Effect of Cinacalcet on the Redox Status of Albumin in Secondary Hyperparathyroidism Patients Receiving Hemodialysis

Tadashi Imafuku, a,b Motoko Tanaka, b,Koki Tokunaga, a Shigeyuki Miyamura, c Hiromasa Kato, a Shoma Tanaka, a Takehiro Nakano, a Kenshiro Hirata, a Daisuke Kadowaki, c Hitoshi Maeda, a Kazutaka Matsushita, b Masaki Otagiri, c Hirotaka Komaba, d Masafumi Fukagawa, d Hiroshi Watanabe,* a and Toru Maruyama*, a

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Chronic kidney disease (CKD) patients with secondary hyperparathyroidism (SHPT) have an increased risk of cardiovascular disease (CVD). Cinacalcet is a calcimimetic that permits impaired endothelial functions to be recovered via inhibiting parathyroid hormone (PTH) production in SHPT patients. However, the underlying mechanism for its action remains unknown. The purpose of this study was to examine the effect of cinacalcet on the redox state of human serum albumin (HSA), a reliable marker for assessing endothelial oxidative damage in SHPT patients who were receiving hemodialysis. Cinacalcet was administered to six SHPT patients for a period of 8 weeks. After 4 weeks of treatment, cinacalcet significantly decreased the oxidized albumin ratio which is a ratio of reduced and oxidized forms of HSA via increasing reduced form of HSA. Moreover, the radical scavenging abilities of HSA that was isolated from SHPT patients were increased by cinacalcet, suggesting the recovery of the impaired vascular anti-oxidant ability. Interestingly, the oxidized albumin ratio in SHPT patients was significantly higher than that in hemodialysis patients. In addition, the changes of intact PTH levels were significantly correlated with the oxidized albumin ratio. It therefore appears that PTH may induce oxidative stress in SHPT patients. In fact, an active analogue of PTH increased the production of reactive oxygen species in human endothelial cells. Thus, cinacalcet exhibits anti-oxidative activity through its pharmacological action. Additionally, cinacalcet itself showed radical scavenging activity. In conclusion, cinacalcet improves the redox status of HSA by inhibiting PTH production and partially by its radical scavenging action.

Key words parathyroid hormone; hemodialysis; oxidative stress; albumin oxidation; secondary hyperparathyroidism; anti-oxidant

INTRODUCTION

Human serum albumin (HSA) is the most abundant protein in the plasma, making up 60–65% of the total plasma proteins. An increasing body of evidence has accumulated to demonstrate that cysteine 34 (Cys34) in HSA, which accounts for approximately 70–80% of the total free thiol (SH) groups in plasma, serves as a major anti-oxidant in plasma and in the extracellular compartment, significantly contributing to the maintenance of vascular homeostasis. 1,2 Data concerning the redox characteristics of HSA which corresponds to a balance between the reduced form of Cys34 (referred to as mercapto-HSA (HMA)) and its oxidized form (referred to as nonmercapto-HSA (HNA)), under various pathophysiological conditions have recently become available. For example, the oxidized albumin ratio which is the ratio of HMA and HNA (HMA/HNA), was well correlated with the anti-oxidative activity of HSA, and hence corresponded to the anti-oxidant function in plasma. 1–6 Interestingly, such an increase in the oxidized albumin ratio has been reported to be significantly associated with disease progression in oxidative stress related diseases, such as chronic liver diseases and chronic kidney disease (CKD) etc. 7–13 Because of this, it has been proposed that the oxidized albumin ratio can serve as a reliable marker for assessing the extent of systematic oxidative damage under various conditions. Based on the findings reported by us and other groups, Colombo et al. proposed a unique concept referred to as “redox albuminomics” in which HSA is oxidized in vivo, and the process is correlated with organ dysfunction. 14 Cinacalcet, a member of calcimometics family, suppresses parathyroid hormone (PTH) secretion by binding allosterically to the calcium-sensing receptor on the membrane surface of parathyroid cells, has been developed to treat secondary hyperparathyroidism (SHPT) which is characterized by the hypersecretion of PTH from the parathyroid gland. 15,16 It should also be noted that SHPT is frequently associated with CKD patients because decreased serum calcium levels that occur during CKD pathogenesis induce the hypersecretion of PTH. 17–21 Therefore, cinacalcet is frequently administered to CKD patients with SHPT. A number of clinical studies have demonstrated that cinacalcet treatment effectively decreases the concentrations of circulating PTH and serum calcium and phosphorus concentrations. 22–24 More importantly, cinacalcet may suppress the onset and progress of cardiovascular disease...
(CVD) in SHPT patients who had a high morbidity rate of CVD that was a major cause of their death.\(^{25–27}\)

