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ORIGINAL ARTICLE

Postmortem biochemistry and immunohistochemistry of chromogranin A as a stress marker with special regard to fatal hypothermia and hyperthermia

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Abstract Chromoganin A (CgA) is widely distributed in the secretory granules of endocrine and neuroendocrine cells and cosecreted with hormones such as catecholamines. The present study investigated postmortem serum and cerebrospinal fluid (CSF) levels of CgA in comparison with those of catecholamines, and also cellular CgA immunopositivity in the hypothalamus, adenohypophysis and adrenal medulla to assess forensic pathological significance. Serial medicolegal autopsy cases (n=298, within 3 days postmortem) were used. Serum and CSF CgA levels were independent of the gender or age of subjects or postmortem time. The most characteristic findings were seen for fatal hypothermia (cold exposure), hyperthermia (heat stroke) and intoxication. Serum CgA levels were lower for hypothermia and intoxication than for other causes of death (p<0.05), while CSF CgA levels were higher for hypothermia (p<0.0001). A negative correlation was detected between serum and CSF CgA levels for hypothermia (R=0.552, p<0.05). Correlations between serum levels of CgA and catecholamines (adrenaline, noradrenaline and dopamine) were evident for hyperthermia (R=0.632–0.757, p<0.05 to <0.01), but there was no significant correlation between CgA and catecholamine levels in CSF. Cellular CgA immunopositivity in the hypothalamus, adenohypophysis and adrenal medulla varied extensively among cases in each group. However, CgA immunopositivity in hypothalamus neurons was lower for hypothermia than other causes of death including hyperthermia and intoxication. These observations suggest characteristic neuroendocrinal activation in fatal cases of hypo- and hyperthermia and also intoxication. CgA may be a useful biochemical and immunohistochemical marker for investigating these causes of death.

Keywords Forensic pathology · Chromogranin A · Catecholamine · Biochemistry · Immunohistochemistry · Hypothermia · Hyperthermia

Introduction

The stress response systems mainly comprise the sympathetic/adrenomedullary (S/A) system and the hypothalamic–pituitary–adrenal (HPA) axis [1]. The activities of these systems can be biochemically evaluated by measuring catecholamine and cortisol levels, respectively, as an index of stress [2, 3]. Previous studies have suggested postmortem serum concentrations of catecholamines (adrenaline (Adr), noradrenaline (Nad), and dopamine (DA)) to be useful biomarkers for investigating the cause of death and magnitude of physical stress responses during the death process [2, 3]. With respect to this, it is known that catecholamines are coreleased with chromogranin A (CgA) [4–6].

CgA is a 49–68-kDa acidic, Ca^{2+}-binding glycoprotein originally isolated as a major soluble protein in adrenal medullary chromaffin granules [7]. However, it has been established that CgA is widely distributed in secretory granules of endocrine and neuroendocrine cells, and secreted with coresident hormones preferentially into the lymph [4, 5]. Therefore, an increase in circulating blood CgA level is closely associated with neuroendocrinal

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activation. Previous studies suggested the increase in CgA level especially due to environmental stress [4, 5, 8–10]. Furthermore, recent findings provided evidence that CgA can also function as a prohormone precursor [11]. In healthy subjects, CgA levels were measured at 39–116 ng/ml (0.6–2.4 pmol/ml) for serum and about 170 ng/ml (2.5–3.5 pmol/ml) for cerebrospinal fluid (CSF) [12]. CgA has a plasma half-life of 18.4 min, fitting a two-compartment model, with a rapid half-life of 16 min followed by a longer half-life of 520 min, suggesting that circulating CgA binds to tissues [13–15]; thus, the half-life in circulating blood is longer than that of catecholamines (several minutes) [16]. These findings suggest CgA to be a potential marker for the postmortem assessment of a "tonic" (lasting) neuroendocrinal activation in death process, especially fatalities due to environmental thermal hazards, rather than single "phasic" responses to stress, for which catecholamines can be major indexes [12]. However, there appears to have been no report of practical data on CgA as a stress marker with medicolegal implications.

