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 \mu mol/L, increasing VSMC proliferation 254 \pm 21% above control. A dose of 10 \mu mol/L methylmalonyl-CoA and 10 \mu mol/L CoA increased the rate of proliferation of VSMCs only by 178 \pm 43% and 50 \pm 42% above control, respectively. Glutathione has no proliferative effect on VSMCs. The growth-stimulating effect of CoA-SSG (1 \mu mol/L) was decreased by the antagonists 3,7-dimethyl-1-propargylxanthine (DMPX; 11 \mu mol/L) (38% compared with CoA-SSG without antagonist) and pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid (PPADS; 10 \mu mol/L) (48% compared with CoA-SSG without antagonist; each \(P<0.05\) versus control), indicating that the effect is mediated partly via \(A_2\) and partly via \(P_2Y_1\) and/or \(P_2Y_4\) 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
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 KCIO₄. 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, 310×65 mm, 65 to 40 µm, Merck) with 40 mmol/L aqueous triethylammonium acetate (TEAA) in water (flow rate 2 mL/min). After nonbinding substances had been removed with aqueous 40 mmol/L TEAA, the remaining substances were eluted with 20% acetonitrile in water (flow rate 2 mL/min) (step 3). The elution was detected by UV absorption at 254 nm. The eluate was lyophilized and stored frozen at −80°C.

High-Performance Liquid-Displacement Chromatography

The lyophilized eluate of the preparative reversed-phase chromatography was dissolved in aqueous 40 mmol/L TEAA solution and injected onto a C18 reversed-phase column (Supersphere, 2.1×100 mm, 4 µm, Merck; step 4) that was equilibrated with aqueous 40 mmol/L TEAA as carrier as before. The carrier was pumped through the system at a flow rate of 50 µL/min during injection of the sample. After the injection was finished, n-butanol (100 µmol/L) in 40 mmol/L TEAA was used as displacer (flow rate 50 µL/min). The displacement chromatography was monitored by UV absorption at 254 nm. The fraction size was 100 µL. Every fraction of the displacement chromatography was lyophilized and further separated by anion-exchange chromatography.

Anion-Exchange Chromatography

Lyophilized fractions of the displacement chromatography were dissolved in 1 mL 20 mmol/L K, HPO₄ in water (pH 8) (elucent A) and chromatographed by use of an anion exchanger (step 5) (column: Mono Q HR 5/5, 50×5 mm, 10 µm, Pharmacia Biotech; eluent B: 20 mmol/L K, HPO₄ and 1 mol/L NaCl [pH 8] in water; gradient: 0 to 10 minutes: 0% to 5% B; 10 to 115 minutes: 5% to 40% B; 115 to 120 minutes: 40% to 100% B; flow rate: 0.1 mL/min; UV absorption wavelength: 254 nm).

Reversed-Phase Chromatography

Each peak of the anion-exchange chromatography with a retention time comparable to the retention time of authentic CoA-SSG was assumed to be 2-tailed if P<0.05. 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.

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.
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 $^{[M+H]}$).

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 $\approx 254 \pm 21\%$ above control. Methylmalonyl-CoA $10^{-6}$ mol/L as well as CoA $10^{-6}$ mol/L increased the rate of

Figure 2. Positive-ion PSD-MALDI mass spectrum of desalted fraction labeled in Figure 1B by arrow. A’ indicates adenine; Ap, AMP; Adp, ADP; Ap3, ATP; Glu, glutamic acid; H, hydrogen; O, oxygen; N, nitrogen; M, protonated parent ion; and P, phosphate group. Abscissa shows relative mass/charge, $M_z/z$, $z=1$; 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.
CoA-SSG as Parathyroid Vasoconstrictive Factor

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-1. In many studies, parathyroidectomy was demonstrated to decrease blood pressure. In patients who were hypertensive before operation, both the systolic and diastolic blood pressure significantly decreased after parathyroidectomy. Therefore, the existence of a vasoconstrictor factor in the plasma was suggested to be a peptide linked to a lysophospholipid. Jankowski et al suggested a parathyroid gland–derived hypertensive factor (PHF) according to the observation that parathyroidectomy had no effect on MAP or hypertensive factor disappearance from the plasma. After partial characterization, the structure of the PHF was suggested to be a peptide linked to a lysophospholipid. In this study, we were able to isolate and identify CoA-SSG from human parathyroid glands, which was identified earlier from bovine adrenal glands. There is some probability that the substance isolated from human parathyroid glands by Schütter et al in 1992 may be attributed to CoA-SSG.

From experiments in adrenal glands, it is known that CoA-SSG can be released by stimulation with carbamyl or A23187. From these findings, it can be inferred that both an increase in cytosolic free Ca2+ concentration and cholinergic stimulation of adrenal medulla are important mechanisms of CoA-SSG release. For parathyroid gland tissue, however, these mechanisms can only be inferred by analogy at present.

Vascular actions of CoA-SSG have been demonstrated in previous studies. CoA-SSG has a direct effect on the renal vascular resistance. 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 A2a antagonist DMPX as well as by the P2x antagonist PPADS. Because the P2x receptor is not involved in cell proliferation and, moreover, this receptor subtype is lost in cultured VSMCs, 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, it seems likely that the growth-stimulating effect of CoA-SSG is mediated by these P2y receptor subtypes. Both the sigmoidal 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. In parathyroid gland, the stimulus of CoA-SSG release is much more difficult to define, because we do not know whether calcium or phosphates on purinoceptors in the rat isolated perfused kidney, Br J Pharmacol. 1997;120:1453–1460.


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