Isolation and Characterization of Coenzyme A Glutathione Disulfide as a Parathyroid-Derived Vasoconstrictive Factor

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Background—Coenzyme A glutathione disulfide (CoA-SSG) was recently isolated from bovine adrenal glands and was shown to be a renal vasoconstrictor. The identification of CoA-SSG in human parathyroid glands and its action on cultured vascular smooth muscle cells (VSMCs) are described here.

Methods and Results—After purification to homogeneity by several chromatographic steps, CoA-SSG was identified by matrix-assisted laser desorption/ionization mass spectrometry and enzymatic analysis. The dose-dependent growth-stimulating effect of CoA-SSG on VSMCs, measured by the [3H]thymidine method, is characterized by a threshold of 10⁻⁸ mol/L and a maximum effect of 10 μmol/L, increasing VSMC proliferation 254±21% above control. A dose of 10 μmol/L methylmalonyl-CoA and 10 μmol/L CoA increased the rate of proliferation of VSMCs only by 178±43% and 50±42% above control, respectively. Glutathione has no proliferative effect on VSMCs. The growth-stimulating effect of CoA-SSG (1 μmol/L) was decreased by the antagonists 3,7-dimethyl-1-propargylxanthine (DMPX; 11 μmol/L) (38% compared with CoA-SSG without antagonist) and pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid (PPADS; 10 μmol/L) (48% compared with CoA-SSG without antagonist; each P<0.05 versus control), indicating that the effect is mediated partly via A₂ and partly via P₂Y₁ and/or P₂Y₄ receptor.

Conclusions—CoA-SSG may play a regulatory role in VSMC growth as a progression factor and thereby could play an important role in development of hypertension. (Circulation. 2000;102:2548-2552.)

Key Words: enzymes ▪ muscle, smooth ▪ cells

The parathyroid glands have long been supposed to contain a yet unidentified vasoactive hormone. Pang et al¹ and others² showed that extracts of parathyroid glands from spontaneously hypertensive rats elicited vasoconstriction in several experimental models.

However, the so-called “parathyroid hypertensive factor” (PHF) has not yet been successfully identified. Interestingly, the PHF was shown to contain both peptide³ and nonpeptide components. Because convincing parathyroid-derived factor had been presented by those authors, we were interested in whether human parathyroid glands contain a recently identified vasoconstrictive substance, coenzyme A glutathione disulfide (CoA-SSG). In an earlier study, we isolated CoA-SSG from bovine adrenal glands and showed that this substance is a potent vasoconstrictor.⁴ In addition to its vasoactive action, the substance potentiates the effects of angiotensin II on vascular tone.

In the present study, we demonstrate that CoA-SSG is also found in human parathyroid glands. There is some probability that CoA-SSG is identical with the PHF, the existence of which had been shown only indirectly in earlier studies.

Furthermore, we extend our knowledge of the physiologic action of CoA-SSG with respect to vascular smooth muscle cell (VSMC) growth. This may be of interest, because disturbances in VSMC growth are known to play an important role in cardiovascular disorders such as hypertension and atherosclerosis.⁵,⁶ The pathogenesis of hypertension is a process that involves both enhanced vasoconstriction⁵ and the remodeling of the arterial wall,⁷ which is characterized by hypertrophy and proliferation of VSMCs. Furthermore, it appeared likely that CoA-SSG exhibits not only vasoconstrictive but also proliferative actions, because several vasoconstrictors, such as angiotensin II,⁸ thromboxane,⁹ and ATP,¹⁰ are known to be potent VSMC growth factors as well.

Methods

All reagents were purchased from Sigma Chemical Co unless otherwise specified. Human parathyroid glands were obtained from patients undergoing parathyroidectomy for parathyroid adenoma or hyperplasia due to secondary or primary hyperparathyroidism. In all patients with secondary hyperparathyroidism, end-stage renal disease was present. The study was approved by the local ethical committee, and written consent was obtained from the patients. For isolation of CoA-SSG, only macroscopically intact tissue was used.
Purification of CoA-SSG From Human Parathyroid Gland Tissue

After excision from the patients, human parathyroid gland tissue (∼2 g) was immediately placed in ice-cooled physiological saline solution and processed within 30 minutes.

The following isolation procedure was designed to exclusively isolate CoA-SSG from human parathyroid glands. The human parathyroid gland tissue was cut into small pieces (~1 cm³), frozen in liquid nitrogen, and stored at −80°C for 1 night. Then the tissue was lyophilized and powdered (step 1). The powder was suspended in 20 mL 0.6 mol/L ice-cold perchloric acid and homogenized at 25 000 rpm 10 times for 30 seconds. The homogenate was ultracentrifuged at 30 000 rpm for 60 minutes at 4°C. The supernatant was adjusted with KOH to pH 8.5 and stored at 4°C for 30 minutes to precipitate KClO₄. After centrifugation at 4000 rpm for 10 minutes at 4°C, the supernatant was titrated to pH 6.5 with HCl and centrifuged again as above (step 2).