The present study investigated postmortem serum and CSF CgA levels in serial medicolegal autopsy cases to examine the feasibility of using CgA as a stress marker with special regard to fatal hypothermia and hyperthermia, compared with catecholamine levels. Cellular CgA immunopositivity in the hypothalamus, hypophysis, and adrenal medulla was also examined.

Materials and methods

Materials

Serial medicolegal autopsy cases (n=298; within 3 days postmortem with a median of 19.5 h; survival time of 0.1 h–45 days with a median of 0.5 h) at our institute were examined. The cases comprised 206 males and 92 females, between 0 and 97 years (median, 62 years) of age (Table 1). The causes of death were classified based on a complete autopsy and macromorphological, micropathological, and toxicological examinations as follows: blunt injury (n=39: head injury, n=19; others, n=20), sharp instrument injury (n=14), asphyxiation (n=29: hanging, n=8; strangulation, n=8; others, n=13), fatal intoxication (n=21: methamphetamine (MA), n=4; psychotropic drugs, n=10; carbon monoxide (CO), n=4; others, n=3), drowning (n=35), fire fatality (n=54), hypothermia (cold exposure, n=20), hyperthermia (heat stroke, n=12), and acute cardiac death (ACD; n=74) including acute myocardial infarction (AMI; n=40) and recurrent myocardial infarction (RMI; n=23) without hemopericardium and those with hemopericardium (HP; n=11). Fire fatality (n=54) was subdivided into cases with a low (<30%), intermediate (30–60%), and high (>60%) carboxyhemoglobin (CO-Hb) level (n=27, n=12, and n=15, respectively). Hypothermia and hyperthermia cases were those due to cold and hot weather, respectively, at night or during the day, and the diagnosis was based on pathology and toxicology in consideration of circumstantial evidence, excluding other causes of death [17–20]. Cases of hypo- and hyperthermia during drug abuse and bathing, respectively, were excluded. Postmortem interval was defined as the time from estimated time of death to autopsy. Survival time was the period from the onset of fatal insult to death. For this study, clearly accountable cases were collected.

Blood in the right cardiac chamber and CSF in the basilar subarachnoid cisterna were collected using sterile syringes after opening the pericardial and cranial cavity, respectively, at autopsy. The blood samples were immediately centrifuged to separate sera. The specimens were stored at −20°C until use and centrifuged before analysis [21]. Tissue specimens of the hypothalamus, hypophysis, and bilateral adrenal glands were collected and fixed in 4% formaldehyde in phosphate-buffered saline (PBS; pH 7.2) for histopathological and immunohistochemical investigation.

Biochemical analyses

CgA measurements

Serum and CSF CgA levels were determined using an enzyme-linked immunosorbent assay kit (YK070 Human Chromogranin A EIA, Yanaihara Institute, Inc., Shizuoka, Japan) using the method of Nishikawa et al. [22]. Briefly, synthetic human CgA (344–374)-I was used as a standard antigen [23]. The standard diluent used in the EIA was 0.01 M phosphate buffer (pH 7.4) containing 0.5% (*w*/*v*) bovine serum albumin, 0.025 M ethylenediaminetetraacetic acid, 0.14 M NaCl, and 0.02% NaN_3. In each assay tube, standard diluent (0.4 ml) was mixed with diluted standard antigen or sample (0.1 ml), diluted antihuman CgA (344–374)-I serum RY76 (final dilution 1:8,400), and *N*α-biotinylglycylglycyl human CgA (344–374)-I (approximately 103 Bq; 0.1 ml). After incubation at 4°C for 24 h, antibody-bound and free-labeled antigens were separated by addition of diluted normal rabbit serum (1:50, Daiichi Radioisotope Labs., Ltd., Tokyo, Japan; 0.05 ml), diluted goat antirabbit IgG serum (1:10, Daiichi Radioisotope Labs., Ltd., Tokyo, Japan; 0.05 ml), and 10% (*w*/*v*) polyethylene glycol 6,000 (M.W. 7,500; 0.5 ml). A standard curve was constructed, and unknown values of immunoreactive human CgA in samples were interpolated with an original computer program that utilized a log-logit transformation to produce a linear dose–response curve.

