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Effects of Uremic Toxin *p*-Cresol on Proliferation, Apoptosis, Differentiation, and Glucose Uptake in 3T3-L1 Cells

*Sayuri Tanaka, †Shozo Yano, †Abdullah M. Sheikh, †Atsushi Nagai, and *Toshitsugu Sugimoto

**Departments of Internal Medicine and †Laboratory Medicine, Shimane University Faculty of Medicine, Izumo, Japan*

Abstract: Malnutrition is a common feature seen in chronic dialysis patients, and the survival rate of obese patients receiving such treatment is higher than that of lean patients. Irrespective of obesity or diabetes, dialysis patients commonly have insulin resistance, and the leading cause of death is cardiovascular (CV) disease. It has been reported that the concentration of *p*-cresol, a uremic toxin, is highly associated with CV events. As uremic toxin levels are high in dialysis patients, they may be involved in the pathogenesis of insulin resistance and CV disease in this population. However, little is known so far. Thus, we focused on this uremic toxin to examine its effects on adipocytes and their precursors. 3T3-L1 cells, a mouse preadipocyte cell line, were cultured until 90% confluency. The cells were then differentiated with 500 μM 3-isobutylmethylxanthine, 250 nM dexamethasone, and 10 μg/mL insulin. Cell proliferation was evaluated by cell counting and bromodeoxyuridine (Brd-U) incorporation assay. Glucose uptake was estimated using radiolabeled 2-deoxyglucose. The range of concentrations of *p*-cresol used in the experiments was from 2 to 200 μM. The investigation of cell proliferation by cell counting revealed that, compared with control, 3T3-L1 cells treated with 100 and 200 μM *p*-cresol were significantly decreased in number at day 3 and day 7 of culture. The Brd-U incorporation assay also demonstrated similar inhibitory effects on cell proliferation, suggesting that *p*-cresol affected the normal cell cycle. Oil Red O staining at day 7 showed that the number of mature adipocytes was decreased by treatment with 200 μM *p*-cresol. Consistent with that finding, the number of apoptotic cells at day 7 was increased by treatment with 100 and 200 μM *p*-cresol. Peroxisome proliferator-activated receptor γ (PPARγ) mRNA expression increased time-dependently during the differentiation process of 3T3-L1 cells. *p*-Cresol dose-dependently decreased differentiation-induced mRNA expression of PPARγ. Uptake of ^{3}H-labeled 2-deoxyglucose was markedly decreased by 200 μM *p*-cresol in the presence or in the absence of insulin, mainly because of the decreased number of mature adipocytes. High concentrations of *p*-cresol disturbed the cell cycle, induced apoptosis, inhibited the differentiation of preadipocytes into mature adipocytes, and decreased glucose uptake at baseline and after insulin stimulation. These findings indicate that accumulated *p*-cresol may induce reduction in adipose tissue, insulin resistance, and malnutrition, eventually leading to poor outcomes in chronic dialysis patients. **Key Words:** Uremic toxin—*p*-Cresol—Adipocyte—Glucose uptake—Insulin resistance.

Malnutrition and sarcopenia are commonly seen in chronic dialysis patients, which might be the result of restricted protein intake and hypercatabolism. In this population, a body fat mass to muscle mass ratio has been reported to be increased (1). However, a prospective study found that, 2 years after the initiation of dialysis therapy, body fat mass was markedly decreased (2). Moreover, dialysis patients with obesity have a better survival rate (3) and a decreased cardiovascular (CV) death rate compared with lean patients (4–6), which is a case of so-called reverse epidemiology.

Chronic kidney disease (CKD) patients exhibit insulin resistance, even without obesity or diabetes (7). Insulin resistance is likely to have a close relationship with arteriosclerosis and CV events (8). According to previous research regarding insulin

doi:10.1111/aor.12252

Received August 2013; revised October 2013.

Address correspondence and reprint requests to Dr. Shozo Yano, 89–1 Enya-cho, Izumo City, Shimane 693–8501, Japan. E-mail: syano@med.shimane-u.ac.jp

Presented in part at the 50th ERA-EDTA Congress held May 18–21, 2013 in Istanbul, Turkey.

resistance in renal failure patients, glucose uptake is decreased, mainly in the muscle and adipose tissue (9). Thus, adipocytes might provide a valuable insight into the underlying mechanisms of malnutrition and insulin resistance in this patient population.

