting [FACSCalibur<sup>TM</sup> (excitation  $\lambda$  = 488 nm, emission  $\lambda$  = 650 nm); Becton, Dickinson and Co., Franklin Lakes, NJ, USA].

### 6. Measurement of caspase-3 activity

Caspase-3 activity was measured using a commercially available kit (CaspACE<sup>TM</sup> Assay System, Promega Corp., Madison, WI, USA), according to the instructions of the manufacturer. Cells were centrifuged at 200 g for 10 min, and cell pellets were kept on ice. The cell pellets were washed with ice-cold PBS, suspended in cell lysis buffer, and kept on ice for 15 min. After centrifugation at 15000 g for 20 min, the supernatants were collected and used as samples. Equal volumes of the assay reagent DEVD pep-

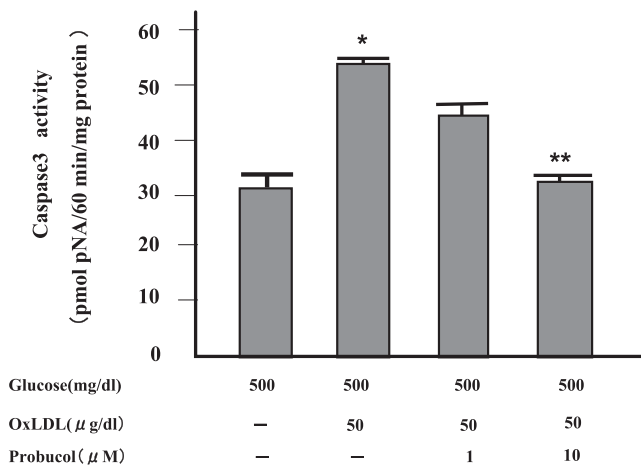


Fig. 2d: Caspase-3 activity in OxLDL-exposed NHMCs incubated with probucol.

NHMCs were incubated with 500 mg/dl glucose with or without OxLDL (50 µg/ml) and treated with probucol at a final concentration of 1 or 10 µM. The fluorescence of cleaved pNA was measured as caspase 3 activity by spectrofluorometry at an excitation wavelength of 405 nm.

Values are expressed as mean ± SD.

\*p < 0.01 vs 500 mg/dl glucose

\*\*p < 0.01 vs 500 mg/dl glucose, OxLDL (+), probucol (-)

OxLDL: oxidized low-density lipoprotein, NHMCs: normal human mesangial cells, PNA: p-nitroaniline

tide conjugated with p-nitroaniline (DEVD-pNA) and a fluorometric substrate of caspase-3 were added to the samples and incubated in a 96-well plate at 37°C for 4 h. Fluorescence of cleaved pNA was measured by spectrofluorometry at an excitation wavelength of 405 nm using an automatic microtiter plate reader (Microplate Reader EZS-ABS; Asahi Techno Glass Corp. Tokyo, Japan). Activity was normalized to total protein content.

## 7. Statistical analysis

All experiments were repeated at least 3 times in triplicate wells. All data are expressed as mean ± SD. StatView J 5.0 software (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test were done to determine if differences between groups were statistically significant. A p value less than 0.01 was considered to indicate statistical significance.

## Results

### 1. High glucose and OxLDL increased NADPH oxidase subunit expression and ROS production, activating caspase-3 in NHMCs

We evaluated oxidative stress induced by high glucose

by measuring 2 NADPH oxidase components, p22phox and p47phox, in NHMCs. First, we examined if high glucose increased expressions of NADPH oxidase subunit p22phox mRNA and p47phox protein in NHMCs. As shown in Fig. 1a, NADPH oxidase subunit p22phox mRNA expression was significantly higher in high-glucose medium than in normal-glucose medium. Similarly, subunit p47phox protein expression was significantly higher in NHMCs exposed to high glucose (Fig. 1b). We also measured intracellular ROS. High-glucose treatment stimulated production of intracellular ROS in NHMCs (Fig. 1c). In the apoptotic signal pathway, high-glucose treatment activated caspase-3 in NHMCs (Fig. 1d).

Co-treatment of OxLDL with high glucose significantly increased mRNA expression of NADPH oxidase subunit p22phox (Fig. 2a) and protein expression of subunit p47 (Fig. 2b) in NHMCs. OxLDL also stimulated production of intracellular ROS (Fig. 2c) and activated caspase-3 (Fig. 2d) in NHMCs.

### 2. Probulcol protected human mesangial cells against oxidative cell damage induced by high glucose and OxLDL

Probulcol treatment dose-dependently inhibited the increased expression of subunit p22phox mRNA and p47phox protein induced by exposure to high glucose in NHMCs (Fig. 1a, b). Probulcol (10 µM) similarly inhibited the increased production of intracellular ROS induced by high glucose (Fig. 1c) and suppressed glucose-induced activation of caspase-3 at concentrations of 1 and 10 µM (Fig. 1d).