Preparative Reversed-Phase Chromatography

Extract of the human parathyroid gland tissue was concentrated on a C18 reversed-phase column (LiChroprep, 310×30 mm, 4 μm, Merck) and processed within 30 minutes.

The lyophilized fractions with a significant UV absorption at 254 nm from the reversed-phase chromatography were examined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and postsource decay (PSD)-MALDI-MS. A reflector-type time-of-flight mass spectrometer (Reflex III, Bruker) was used according to Hillenkamp and Karas. The sample was mounted on an x, y, z movable stage allowing irradiation of selected sample areas. In this study, a nitrogen laser (VSL-337 ND, Laser Science) with an emission wavelength of 337 nm and 3-ns pulse duration was used. The laser beam was focused to a diameter of typically 50 μm at an angle of 45° to the surface of a target. Microscopic sample observation was possible. Ten to 20 single spectra were accumulated for a better signal-to-noise ratio. In MALDI-MS, large fractions of the desorbed analyte ions undergo PSD during flight in the field free drift path. With a reflector-type time-of-flight setup, sequence information from PSD fragment ions of precursors produced by MALDI were obtained. Sample preparations for MALDI and PSD-MALDI experiments were identical. The concentrations of the analyzed substances were 1 to 10 μmol/L in bidistilled water. Analyte solution (1 μL) was mixed with 1 μL of matrix solution (50 mg/mL 3-hydroxy-picolinic acid in water). To this mixture, cation exchange beads (AG 50 W-X12, 200 to 400 mesh, Bio-Rad) equilibrated with H⁺ as counterion were added to remove Na⁺ and K⁺ ions. The mixture was gently dried on an inert metal surface before introduction into the mass spectrometer. The mass accuracy was in the range of ±0.01%.

Enzymatic Analysis and Reduction With Mercaptoethanol

To test for the presence of disulfide bonds, 5 μL of the fraction was incubated with 1 μL glutathione reductase (EC 1.6.4.2, Type VII, Sigma; 10 U/L) at room temperature for 30 minutes. In addition, 5 μL of the fraction was boiled with 5 μL mercaptoethanol for 20 minutes. The split products were identified by their molecular masses with the use of MALDI-MS and PSD-MALDI-MS, respectively.

Effect of CoA-SSG on Rate of Proliferation of VSMCs

Aortic VSMCs from normotensive Wistar-Kyoto rats were subcultured in 96-well dishes (Falcon) at a density of 5×10⁴ cells/mL and kept in culture medium containing 10% FCS to reach a subconfluent monolayer. After 24 hours, the cells were growth-arrested in 0.1% FCS for 48 hours without affecting cell adherence to culture wells or viability as checked by trypan blue vital dye exclusion.

Quiescent VSMCs were then exposed to fresh culture medium with 0.1% FCS with CoA-SSG, CoA, methylmalonyl–coenzyme A, and glutathione for a 48-hour incubation period. Moreover, in the presence of CoA-SSG, the receptor antagonist pyridoxal-phosphate-6-azophenyl-2,4-disalicylic acid (PPADS, 10 μmol/L) and as a potent and selective A₁ antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX, 11 μmol/L) were added to the culture medium. Cell proliferation was measured by use of the [³H]thymidine incorporation rate as described elsewhere.

Control Experiment: Incubation of CoA and Glutathione in the Presence of Perchloric Acid

CoA 1 μmol and glutathione 1 μmol were dissolved in 20 mL water. Perchloric acid was added to a final concentration of 0.6 mol/L. The sample was treated like the tissue extract described above. The supernatant passed the same chromatographic steps as described above. Fractions from the last chromatographic step with a significant UV absorption were analyzed by MALDI-MS.

Statistical Analysis

The data were expressed as mean±SEM. The data of the proliferation experiments were analyzed by ANOVA. Statistical significance was assumed to be 2-tailed if P<0.05.

Results

Figure 1A shows the chromatogram of a reversed-phase displacement chromatography with TEAA as an ion-pair reagent of the human parathyroid gland extract after extrac-
tion with perchloric acid and concentration on a reversed-phase chromatography gel. CoA-SSG eluted within 88 to 90 minutes, labeled in the figure by an arrow and a gray bar.

Each fraction of the displacement chromatography with a significant UV absorption at 254 nm underwent anion-exchange chromatography (step 5; Figure 1B). Each fraction of the anion-exchange chromatography with a retention time comparable to that of CoA-SSG was desalted by reversed-phase chromatography (step 6). This lyophilized eluate of the reversed-phase chromatography fraction was subjected to the analytical procedures described below.