Uremic toxins are believed to be involved in the development of insulin resistance and CV events in CKD patients. For example, the concentration of the uremic toxin *p*-cresol, which is reported to be associated with CV events, is high in the blood of CKD patients (10). Moreover, due to its high protein-binding ability, its concentration barely decreases in dialysis patients (24 mg/L = 200 μM) even after a hemodialysis session, while it is hardly detectable in healthy subjects (11). Taken together, these findings suggest that *p*-cresol might play a vital role in the development of insulin resistance and CV events in dialysis patients. However, the underlying mechanism remains to be explored. In this study, we have investigated the role of *p*-cresol in adipocytes with respect to proliferation, differentiation, and glucose uptake. We found that *p*-cresol decreases the number of mature adipocytes by inhibiting proliferation and differentiation and inducing apoptosis. These findings suggest that *p*-cresol is involved in the reduction of fat mass and the development of insulin resistance in dialysis patients.

MATERIALS AND METHODS

Materials

3T3-L1 mouse preadipocytes were purchased from the American Type Culture Collection (Rockville, MD, USA). Cell culture medium and supplements were purchased from Gibco-BRL (Rockville, MD, USA). All other chemicals were of the highest grade available commercially.

Cell culture

3T3-L1 mouse cells were cultured in 5% CO_2 at 37°C in humid conditions in high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco-BRL). The medium was changed twice a week. For induction of differentiation, after reaching confluency the cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, 1 μg/mL insulin, 0.25 μM dexamethasone, and 0.5 mM 3-isobutyl-l-methylxanthine.

Oil Red O staining

After appropriate treatment, cells were fixed using 10% formalin in phosphate-buffered saline (PBS). Then the cells were treated with 10% isopropanol for 1 min and stained with 60% filtered Oil Red O (Sigma, St. Louis, MO, USA) for 20 min. After every step, the cells were washed with PBS. After staining, the cells were analyzed under a microscope.

Evaluation of cell growth

Cell proliferation was evaluated by cell counting with the use of an automatic cell counter (145-0001J1, Ikeda Scientific, Tokyo, Japan) and a bromodeoxyuridine (Brd-U) cell proliferation assay kit (Roche Molecular Biochemicals, Mannheim, Germany). 3T3-L1 cells were seeded on 96-well plates at a density of 2×10^4 cells/well. The cells were cultured for 24 h in DMEM supplemented with 10% FBS and antibiotics. Then the cells were treated with dimethyl sulfoxide (DMSO; control) or *p*-cresol at various concentrations (2, 20, 100, and 200 μM). On the next day, cells were pulse-labeled for 2 h with 10 M Brd-U. Then the cells were incubated with diluted peroxidase-conjugated anti-Brd-U antibody for 30 min. The absorbance was measured at 450 nM using a microplate reader (Model 3550, Bio-Rad, Richmond, CA, USA).

Measurement of apoptotic cell death

3T3-L1 cells were seeded on 96-well plates at a density of 2×10^4 cells/well and cultured overnight in DMEM with 10% FBS and antibiotics. On the next day, the cells were treated with differentiation medium. At the same time, the cells were treated with DMSO control or *p*-cresol at various concentrations (2, 20, 100, and 200 μM) for 1, 3, or 7 days. Then, the cells were lysed, and the supernatant was analyzed for DNA fragments using an enzyme-linked immunosorbent assay (ELISA) system (Cell Death Detection ELISA Plus, Roche Molecular Biochemicals).

Real-time PCR quantification of gene expression

SYBR Green chemistry was used to perform quantitative determination of the mRNA of peroxisome proliferator-activated receptor γ (PPARγ) and a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), following an optimized protocol (12–14). Briefly, total RNA was isolated with TRIzol reagent (Invitrogen, San Diego, CA, USA) and further cleaned by two successive phenol/chloroform extractions. First-strand cDNA was synthesized with an oligo-dT primer and a SuperScript III cDNA synthesis kit (Invitrogen). The design of sense and antisense oligonucleotide primers was done with Primer Express software (version 2.0.0,

Applied Biosystems, Carlsbad, CA, USA) using published PPARγ and GAPDH mRNA sequences. Gene-specific cDNA was amplified with an ABI PRISM 7000 sequence detection system (Applied Biosystems). The DNA-specific SYBR Green was added into the PCR buffer provided in the QuantiTect SYBR PCR kit (QIAGEN, Valencia, CA, USA) to allow for quantitative detection of the PCR product in a 25-μL reaction volume. The temperature profile of the PCR reaction was 60°C for 2 min, followed by 95°C for 15 min, then 40 cycles of denaturation at 94°C for 15 s, and annealing and extension at 60°C for 1 min.