Oxidative stress has been accepted as a non-classical factor for the onset and development of CVD in CKD.\(^{28,29}\) In particular, the development of oxidative stress is closely related to vascular damage, which included the induced thickening of vascular smooth muscle, the migration of immune cells, atherosclerosis and vascular restenosis.\(^{30,31}\) Interestingly, Terawaki et al. reported that a lower level of HMA was associated with mortality due to cardiovascular disease in hemodialysis patients.\(^{32}\) These findings lead us to hypothesize that recovering of redox albuminomics by reducing oxidative stress may be an attractive strategy for the treatment of CVD in CKD patients with SHPT. Kuczera et al. and Ari et al. recently reported that cinacalcet treatment reduced oxidative stress in hemodialysis patients with SHPT, thus permitting their endothelial dysfunction to be recovered.\(^{33,34}\) However, whether cinacalcet affects redox albuminomics in SHPT patients, and whether it exerts its anti-oxidative action directly or indirectly remains unknown.

The purpose of this study was to examine the effect of cinacalcet on the redox status of HSA in SHPT patients who were receiving hemodialysis. Additionally, the mechanism by which cinacalcet exerts its anti-oxidative activity was also investigated.

**MATERIALS AND METHODS**

**Study Population** The study candidates were 6 stable hemodialysis patients with SHPT (2 men and 4 women) who were at least 54 years of age and who had required for maintenance dialysis for at least 4 years. They had been admitted to the Department of Nephrology of the Akebono Clinic of Japan. The eligibility criteria were serum intact parathyroid hormone (iPTH) level of at least 250 pg/mL. In this study, the daily dose of cinacalcet was adjusted to between 25 to 50 mg. Patients who had a history of surgical parathyroidectomy were excluded. All the patients had been previously administered an angiotensin receptor antagonist (ARB), such as olmesartan or telmisartan, and a vitamin D receptor activator (VDR), such as alfacalcidol or calcitriol at least for one year, and the doses of those drugs were not altered during the study period. Table 1 lists the baseline clinical characteristics of the study patients.

Healthy volunteers (4 men and 2 women) were common adult (24–28 years old) in our laboratory. The average of age and time on hemodialysis (HD) are 66.7 ± 8.2 years old and 11.9 ± 7.6 years in hemodialysis patients without SHPT (3 men and 3 women).

The protocol used in this study was approved by the institutional review board and informed consent was obtained from all subjects. This study is registered with the Cochrane Renal Group Registry, number CRG120700131. The study was approved by “Ethics Committee of the Akebono Clinic.” All samples were obtained with written informed consent and reviewed by the ethical board of the corresponding clinic.

**Determination of Biochemical Markers** Approximately 10 mL blood was drawn just prior to dialysis, and serum was collected by centrifugation at 3000 rpm for 15 min at 4°C. The specimens were immediately stored at −80°C until used in analyses. Serum levels of calcium and phosphorus were measured at a commercial laboratory (SRL, Tokyo, Japan). The serum iPTH levels were measured by an Elecsys PTH assay (Roche Diagnostics, Mannheim, Germany; normal range, 15–65 pg/mL).

**Determination of the Oxidized Albumin Ratio** The redox status of HSA was determined by an HPLC system, as reported previously\(^{35}\) at 0, 2, 4, 6 and 8 weeks after oral cinacalcet treatment. Briefly, 10 folds diluted serum samples were analyzed using a Shodex Asahipak ES-502N column (Showa Denko, Tokyo, Japan). From the HPLC profile, the content of HMA and HNA was estimated by dividing the area of each fraction by the total area corresponding to HSA. Using these values, we estimated the oxidized albumin ratio.