Probulcol also protected NHMCs against oxidative stress induced by OxLDL and high glucose. Both 1 and 10 µM of probucol reduced NADPH oxidase subunit p47phox protein expression to baseline (Fig. 2a, b) and inhibited the increased production of ROS induced by OxLDL in NHMCs (Fig. 2c). In the apoptotic signal pathway, OxLDL-enhanced caspase-3 activity was significantly reduced by probucol treatment (10 µM) in NHMCs (Fig. 2d).

## Discussion

Oxidative stress contributes to progression of diabetic nephropathy. Especially in end-stage diabetic nephropathy, complications may progress even when blood pressure, glucose, and lipids are well controlled. One proposed mechanism for such progression is that the direct cytotoxic effect of oxidative stress leads to renal dysfunction and renal fibrosis. Within the cell, NADPH oxidase and protein kinase C mediate the oxidation reaction. In particu-



lar, elevation of NADPH oxidase expression increases ROS production. Multiple lines of evidence show that ROS is an important oxidative mediator in diabetic nephropathy.<sup>7,14,15</sup> Although NADPH oxidase has several subunits, and its action and distribution vary by cell and tissue, NADPH oxidase subunits are expressed abundantly in renal vessels, glomerular mesangial cells, and podocytes.

NADPH oxidase is composed of 2 membrane-associated components (p22phox and p91phox) and 4 major cytosolic components (p47phox, p40phox, p67phox, and rac-1/2). When activated, p47phox is phosphorylated, and the other cytosolic components translocate to the membrane. Several studies have shown that high glucose-induced mesangial damage is mediated through activation of NADPH oxidase subunit expression.<sup>4,16</sup> Although we did not measure NADPH oxidase activity in the present study, it is known that NADPH oxidase is activated through phosphorylation of the cytosolic subunit of p47phox and membrane subunit p22phox, and that increased expression of these subunits is sufficient to up-regulate NADPH oxidase activity.<sup>17,18</sup>

In the present study, NADPH oxidase, an index of oxidation reaction, was elevated in mesangial cells treated with high glucose. High glucose concentrations can result in direct generation of ROS from NADPH oxidase as well as activation of the polyol pathway through NADPH oxidase to produce peroxynitrite.<sup>4</sup> Both mechanisms eventually produce oxidants and cause cell injury. We also found that, as compared with high-glucose treatment alone, co-treatment with high glucose and OxLDL further up-regulated expression of NADPH oxidase in mesangial cells. OxLDL is an important cause of oxidative stress and plays a key role in the development of atherosclerotic lesions by activating and generating oxidative mediators, including ROS and nuclear factor- $\kappa$ B (NF- $\kappa$ B).<sup>19</sup> NADPH oxidase is an activator of these mediators. Although previous studies found that OxLDL induces NADPH oxidase expression, resulting in oxidative cellular damage in endothelial cells,<sup>20,21</sup> this association has not been reported in the kidney. This is the first report of the direct action of OxLDL on NADPH oxidase expression in mesangial cells.

In this study, we also showed that co-treatment with OxLDL and high glucose further elevated caspase-3 activity in the apoptotic pathway. Activation of caspases has an important role in apoptosis<sup>22,23</sup> because caspases are key proteolytic mediators in the initiation and execution of the apoptotic response.<sup>24-27</sup> Kang et al.<sup>7</sup> reported that high am-

bient glucose induces mitochondrial dysfunction and caspase-3 activation as the terminal event in NHMCs. The mechanisms of apoptosis development are complex, particularly those that involve mitochondrial dysfunction and activation of multiple apoptotic signaling pathways, such as the caspase pathway. Many studies of high glucose-induced apoptosis have suggested that high glucose triggers production of ROS, superoxide, and peroxynitrite, and activates NF- $\kappa$ B, in mesangial cells.<sup>4,7,28-30</sup> In particular, 1 report found that ROS activated caspase via NF- $\kappa$ B signal.<sup>7</sup> ROS is produced by NADPH oxidase, and our results showed that high glucose increased NADPH oxidase expression and caspase activity, which suggests that ROS generation via NADPH oxidase is a major pathway of glucose-induced mesangial cell apoptosis. Our findings showed that, in high-glucose medium, OxLDL increased caspase-3 activity in mesangial cells. Many studies reported that lipid abnormalities could have a role in the pathogenesis of glomerular oxidative injury. OxLDL contributes to these changes by producing superoxide and stimulating transforming growth factor- $\beta$  (TGF- $\beta$ ) and Ras-dependent NADPH oxidase via Rac protein in mesangial cells. This process leads to increased ROS production, culminating in mesangial cell death.<sup>31-33</sup>