^{3}H-labeled 2-deoxyglucose uptake

Differentiated 3T3-L1 adipocytes were treated with various concentrations (2, 20, 100, and 200 μM) of *p*-cresol or DMSO for 7 days in 12-well plates. After washing with 2 mL/well of serum-free DMEM, cells were incubated in 2 mL/well of serum-free DMEM with the indicated concentrations of *p*-cresol or DMSO for 3 h at 37°C. Cells were then washed twice with 1 mL/well of glucose-free serum-free DMEM. Cells were further incubated at 37°C for 30 min in glucose-free serum-free DMEM for each concentration of *p*-cresol and DMSO in the absence (baseline) or presence (100 nM) of insulin. The glucose uptake was determined after the addition of ^{3}H-labeled 2-deoxyglucose (2-DOG; final concentration of 50 mM, 0.037 MBq/mL; Amersham Biosciences, Piscataway, NJ, USA).

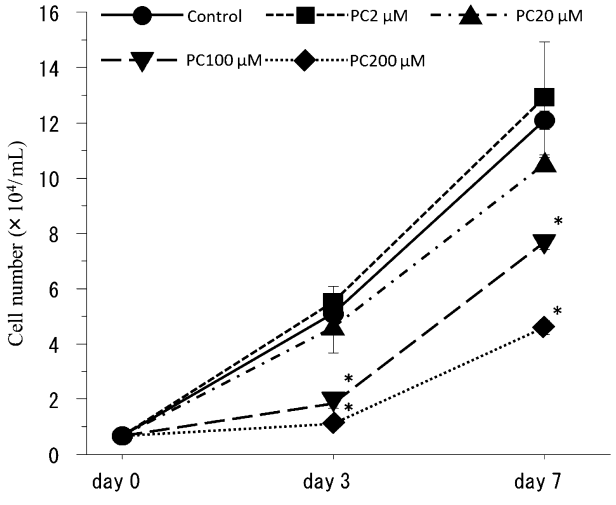

FIG. 1. Effect of *p*-cresol on the growth of 3T3-L1 cells. The number of cells cultured with DMSO control or *p*-cresol at each concentration (2, 20, 100, and 200 μM) was measured at day 0, day 3, and day 7. **P* < 0.0001 compared with the control. The results are expressed as the mean ± SEM over control values (*n* = 4).

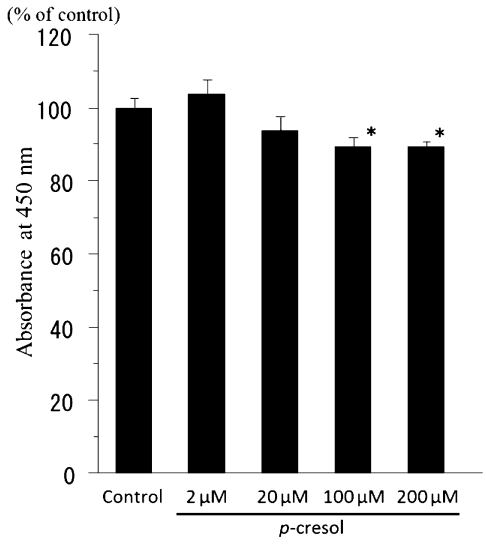

FIG. 2. Effect of *p*-cresol on the proliferation of 3T3-L1 cells. A Brd-U cell proliferation assay was performed in cells cultured with or without *p*-cresol. The details are described in the Materials and Methods section. Each group was compared with the control group (% of control). **P* < 0.05 compared with the control. The results are expressed as the mean ± SEM over control values (*n* = 6).

Statistical analysis

Results are expressed as mean ± SEM. Statistical evaluation of the differences between the groups was carried out with one-way ANOVA followed by Fisher's protected least significant difference, or with paired *t*-test, if appropriate. For all statistical analysis, a value of $P < 0.05$ was considered a statistically significant difference.