Table 1 Case profiles (n=298)

Cause of death	Male/female	Age (years) Range (median)	Survival time (h) Range (median)	Postmortem interval (h) Range (median)
Blunt injury				
Head injury	17/2	1–96 (36.0)	0.1–1,080.0 (23.0)	6.1–35.3 (18.5)
Others	14/6	22–84 (55.0)	0.3–480.0 (2.0)	10.3–34.2 (20.1)
Sharp instrument injury	9/5	19–75 (46.0)	0.3–4.0 (0.5)	10.0–30.2 (14.2)
Asphyxiation				
Hanging	6/2	0–72 (59.0)	0.1–3.0 (0.5)	9.0–45.7 (23.5)
Strangulation	2/6	32–87 (61.5)	0.5–108.0 (0.5)	13.6–34.0 (25.2)
Others[a]	6/7	23–96 (45.0)	0.5–96.0 (0.5)	6.4–37.0 (19.5)
Drowning	19/16	34–92 (65.0)	0.3–16.0 (0.5)	3.8–47.2 (18.6)
Fire fatality	38/16	5–97 (64.0)	0.1–1,080.0 (0.5)	7.9–46.7 (16.9)
Fatal intoxication				
Methamphetamine	4/0	30–43 (35.5)	3.0–36.0 (21.0)	60.–26.2 (18.1)
Psychotropic drugs	4/6	30–74 (36.0)	1.0–26.0 (6.0)	6.8–38.0 (21.6)
Carbon monoxide	3/1	27–56 (39.5)	3.0–6.0 (3.0)	24.0–48.0 (24.5)
Others[b]	3/0	46–66 (52.0)	3.0–36.0 (4.0)	16.8–27.6 (18.4)
Hyperthermia	8/4	0–92 (69.5)	3.0–168.0 (4.5)	11.0–41.0 (27.0)
Hypothermia	16/4	26–90 (65.0)	6.0–24.0 (6.0)	6.0–52.7 (22.3)
Acute cardiac death	57/17	30–94 (65.0)	0.5–336.0 (0.5)	5.0–48.0 (19.5)

[a] Aspiration (n=8), smothering (n=2), and traumatic asphyxia (n=3)

[b] Organic solvents (n=2) and alcohol (n=1)

Catecholamine measurements

Serum and CSF catecholamine concentrations were measured using high-performance liquid chromatography [2, 24]. The ranges of measurements were 2.0–10,500 pg/ml for Adr, 1.8–19,500 pg/ml for Nad, and 2.1–18,750 pg/ml for DA. Serum samples were diluted with saline before measurement (10- to 1,000-fold for serum).

Immunohistochemistry

Immunoenzyme procedure

Routine specimens of the hypothalamus, hypophysis, and adrenal glands were fixed in 4% formaldehyde in PBS (pH 7.2) for 12 h, embedded in paraffin, and then sectioned at a thickness of 4 μm. After deparaffinization, the sections were immersed in 0.3% H_2O_2–methanol for 15 min to inactivate endogenous peroxidase. After a wash with PBS (3–5 min), blocking was performed with PBS containing 1% normal goat serum and 1% bovine serum albumin at room temperature for 30 min [25, 26]. The primary antibody used was rabbit antihuman CgA antibody (1:500; DAKO, Glostrup, Denmark) [27]. Following an overnight incubation with the primary antibody at room temperature, immunoreactions were visualized by the avidin–biotin complex (ABC) method using the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and color development with 3,3-diaminobenzidine tetrahydrochloride according to the manufacturer's instructions (counterstaining with hematoxylin) [28].

Quantitative analyses of cellular CgA immunopositivity

Total numbers of neurons in the hypothalamus and endocrine cells in the adenohypophysis and adrenal medulla and the number of cells showing CgA immunoreactivity were counted under ×200 magnification: Five random fields were independently examined by two observers using the Lumina Vision system (Mitani, Osaka, Japan) according to standard procedures [28], and mean values were estimated. Interobserver variability was usually less than 10% of the measured mean values. Percent positivity was estimated as the percentage of CgA-positive cells = number of CgA-positive cells/total number of hematoxylin-positive cells ×100, respectively. For the adrenal medulla, mean values of bilateral specimens were used.