The present results showed that treatment with high glucose and OxLDL upregulated NADPH oxidase expression, intracellular ROS production, and caspase-3 activation. These changes were inhibited by probucol treatment. Although the mechanism by which probucol protects against oxidative cell damage induced by high glucose and OxLDL in NHMCs is unclear, Umeji et al. suggest that probucol decreases NADPH oxidase activity by activating copper/zinc superoxide dismutase (Cu/ZnSOD).<sup>34</sup> This effect has been described as a specific antioxidant effect of probucol. In addition, Jiang et al. reported that probucol decreased NADPH oxidase activity via hemoxygenase-1 (HO-1).<sup>35</sup> Taken together, these findings suggest that probucol decreases NADPH oxidase activity via other enzymes associated with oxidation. In addition, probucol was shown to exert antioxidant effects by inhibiting LDL oxidation.<sup>36</sup> The main components in atherosclerotic lesions are foamy cells originating from macrophages. In other words, atherosclerotic lesions are produced from macrophages transformed by OxLDL. Thus, inhibition of OxLDL production by probucol could lead to regression of atherosclerotic lesions. However, this mechanism alone is not adequate to explain the inhibited progression of diabetic



nephropathy. We therefore examined other potential anti-oxidant mechanisms of probucol. Several mechanisms might be involved in the anti-apoptotic effects of probucol, e.g., inhibition of intracellular lipid peroxidation, NF- $\kappa$ B activation and p53 expression, reduction of ROS production, and upregulation of Bcl-2 level.<sup>37-40)</sup> Su et al.<sup>11)</sup> proposed that Daxx is involved in OxLDL-induced macrophage apoptosis and that probucol inhibits apoptosis through down-regulating Daxx expression. However, the mechanisms responsible for the anti-apoptotic effects of probucol in kidney remain unknown.

There was some discrepancy regarding the dose-effect of probucol in the present assays. A low dose (1  $\mu$ M) suppressed activation of caspase-3 but not the expression of NADPH subunit p22phox. NADPH oxidase is an electron transport system with membrane-bound (p22phox, gp91phox, Nox4) and cytosolic subunits (p47phox, p40phox, p67phox). The cytosolic subunits translocate to the membrane and associate with membrane subunits, resulting in rapid activation of NADPH oxidase. Translocation of p47phox and p67phox is required for NADPH oxidase activity. P47phox might be a more important element in NADPH oxidase activity and apoptosis. We did not directly measure NADPH oxidase activity in this study, and it may not be sufficient to evaluate NADPH oxidase activity by measuring the expression of NADPH oxidase subunits (i.e., p47phox and p22phox). This may be a reason for the observed discrepancy. Further studies are clearly needed to resolve this question.

### Conclusion

Both high glucose and OxLDL upregulated mRNA and protein expressions of NADPH oxidase subunits, ROS production, and activation of caspase in NHMCs. These changes were inhibited by probucol treatment, which suggests that probucol suppresses the oxidation reaction, and thus apoptotic change, in NHMCs exposed to high glucose and OxLDL. These findings support our previous clinical finding that probucol decreases a marker of systemic oxidative stress and delays progressive loss of renal function in diabetic nephropathy.

**Conflicts of interest:** None declared.

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# 高血糖および酸化 LDL 培養下のメサンギウム細胞における NADPH oxidase 発現とアポトーシスに対する プロブコールの効果

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## 要約

**目的:** われわれは 2 型糖尿病患者においてプロブコールが酸化ストレスを減少させ腎機能障害の進展を抑制する報告をした。アポトーシスは糖尿病の微小血管障害の主要病因であり本研究では nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 発現と reactive oxygen species (ROS) の産生およびアポトーシスに対するプロブコールの効果を正常人メサンギウム細胞 (normal human mesangial cells : NHMCs) で検討した。

**方法:** NHMCs は正常糖 (100 mg/dl), 高糖状態 (500 mg/dl) にて培養し酸化 low-density lipoprotein (LDL), プロブコール (1,10 μM) を添加した。NADPH oxidase サブユニットの messenger ribonucleic acid (mRNA) “p22phox”, 蛋白量 “p47phox” はそれぞれ reverse transcriptase-polymerase chain reaction (RT-PCR), ウェスタンブロット法で, ROS 産生は fluorescence activated cell sorting (FACS) を用いアポトーシスはカスパーゼ 3 活性で評価した。

**結果:** 高糖状態および酸化 LDL いずれも p22phox, p47phox の発現が増加し ROS の産生およびカスパーゼ 3 の活性も高めた。プロブコールはこれらの変化を抑制した。

**考察:** 本研究は高糖, 酸化 LDL 添加状態の NHMCs で, プロブコールが酸化反応とそれによるアポトーシスを抑制することを示唆した。

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