RESULTS

Effects of *p*-cresol on proliferation of 3T3-L1 cells

We examined the time-dependent changes in 3T3-L1 cell number during proliferation phase. The cell count showed that the time-dependent increase in cell number was significantly inhibited by treatment with 100 and 200 μM *p*-cresol ($P < 0.0001$) at day 3 and day 7, while no significant decrease was observed at 20 μM or lower concentrations of *p*-cresol (Fig. 1). In agreement with the cell count, the Brd-U cell proliferation assay also demonstrated a decrease in Brd-U incorporation in cells treated with 100 and 200 μM *p*-cresol, suggesting an inhibition of cell proliferation (Fig. 2). Although there seems to be a discrepancy in terms of the rate of proliferation suppression between Figs. 1 and 2, this may have resulted from apoptotic cell death, as explained further on.

Inhibition of adipogenesis in 3T3-L1 cells by *p*-cresol

Effects of p-*cresol on morphological change and adipogenesis in 3T3-L1 cells*

After differentiation for 7 days, Oil Red O staining revealed multiple lipid droplets in mature adipocytic 3T3-L1 cells (Fig. 3a). In the case of the cells treated with 2 and 20 μM *p*-cresol, no significant change was found with respect to adipocytic differentiation (Fig. 3b,c). In contrast, treatment with 200 μM *p*-cresol led to a marked decrease in the number of lipid-laden mature adipocytes (Fig. 3d), suggesting that a high concentration of *p*-cresol inhibited the differentiation of preadipocytes into adipocytes. As the total cell number was decreased in this group, apoptotic cell death most probably occurred.

p-*Cresol increases apoptosis of 3T3-L1 cells*

Next, we examined the effect of *p*-cresol on apoptosis of 3T3-L1 cells. The number of apoptotic cells during differentiation was determined by an ELISA kit for DNA fragments. Apoptotic cell death was significantly increased by 100 and 200 μM *p*-cresol at day 3 and day 7 ($P < 0.05$) (Fig. 4). Thus, in addition to suppressed proliferation (Fig. 2), increased apoptotic cell death could be involved in the marked decrease in cell number at high concentrations of *p*-cresol, as shown in Figs. 1 and 3d.

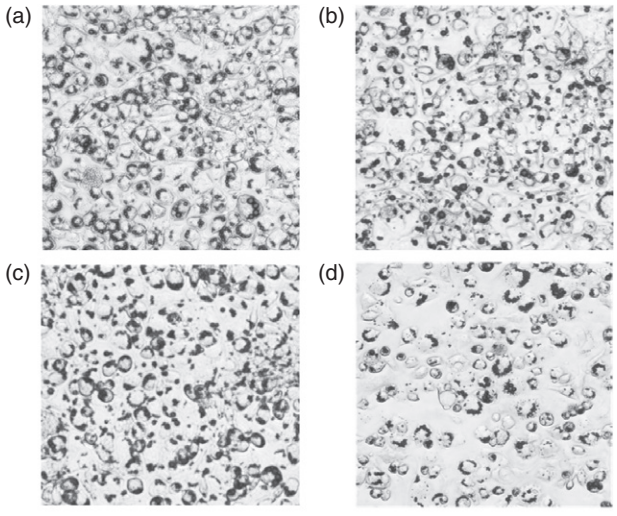

FIG. 3. Effects of *p*-cresol on morphological change and adipogenesis of 3T3-L1 cells. 3T3-L1 preadipocytes were subjected to a differentiation protocol in the presence of DMSO control (a), 2 μM *p*-cresol (b), 20 μM *p*-cresol (c), or 200 μM *p*-cresol (d), and stained with Oil Red O.