Statistical analyses

The Fisher exact test was used to compare two parameters including CgA and catecholamine levels, gender and age of subjects, survival time, and postmortem time. For compar-

isons between groups, the nonparametric test (Mann–Whitney *U* test) and Scheffe test were used for analyses involving multiple comparisons. These analyses were carried out using Microsoft Excel and StatView (version 5.0; SAS Institute Inc.), and a p value of less than 0.05 was considered significant. The line in each box represents the median, and the lines outside each box represent the 90% confidence interval. The maximum CgA and catecholamine concentrations in serum and CSF were log-transformed for graphical presentation only [28]. The sensitivity and specificity in distinguishing two groups using cutoff CgA and catecholamine values were estimated by means of a receiver-operating characteristics analysis [29]. The areas under the curves were calculated and analyzed by the one-tailed test. The optimal compromise between sensitivity and specificity was determined graphically.

Results

Relationship of CgA and catecholamine levels to gender, age, and postmortem period

In all cases, postmortem serum CgA levels (3.0–3,678.83 pmol/ml) were higher than clinical reference values (0.6–2.4 pmol/ml), and CSF levels (0.02–98.50 pmol/ml) were mostly lower than serum levels and approximated clinical reference values (2.5–3.5 pmol/ml). Serum and CSF CgA levels showed no significant tendency toward a postmortem change, a gender-related difference, or age dependency.

Serum catecholamine (Adr, Nad, and DA) levels were markedly higher in most cases (about 100–1,000,000 pg/ml), compared with their clinical reference values (Adr, <100 pg/ml; Nad, 100–450 pg/ml; DA, <20 pg/ml), with extensive variation among cases and a slight tendency toward a postmortem increase for serum Adr (r=0.211, p<0.001). CSF levels showed a similar distribution, with a slight age-dependent decrease for CSF Adr (r=0.239, p<0.001) and Nad (r=0.224, p<0.0001).

Relationship of CgA and catecholamine levels to survival time and influence of critical medical care

Survival time-dependent change was not evident (r<0.2 and/or p>0.05) for serum and CSF levels of CgA and catecholamines (Adr, Nad, and DA). When subjects with and without medical care at a hospital before determination of death were compared, serum and CSF CgA levels were found to show no differences. Serum DA and CSF Adr levels were slightly higher (p<0.01) for deaths under critical medical care; thus, for these markers, cases without medical care were used in further analyses.

Relationship of CgA and catecholamine levels to cause of death

Serum and CSF CgA concentrations

Serum CgA CgA levels were significantly lower for intoxication (3.25–37.37 pmol/ml; median 6.00 pmol/ml) and hypothermia (3.10–85.00 pmol/ml; median, 9.40 pmol/ml) than for the other causes of death (Fig. 1a). Most cases of drowning (28.58–430.54 pmol/ml; median, 171.50 pmol/ml) and hyperthermia (102.50–465.30 pmol/ml; median, 272.00 pmol/ml) had a higher level, and a large difference among cases was seen for the other causes of death: blunt injury (3.00–455.90 pmol/ml; median, 60.65 pmol/ml), sharp instrument injury (7.50–268.46 pmol/ml; median, 43.90 pmol/ml), asphyxiation (16.80–547.50 pmol/ml; median, 115.80 pmol/ml), fire fatality (8.00–390.00 pmol/ml; median, 44.00 pmol/ml), and ACD (11.80–3,678.83 pmol/ml; median, 98.09 pmol/ml; p<0.05 to <0.001). For blunt injury, however, no difference was detected between head injury (20.50–455.90 pmol/ml; median 125.50) and nonhead injury (3.00–398.50 pmol/ml; median 55.46 pmol/ml). There was no difference between the subgroups of asphyxiation (hanging, 47.50–547.50 pmol/ml with a median of 85.87; strangulation, 137.50–512.50 pmol/ml with a median of 225.50 pmol/ml; others, 16.80–159.20 pmol/ml with a median of 36.20 pmol/ml), between the subgroups of intoxication (MA, 3.25–8.46 pmol/ml with a median of 4.93 pmol/ml; psychotropic drugs, 4.53–37.37 pmol/ml with a median of 7.93 pmol/ml; CO, 3.98–29.58 pmol/ml with a median of 5.29 pmol/ml; others, 6.76–17.57 pmol/ml with a median of 12.08 pmol/ml), among fire fatalities with a low (<30%), intermediate (30–60%), and high CO-Hb level (>60%; 8.00–390.00 pmol/ml with a median of 59.80 pmol/ml, 10.60–358.50 pmol/ml with a median of 34.03 pmol/ml, and 12.08–385.20 pmol/ml with a median of 28.50 pmol/ml, respectively), or between the subgroups of ACD (AMI, 11.80–659.80 pmol/ml with a median of 86.49 pmol/ml; RMI, 11.80–3,678.83 pmol/ml with a median of 108.71 pmol/ml; HP, 16.50–659.70 pmol/ml with a median of 132.50 pmol/ml). The cutoff value for distinguishing lower and higher serum CgA levels (hypothermia and intoxication vs. drowning and hyperthermia) was 20 pmol/ml (sensitivity, 0.81; specificity, 1.00).