**Measurement of Thiol Content in Serum Samples** Total thiol content in serum samples obtained from subjects were estimated by the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) method, as previously reported.\(^{36}\) Briefly, the increase in absorbance at 405 nm was monitored against time after the addition of DTNB (Dojin Kagaku, Kumamoto, Japan). A chloramine-T (Nacalai Tesque, Kyoto, Japan) solution was used as the external calibration standard.

**Measurement of Advanced Oxidation Protein Products (AOPPs) in Serum Samples** Serum AOPPs levels were determined by spectrometry (340 nm) as described previously.\(^{37}\) In a typical experiment, a 100 µL aliquot serum was diluted ten-fold with phosphate-buffered saline (PBS) transferred to 96-well plate and 20 µL of acetic acid and 10 µL of 1.16 M potassium iodide solution added. A chloramine-T (Nacalai Tesque, Kyoto, Japan) solution was used as the external calibration standard.

**Measurement of Radical Scavenging Ability of HSA Isolated from Serum Samples of SHPT Patients with Cinacalcet and of Cinacalcet Itself** The radical scavenging ability of isolated HSA or cinacalcet was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azobis-2-amidinopropane dihydrochloride (APPH) according to a previous report.\(^{38,39}\)

HSA was purified from serum samples using a previously reported method in which a Blue Sepharose 6 Fast Flow column (GE Healthcare, Tokyo, Japan) was used. The samples were then dialyzed against deionized water.

**Measurement of Reactive Oxygen Species (ROS) Production by PTH Using in Vitro Study** Human umbili-
cal vein endothelial cells (HUVEC) (American Type Culture Collection, Manassas, VA, U.S.A.) were cultured according to previous reports. To measure the production of ROS, CM-H2DCFCA (Thermo) was used as an ROS probe. HUVEC were incubated in 96-well plates (1 × 10^4 cells/well) in their medium at 37°C for 24 h, and then with 5 μmol/L CM-H2DCFCA for 30 min in D-PBS. After removing the supernatant from the wells, the HUVEC were incubated with different concentrations of PTH in D-PBS for 30 min. To determine the effect of inhibitors of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on PTH-induced ROS production, DPI (50 μmol/L) was added to the well 30 min before the addition of PTH. Fluorescence intensity was measured at an excitation of 485 nm and an emission of 535 nm using a fluorescence microplate reader SPECTRAfluor Plus (Tecan, Männedorf, Switzerland).

**Statistical Analysis** All data are expressed as the mean ± standard deviation (S.D.). Differences between groups were examined for statistical significance using the Wilcoxon signed-rank test and Tukey’s test. A *p*-value <0.05 denoted the presence of a statistically significant difference.

**RESULTS**

**Therapeutic Effect of Cinacalcet in SHPT Patients Receiving Hemodialysis** In the present study, 6 SHPT patients undergoing dialysis were enrolled (Table 1). The baseline mean ± S.D. iPTH level was 758 ± 553 pg/mL. These patients were administered cinacalcet for a period of 8 weeks. As expected, their serum iPTH levels were gradually decreased, and finally reached nearly half at the end of study period (mean ± S.D. 293 ± 138 pg/mL at week 8) (Fig. 1A). In addition, serum calcium (Fig. 1B) and phosphorus (Fig. 1C) concentrations showed a tendency to decrease, and the calcium-phosphate product (Ca × P) value, which is associated with vascular calcification, was significantly reduced (Fig. 1D). These data indicate that cinacalcet clearly exhibits therapeutic effects on SHPT patients who are receiving hemodialysis under present experimental conditions.