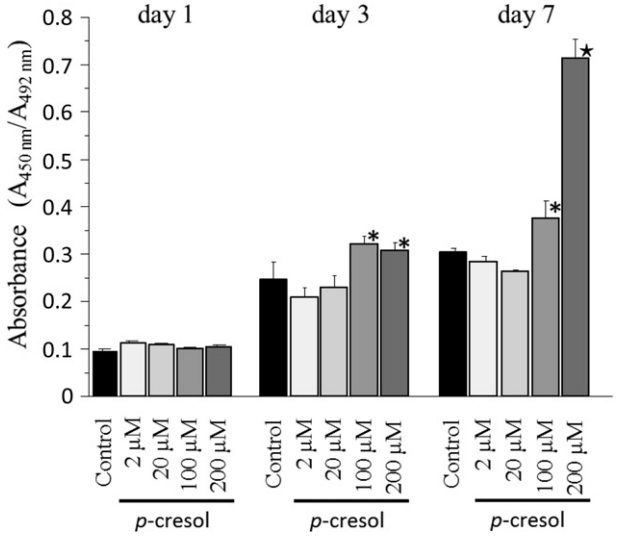

FIG. 4. Dose-dependent effect of *p*-cresol on apoptosis during differentiation of 3T3-L1 cells. Cells were cultured with DMSO control or *p*-cresol (2, 20, 100, and 200 μM). Apoptosis was evaluated by ELISA for DNA fragments at day 1, day 3, and day 7.*$P < 0.05$, ★$P < 0.0001$, compared with control. The results were expressed as the mean ± SEM over control values ($n = 6$).

PPARγ expression during the differentiation of 3T3-L1 cells to adipocytes

PPARγ is one of the main regulators of adipocyte differentiation. Hence, we examined time-dependent changes in PPARγ mRNA expression in 3T3-L1 cells during the process of adipocytic differentiation. The expression of mRNA is known to be increased during differentiation, reaching a peak at 3 to 4 days after induction, followed by a steady state. Thus, we isolated total RNA from postconfluent 3T3-L1 cells at days 0, 1, 3, and 7 after the induction of standard differentiation. In the control cells, an approximately eightfold increase in PPARγ mRNA over day 0 was observed at day 3, and a similar level was maintained up to day 7 (Fig. 5a). While similar time course and expression level were observed in the cells treated with 2 and 20 μM *p*-cresol, PPARγ mRNA expression was significantly decreased with 100 and 200 μM *p*-cresol treatment compared with the control at the same time point (Fig. 5b). This indicates that a high concentration of *p*-cresol may delay the differentiation and maturity of adipocytes.

Effects of *p*-cresol on glucose uptake

We next examined the effect of *p*-cresol on glucose uptake in 3T3-L1 adipocytes, using 2-DOG. After reaching subconfluency, 3T3-L1 adipocytes were incubated with differentiation medium in the presence of increasing doses of *p*-cresol (from 2 to 200 μM) or DMSO for 7 days. The glucose uptake

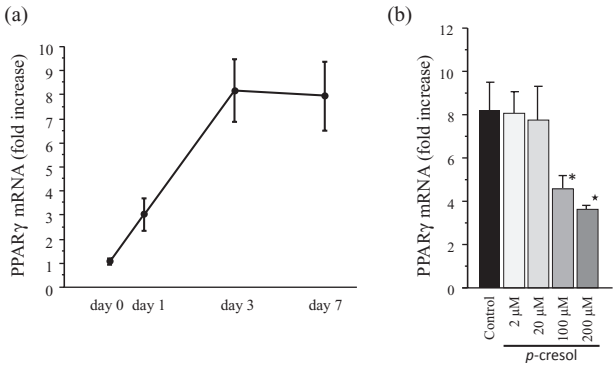

FIG. 5. Time course of PPARγ mRNA expression in 3T3-L1 adipocyte precursor cells during maturation. (a) PPARγ mRNA levels in cells treated with DMSO control ($n = 10$) at day 0, day 1, day 3, and day 7. (b) PPARγ mRNA levels in cells treated with *p*-cresol (2, 20, 100, and 200 μM) and DMSO control at day 3 ($n = 8$). The values were expressed as the fold increase over DMSO control at day 0.*$P < 0.05$, ★$P < 0.005$, compared with the control. The results were expressed as the mean ± SEM over control values ($n = 8$).

experiment showed that the baseline glucose uptake was significantly inhibited in the cells treated with *p*-cresol concentrations of 100 and 200 μM (Fig. 6a). Such inhibition of glucose uptake by high concentrations of *p*-cresol was also observed when 3T3-L1 cells were stimulated with insulin. To evaluate the response to insulin, we calculated the ratio of glucose uptake between the presence and the absence of insulin. As a result, a significant reduction was observed at 200 μM *p*-cresol (Fig. 6b), indicating that resistance to insulin might be developed in response to high concentrations of *p*-cresol.