CSF CgA CgA levels were significantly higher for hypothermia (9.00–98.50 pmol/ml; median, 35.60 pg/ml) than for the other causes of death (p<0.0001), with cases of sharp instrument injury (0.16–2.79 pmol/ml; median, 0.51 pmol/ml) and asphyxiation (0.02–3.26 pmol/ml; median, 0.46 pmol/ml) having significantly lower levels (p<0.05 to <0.0001; Fig. 1b). Most cases of drowning

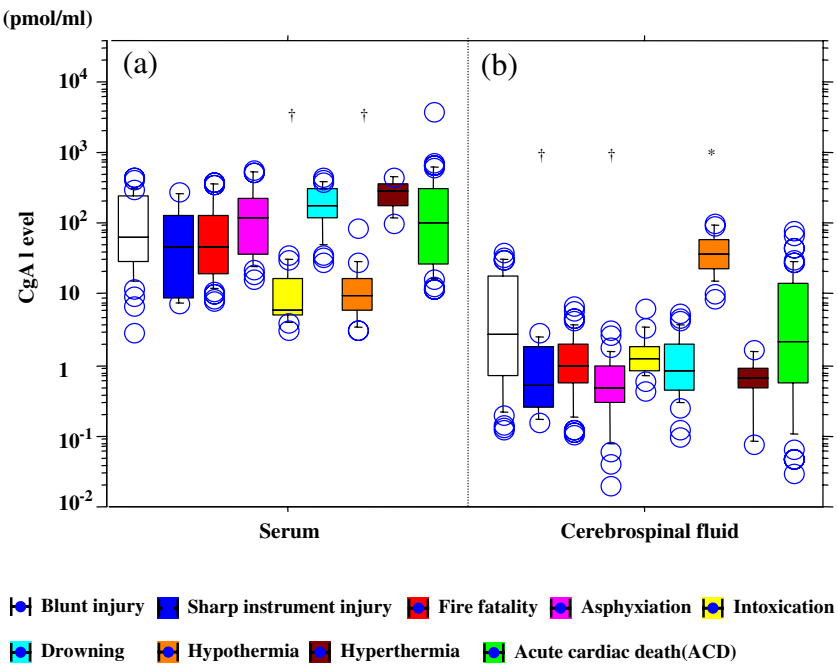

Fig. 1 Chromogranin A (*CgA*) levels in serum (**a**) and cerebrospinal fluid (**b**) with regard to cause of death. **a** Significantly lower: †$p<0.05$ to <0.001, hypothermia and intoxication vs. other causes of death. **b** Significantly higher: *$p<0.0001$, hypothermia vs. other causes of death. Significantly lower: †$p<0.05$–0.0001, sharp instrument injury and asphyxiation vs. other causes of death