First, the molecular mass of the substance underlying the peak of the reversed-phase chromatography with a retention time comparable to that of authentic CoA-SSG was determined by MALDI-MS as 1073.4 Da (1074.6 Da $\pm 1$).

Figure 2 shows the corresponding PSD-MALDI mass spectrum of the desalted fraction of anion-exchange chromatography, labeled by an arrow in Figure 1B. The fragmentation pattern of the isolated substance obtained by this PSD-MALDI mass spectrum was identical to that from commercially available CoA-SSG.

Figure 3 shows the MALDI mass spectra of the isolated fraction before (Figure 3A) and after (Figure 3B) incubation with glutathione reductase, indicating the presence of glutathione in the molecule and a disulfide bond of CoA and glutathione. Boiling with mercaptoethanol yields an identical MALDI spectrum.

To prove whether perchloric acid may oxidize CoA and glutathione, resulting in CoA-SSG, both substances were incubated with perchloric acid. The mixture passed the same purification procedure as the tissue extract. With MALDI-MS, no CoA-SSG was detectable.

CoA-SSG induced a dose-dependent increase in DNA synthesis in VSMCs, as determined by $[^3H]$thymidine uptake (Figure 4). The threshold of the growth-stimulating effect of CoA-SSG was $10^{-8}$ mol/L. The maximum effect was obtained at CoA-SSG concentrations of $10^{-6}$ mol/L, which increased VSMC proliferation $\approx 254\pm21\%$ above control. Methylmalonyl-CoA $10^{-6}$ mol/L as well as CoA $10^{-6}$ mol/L increased the rate of

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**Figure 1.** A, Chromatogram of reversed-phase displacement chromatography of lyophilized eluate of preparative reversed-phase chromatography (column: Supersphere, 2.1 x 100 mm, 4 $\mu$m, Merck; carrier: 40 mmol/L TEAA in water; displacer: 100 mmol/L n-butanol in carrier; flow rate: 50 $\mu$L/min; UV detection: 254 nm; fraction size: 100 $\mu$L). Abscissa shows retention time (minutes); ordinate, UV absorption (arbitrary units). B, Chromatogram of anion-exchange chromatography of fraction labeled by arrow in A (column: Mono Q HR 5/5, 50 x 5 mm, 10 $\mu$m, Pharmacia; eluent E: 20 mmol/L K$_2$HPO$_4$ and 1 mol/L NaCl [pH 8] in water; gradient: 0 to 10 minutes: 0% to 5% E; 10 to 115 minutes: 5% to 40% E; 115 to 120 minutes: 40% to 100% E; flow rate: 1.0 mL/min; UV absorption wavelength: 254 nm).

**Figure 2.** Positive-ion PSD-MALDI mass spectrum of desalted fraction labeled in Figure 1B by arrow. A’ indicates adenine; Ap, AMP; Ap$_2$, ADP; Ap$_3$, ATP; Glu, glutamic acid; H, hydrogen; O, oxygen; N, nitrogen; M$,^+$, protonated parent ion; and P, phosphate group. Abscissa shows relative mass/charge, $M/z$, $z$=1; ordinate, relative intensity (arbitrary units).

**Figure 3.** Positive-ion MALDI mass spectra of isolated fraction before (A) and after (B) incubation with glutathione reductase.

**Figure 4.** Rate of $[^3H]$thymidine incorporation of VSMCs stimulated by CoA-SSG (●), methylmalonyl-CoA (▲), and CoA (○). Ordinate shows rate of $[^3H]$thymidine incorporation in percent above control (0.1% FCS); data are mean±SEM from 10 independent experiments.
proliferation to 178±43% and 50±42%, respectively, above control. As shown in Figure 4, significant effects on the proliferation rate of VSMCs were observed with 10 nmol/L CoA-SSG. Glutathione had no effect on the rate of proliferation of VSMCs. The threshold concentration for the direct growth-stimulatory effect of CoA-SSG was 10⁻⁸ mol/L, and for that of CoA 10⁻⁷ mol/L. The EC₅₀ value (in log mol/L) of CoA-SSG was −8.48±0.15, that of methylmalonyl-CoA −8.15±4.3, and that of CoA −7.41±0.48.

The growth-stimulatory effect of CoA-SSG was inhibited by 2 different purinoceptor antagonists (Figure 5). In the presence of 11 μmol/DMPX blocking the A₂ receptor, the rate of proliferation decreased ~38% compared with CoA-SSG alone (P<0.05 versus control). The growth-stimulating effect induced by CoA-SSG was, moreover, significantly inhibited by the P₂ antagonist PPADS (rate of proliferation [% above control], CoA-SSG versus CoA-SSG+PPADS: 469±59 versus 244±71 [P<0.05 versus control]) (Figure 5).