DISCUSSION

In nonuremic subjects with normal glucose tolerance, insulin resistance increases the CV event risk, even after adjustment for blood pressure and the presence of hyperlipidemia (15,16). In healthy subjects, excess weight resulting from an increase in adipose tissue is the leading cause of insulin resistance (17–19). Regardless of the existence of obesity, CKD patients have been found to be insulin-resistant as compared with healthy subjects (2). Moreover, in dialysis patients, the rate of death due to CV event is approximately 30 times higher than that of the general population (20). It should be noted that in chronic dialysis patients, the rate of mortality due to CV event is higher in lean patients compared with obese ones (7–9). Insulin resistance might be involved in the high mortality of dialysis patients, although the exact cause remains to be elucidated.

Therefore, we investigated whether uremic toxins contribute to insulin resistance. In the present study, we found that *p*-cresol at a concentration found in the blood of dialysis patients inhibited proliferation, adipocytic maturation, and glucose uptake of 3T3-L1 cells, suggesting that it may be responsible for the loss of fat mass and the development of insulin resistance in nonobese chronic dialysis patients. Moreover, the toxic effect was also accompanied by an increase in apoptosis and decrease in the number of mature adipocytes, which are most likely attributable to reduction in glucose uptake. In addition, high concentrations of *p*-cresol inhibited insulin-induced glucose uptake. This finding suggests that *p*-cresol reduces glucose uptake not only by decreasing the number of mature adipocytes but also by attenuating insulin sensitivity. A previous report showed that urea, a uremic toxin, causes insulin resistance through generating reactive oxygen species in mice with renal failure (21), which is consistent with our findings.

The toxicity of *p*-cresol has previously been shown by various in vitro experiments. For instance, *p*-cresol decreases the endothelial cell response to

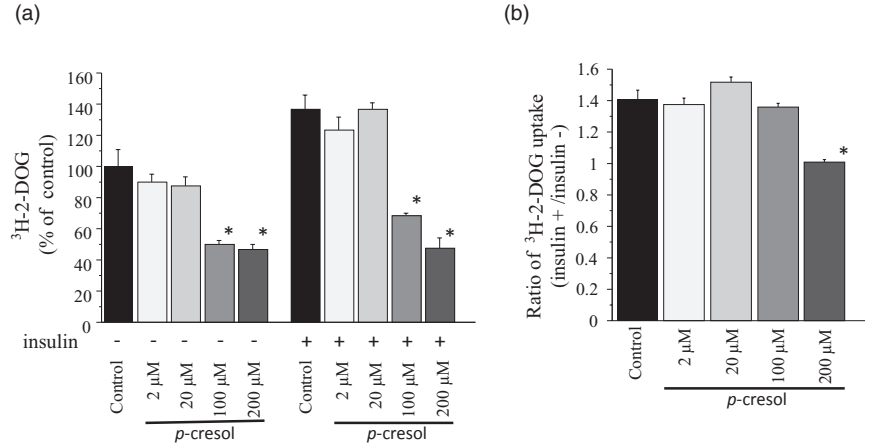

FIG. 6. Effects of *p*-cresol on glucose uptake in 3T3-L1 adipocytes. (a) 3T3-L1 cells were treated with indicated concentrations of *p*-cresol or DMSO control for 7 days during differentiation, followed by the measurement of either the baseline or insulin-stimulated (100 nM) 2-DOG uptake. (b) The ratio of 2-DOG uptake with insulin pretreatment to that without. *$P < 0.001$ compared with the individual control.

inflammatory cytokines (22), and this may contribute to the pathogenesis of CV events in uremic patients. Moreover, in a prospective clinical study, multivariate analysis showed that higher concentrations of free *p*-cresol were significantly associated with CV events in patients with mild to moderate kidney disease, indicating that *p*-cresol may be a good predictor of CV events independent of kidney function (3). In our study, we found that *p*-cresol might be involved in reduced fat mass and the development of insulin resistance. Hence, these findings are compatible with the concept of "reverse epidemiology," whereby lean patients undergoing dialysis therapy have a higher mortality rate than obese patients.

CONCLUSION

p-Cresol inhibited proliferation and differentiation and induced apoptosis in 3T3-L1 cells. These findings indicate that the accumulation of uremic toxins may induce reduction in adipose tissue, insulin resistance, and eventually a poor outcome in chronic dialysis patients. New therapeutic methodologies that target the reduction or removal of uremic toxins such as *p*-cresol may lead to better prognosis in dialysis patients.

Acknowledgments: The authors thank Ms. Keiko Nagira for her technical help. This work is partly supported by Grant-In-Aids for Scientific research, Kakenhi (C) (24591230) (to SY).

Conflict of Interest: All authors have no conflicts of interest.

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