(0.10–5.26 pmol/ml; median, 0.82 pmol/ml), intoxication (0.43–6.26 pmol/ml; median, 1.26 pmol/ml), fire fatality (0.10–6.7 pmol/ml; median, 0.98 pmol/ml), and hyperthermia (0.08–1.65 pmol/ml; median, 0.66 pmol/ml) also had lower levels. A large difference among cases was seen for blunt injury (0.13–40.13 pmol/ml; median, 2.77 pmol/ml) and ACD (0.03–82.3 pmol/ml; median, 2.17 pmol/ml). For blunt injury, however, no difference was detected between head injury (0.66–40.13 pmol/ml; median, 8.38 pmol/ml) and nonhead injury (0.13–31.5 pmol/ml; median, 0.71 pmol/ml) or between the subgroups of ACD (AMI, 0.05–82.3 pmol/ml with a median of 1.69 pmol/ml; RMI, 0.05–45.90 pmol/ml with a median of 5.27; HP, 0.03–18.70 pmol/ml with a median of 1.70 pmol/ml). There was no difference between the subgroups of asphyxiation (hanging, 0.02–1.25 pmol/ml with a median of 0.39; strangulation, 0.43–2.60 pmol/ml with a median of 0.8 pmol/ml; others, 0.10–3.26 pmol/ml with a median of 0.33 pmol/ml), between the subgroups of intoxication (MA, 0.43–1.00 pmol/ml with a median of 0.84 pmol/ml; psychotropic drugs, 0.59–3.5 pmol/ml with a median of 1.41 pmol/ml; CO, 0.78–3.26 pmol/ml with a median of 1.3 pmol/ml; others, 1.46–6.26 pmol/ml with a median of 1.80 pmol/ml), and among fire fatalities with a low (<30%), intermediate (30–60%), and high CO-Hb level (>60%; 0.10–4.65 pmol/ml with a median of 1.07 pmol/ml, 0.11–6.7 pmol/ml with a median of 0.31 pmol/ml, and 0.26–4.26 pmol/ml with a median of 1.93 pmol/ml, respectively). The cutoff value for distinguishing higher and lower CSF CgA levels (hypothermia vs. sharp instrument injury, asphyxiation, drowning, intoxication, fire fatality, and hyperthermia) was 10 pmol/ml (sensitivity, 0.90; specificity, 1.00).

Serum and CSF catecholamie concentrations

Serum and CSF catecholamine levels presented a large difference among cases in each group of the cause of death. Hypothermia cases showed lower serum Adr and Nad levels (420.00–162,331.00 pg/ml with a median of 25,681.00 pg/ml and 594.00–103,585.00 pg/ml with a median of 18,897.50 pg/ml, respectively), compared with other groups except for fire fatality, intoxication, and hyperthermia (5.00–1,917,396.00 pg/ml with a median of 171,163.00 pg/ml, $p<0.01$ and 73.00–601,818.00 pg/ml with a median of 105,382.00 pg/ml, $p<0.0001$, respectively). CSF Nad levels were higher for intoxication (9,196.00–89,818.00 pg/ml with a median of 34,331.00 pg/ml) than other cases (94.00–298,938.00 pg/ml with a median of 18,457.00 pg/ml, $p<0.05$).

Relationship between CgA and catecholamine levels

Serum Positive correlations between CgA and catecholamine levels were detected for hyperthermia (Adr, $r=0.757$, $p<0.01$; Nad, $r=0.695$, $p<0.05$; DA, $r=0.632$, $p<0.05$).

CSF There was no significant correlation between CSF CgA and catecholamine levels. The ratios of CSF CgA to Adr and Nad were relatively higher for fatal hypothermia

(CgA/Adr, 0.00–1.50; median 0.04, $p<0.0001$, CgA/Nad, 0.00–0.02; median 0.00, $p<0.0001$), compared with other causes of death.

Relationship between serum and CSF levels of CgA

In most cases, the serum CgA level was higher than the CSF level (serum/CSF ratio>1.0; Fig. 2). The serum/CSF CgA ratio was significantly higher for asphyxiation (5.15–21,280.00; median 269.38), and a high ratio was also seen for drowning (22.35–1,743.88 with a median of 192.46) and hyperthermia (83.80–4,960.47 with a median of 415.23). Intoxication had a lower serum/CSF CgA ratio (1.08–49.30 with a median of 6.71), and the CSF level was higher than the serum level in most cases of hypothermia, with a serum/CSF ratio of 0.03–8.61 and a median of 0.28. Serum and CSF CgA levels showed a moderate negative correlation for hypothermia ($r=0.552$, $p<0.05$) and sharp instrument injury ($r=0.541$, $p<0.05$) and a mild positive correlation for fire fatality ($r=0.334$, $p<0.05$).