**Effect of Cinacalcet on the Redox Status of HSA in SHPT Patients Receiving Hemodialysis** As previously reported, the oxidized albumin ratio in CKD patients who were receiving hemodialysis was significantly higher than that in healthy subjects (Fig. 2A). Interestingly, it was significantly lower than that in SHPT patients who were receiving hemodialysis. This suggests that Cys34 in HSA was more extensively oxidized under SHPT conditions. As shown in Fig. 2B, this enhanced oxidized albumin ratio in SHPT patients with hemodialysis were gradually decreased by the cinacalcet treatment, and the levels were reduced to nearly half at 4 weeks after cinacalcet treatment. The oxidized albumin ratio then reached a plateau until the end of the study period. Such a decrease in the oxidized albumin ratio can be interpreted by either decreasing the levels of HNA or increasing the levels of HMA. To clarify this, we estimated the changes in HNA and HMA levels during the cinacalcet treatment. As compared to each value at the start of study, HMA levels were increased by about 20% with the cinacalcet therapy while HNA levels were slightly decreased (Fig. 2C). This indicates that cinacalcet therapy increases HMA rather than decreases HNA. Since the radical scavenging ability of HMA is superior to that for HNA, we isolated and purified HSA from serum samples at 4 weeks after the start of the cinacalcet treatment, and evaluated its anti-oxidant capacity against both DPPH and AAPH radicals. As a result, the radical scavenging ability of purified HSA began to increase after the cinacalcet treatment (Figs. 2D, E).

**Effect of Cinacalcet on Oxidative Stress in Serum Samples Obtained from SHPT Patients Receiving Hemodialysis** We further examined the effect of cinacalcet on oxidative stress in SHPT patients who were receiving hemodialysis. The changes in serum thiol content, an index of anti-oxidant ability in the blood circulation, was significantly reduced after initiation of cinacalcet. Data are the mean ± S.D. (n = 6). *p < 0.05 compared with 0 week.
increased after the cinacalcet treatment at 4 weeks (Fig. 3A).

On the other hand, the serum levels of AOPPs, an oxidative stress marker, were decreased significantly (Fig. 3B). These data indicate that cinacalcet induces the inhibition of oxidative stress and recovering anti-oxidant defense system in the blood circulation in SHPT patients who are receiving hemodialysis.

**Correlation between the Pharmacological Effects of Cinacalcet and the Oxidized Albumin Ratio in SHPT Patients** To clarify the mechanism responsible for the anti-oxidant effect of cinacalcet, we investigated the relationship between the pharmacological effects of cinacalcet as shown in Fig. 1 and the oxidized albumin ratio as shown in Fig. 2B at each time point. As a result, oxidized albumin ratios were significantly correlated with the percent change in serum...
iPTH levels from baseline (Fig. 4A). On the other hand, no correlation was observed between the changes in serum phosphorus levels (Fig. 4B) or serum calcium levels (Fig. 4C) and oxidized albumin ratios.

The radical scavenging activity of cinacalcet was also examined using DPPH radicals. As shown in Fig. 4D, cinacalcet scavenged DPPH radicals in a dose-dependent manner.

Effect of PTH on ROS Production in HUVEC Finally, we examined whether PTH induces the production of ROS in endothelial cells through the PTH receptor using HUVEC, which has been reported express PTH type 1 receptors (PTH1R). Here, 1-34 PTH, an active PTH analogue that binds to PTH1R was used in vitro experiment instead of iPTH due to the limited availability of active PTH analogue commercially. In fact, there have been many reports that used 1-34 PTH as intact PTH in vitro and in vivo experiments.

As shown in Fig. 5, a 40 ng/mL solution of PTH significantly induced the production of intracellular ROS in HUVEC. This induction was suppressed by the presence of DPI, an NADPH oxidase inhibitor, and 13-34 PTH, an inactive PTH analogue which lacks PTH1R binding ability (Fig. 5). These data suggest that PTH may enhance oxidative stress by activating NADPH oxidase through PTH receptors.

DISCUSSION

The present study demonstrated for the first time, that in hemodialysis patients with SHPT, cinacalcet treatment significantly decreased the oxidized albumin ratio and that this was accompanied by an increase in the levels of HMA, which ultimately permitted the vascular anti-oxidant defense system to be recovered through the direct and indirect anti-oxidative action of cinacalcet.