Discussion

In the parathyroid gland, several known and unknown vasoactive hormones exist. The parathyroid hormone (PTH) causes dose-dependent transient vasodilatation in various vascular beds. Rat parathyroid cells were shown to synthesize and secrete endothelin I. In many studies, parathyroidectomy was demonstrated to decrease blood pressure. Therefore, the existence of a parathyroid vasoconstrictive factor (PHF) according to the observation that parathyroidectomy decreased mean arterial blood pressure (MAP) in intact animals. Intra-aortic injections of CoA-SSG increase blood pressure in intact animals. The vasopressor effect of CoA-SSG was demonstrated in both renal and mesenteric vasculature. CoA-SSG contracts renal vasculature, increases systemic blood pressure, and increases [Ca²⁺]i in VSMCs. Furthermore, with respect to the vasculature, an interesting interaction with angiotensin II was noted: The vasoconstrictor effect of angiotensin II on renal vasculature was potentiated in the presence of CoA-SSG.

Is the PHF identical with CoA-SSG? Obviously, this question cannot be decided definitively at present. There are several similarities between the 2 agents, such as the peptide and nonpeptide moiety. In contrast, the hypertensive action of PHF is much more delayed and prolonged than that of CoA-SSG. However, the indirect effects of CoA-SSG, potentiating the vasoconstrictive effects of angiotensin II, show a latency similar to the vascular effect of PHF. With respect to both similar and different properties of the 2 agents, it may also be considered whether PHF is a derivative of CoA-SSG. Furthermore, it must be remembered that rat and human parathyroid glands may produce similar, but not identical, vasoactive factors. PHF has not been demonstrated in human parathyroid glands, and conversely, we did not examine rat parathyroid tissue.

At present it is not known whether CoA-SSG is produced or secreted in increased amounts in parathyroid adenomas or hyperplastic parathyroid glands. Several clinical observations may support a role of CoA-SSG in human pathology. First, primary hyperparathyroidism is accompanied by an increase in blood pressure, although PTH is a vasodilator. Given that part of the vascular changes in primary hyperparathyroidism are due to elevated serum Ca²⁺ levels, an increased secretion of CoA-SSG may present an alternative explanation for this clinical finding unexplained by PTH. Second, parathyroid hyperplasia in renal failure could lead to an increased release of CoA-SSG. Although PTH has been postulated to act as a uremic toxin, several features of uremic toxicity, such as neurotoxicity and the cardiovascular uremic changes, could never be thoroughly explained by the physiological actions of PTH. Therefore, it may be speculated that CoA-SSG could also play a role in secondary hyperparathyroidism, although at present, firm data are lacking.
In addition to the vasopressor properties of CoA-SSG, the results of the proliferation assay show that CoA-SSG has a direct effect on the rate of proliferation of VSMCs. The dose-dependent growth-stimulating effect of CoA-SSG is obviously determined by the CoA component of CoA-SSG (Figure 4) and not by the glutathione component. Glutathione amplifies the growth-stimulating effect of the CoA component of CoA-SSG. The stimulating effect of CoA-SSG is mediated by a receptor that recognizes CoA.

As shown in Figure 5, the growth-stimulating effect of CoA-SSG is partially inhibited by the potent and selective A2 antagonist DMPP19 as well as by the P2 antagonist PPADS.20 Because the P2X receptor is not involved in cell proliferation and, moreover, this receptor subtype is lost in cultured VSMCs,10 it seems reasonable that the mitogenic effect of CoA-SSG is mediated by the P2Y receptor. Because only the P2Y1 and P2Y4 subtypes of the P2Y receptor are inhibited by PPADS,20,21 it seems likely that the growth-stimulating effect of CoA-SSG is mediated by these P2 receptor subtypes. Both the sigmooidal concentration-dependency of the effects and the specific blockade by purinoceptor antagonists make a nonspecific proliferative effect rather unlikely.

The role of CoA-SSG in the regulation of vasomotor tone and growth is still largely speculative. First, the adequate stimulus to release CoA-SSG from an endocrine organ has not been defined. With respect to adrenal medulla, sympathetic stimulation may be one mechanism of release, because CoA-SSG has been found in exocytotic granules together with catecholamines.4 In parathyroid gland, the stimulus of CoA-SSG release is much more difficult to define, because we do not know whether calcium or phosphate homeostasis can affect CoA-SSG release in a manner similar to that of PTH. Nevertheless, some data indicate a role of parathyroid glands in vascular control. For example, calcium intake is known to affect blood pressure.32 This observation, among others, led to the concept of a PHE.33 From its biological properties, CoA-SSG can clearly be regarded as a parathyroid-derived hypertensive substance.

In summary, the findings show that human parathyroid glands contain a vasocostrictor factor, as was suggested from studies in rat parathyroid tissue. In addition to its vasocostrictor action, CoA-SSG also acts as a growth factor in VSMCs.

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