The morbidity and mortality of CVD is strongly associated with CKD patients with SHPT patients who are receiving hemodialysis, and the association is more pronounced compared to predialysis CKD patients. Oxidative stress is one of the leading factors contributing to increased mortality in CKD patients with SHPT. Therefore, it can be assumed that oxidative stress is a potential pathogenic mechanism of vasculopathies which contributes to the increased mortality in...
these patients. Based on these notions, it would be expected that medication that inhibits oxidative stress and improves the vascular anti-oxidant defense system would reduce the incidence of CVD risks. In this study, we found that a cinacalcet treatment for at least 4 weeks resulted in a substantial decrease in the oxidized albumin ratio (Fig. 2B) and increased the total serum thiol content (Fig. 3A). Moreover, the radical scavenging activities of HSA purified from the serum samples of SHPT patients were increased by the cinacalcet treatment (Figs. 2D, E). These results imply that cinacalcet improves the anti-oxidant defense system in the blood circulation of these patients. Interestingly, Terawaki et al. reported that a lower level of HMA is closely related to an increased incidence of CVD among peritoneal dialysis patients. Moreover, Matsuyama et al. reported that an elevation in the levels of HMA reflected the recovery of impaired endothelial cell activity. It would be expected that cinacalcet might recover the impaired endothelial function in SHPT patients, and thereby, reduce the risks of CVD, because the oxidized albumin ratio was decreased after initiation of the cinacalcet treatment and this decrease was accompanied by an increase in the levels of HMA. In fact, Ari et al. found that cinacalcet improved endothelial function as estimated by flow mediated dilation in hemodialysis patients with SHPT, although their carotid artery intima-media thickness remained stable.

In SHPT patients on hemodialysis, the oxidized albumin ratio was significantly higher than that in CKD patients who were on hemodialysis (Fig. 1), while it was significantly reduced by cinacalcet treatment. Similar results were also shown in patients with primary hyperparathyroidism (PHPT) who underwent parathyroidectomy, which is the surgical removal of one or more of the parathyroid glands to drastically reduce the accumulation of PTH. In fact, after surgery, the oxidized albumin ratio was significantly decreased and this decrease was associated with a reduction in serum PTH levels, in generally agreement with the findings reported herein. These findings suggest that the accumulation of PTH appears to contribute to the induction of oxidative stress, as shown by the elevation of oxidized albumin ratio. Actually, this conclusion was supported by in vitro experiments using HUVEC in which PTH induced the intracellular ROS production, possibly via the PTHR-NADPH oxidase pathway (Fig. 5). Further studies will be necessary to clarify the detailed mechanism of this event because the concentration of 1-34 PTH used in this study was higher than the plasma level of PTH in SHPT patients to consider its chronic stimulation to endothelial cells in vivo. Moreover, cinacalcet itself showed radical scavenging ability and this scavenging occurred in a dose-dependent manner between 10 to 40 ng/mL (Fig. 4D), which corresponds to the range of its maximum drug concentration in SHPT patients. Therefore, it would be expected that cinacalcet would mainly exhibit anti-oxidant activity via inhibiting the accumulation of PTH, and its direct radical scavenging action is likely also partially involved. Additionally, it would be interesting to clarify whether other calcimimetics such as etelcalcetide and evocalcet, which have recently been launched, could also reduce the oxidized albumin ratio in SHPT patients because the pharmacological effects of those calcimimetics are comparable. If so, in terms of class effect, calcimimetics may exert anti-oxidant action via inhibiting the PTH production.

Of note, all patients enrolled in this study had been receiving ARB, such as olmesartan or telmisartan, and VDRA, such as alfacalcidol or calcitriol for at least one year. Our previous studies demonstrated that these drugs exhibit anti-oxidative activity, thereby reducing the oxidized albumin ratio in hemodialysis patients. To take this into consideration, it is likely that the anti-oxidative effect of cinacalcet is additive in terms of recovering the redox status of HSA in SHPT patients who are administered ARB and VDRA. This is important to consider the clinical situation because ARB and VDRA are frequently administrated to SHPT patients who are receiving hemodialysis.

In conclusion, cinacalcet treatment suppresses oxidative stress in SHPT patients who are receiving hemodialysis via direct and indirect mechanisms, and consequently improves the redox status of HSA. Such an anti-oxidative action of cinacalcet may, in part, explain the reduced risk for cardiovascular and all-cause mortality during cinacalcet treatment.

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Conflict of Interest The authors declare no conflict of interest.

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