CgA immunopositivity in the hypothalamus, adenohypophysis, and adrenal glands

CgA was clearly detected in specific cell components: neurons in the hypothalamus (Fig. 3a), endocrine cells in the adenohypophysis (Fig. 3b), and chromaffin cells in the adrenal medulla (Fig. 3c). Cellular CgA immunopositivity in each tissue varied extensively among cases for each cause of death, without any relationship to the postmortem period, or age or gender of subjects.

Hypothalamus neuronal CgA immunopositivity showed an extensive variation among cases in each cause of death, but was lower in most cases of hypothermia (0.00–100.0%; median 0.0%), compared with other groups including intoxication (0.0–100.0%; median 50.0%) and hyperthermia (0.0–100.0%; median 65.1 %; Fig. 4a). CgA immunopositivity (y) showed a positive correlation with the CSF CgA level (x) for hypothermia ($y=0.502x-9.551$, $r=0.525$, $p<0.05$).

CgA immunopositivity in the adenohypophysis and adrenal medulla was similar for each cause of death. However, CgA immunopositivity in the adenohypophysis was higher for asphyxia (45.90–82.51%; median 70.19%) than in any other group (13.88–98.76%; median 58.28%) excluding intoxication and hypothermia ($p<0.05$ to <0.0001; Fig. 4b). CgA immunopositivity in the adrenal medulla was independent of the cause of death (Fig. 4c). No significant relationship was detected between CgA immunopositivity in the adenohypophysis or adrenal medulla and serum or CSF CgA levels.

Discussion

In the present study, serum and CSF CgA levels were independent of postmortem time, or the gender or age of subjects, although catecholamines (Adr, Nad, and DA) partly showed a slight postmortem time and age dependency or an influence of critical medical care before death, as mentioned previously [2]. However, further investigation involving a serial sampling from the same bodies after death might be needed to establish postmortem stability of CgA. Postmortem serum CgA levels were higher than clinical reference values [12], similarily to catecholamine levels [2], suggesting huge physical stress in death process. Meanwhile, postmortem CSF CgA levels were mostly lower (about $\times 10^{-1}$

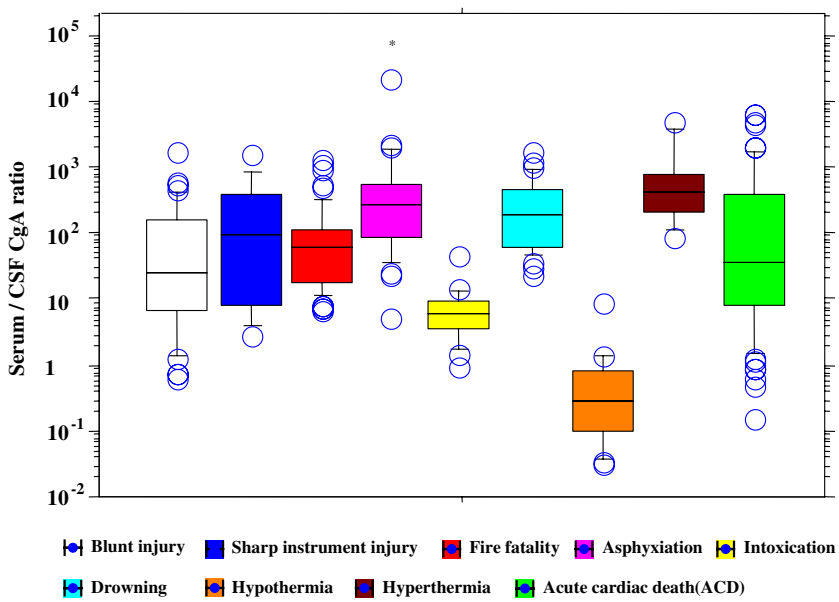

Fig. 2 Serum to cerebrospinal fluid (*CSF*) chromogranin A (*CgA*) ratios with regard to cause of death. Significantly higher: *$p<0.05$ to <0.01, asphyxiation vs. other causes of death except for sharp instrument injury and hyperthermia

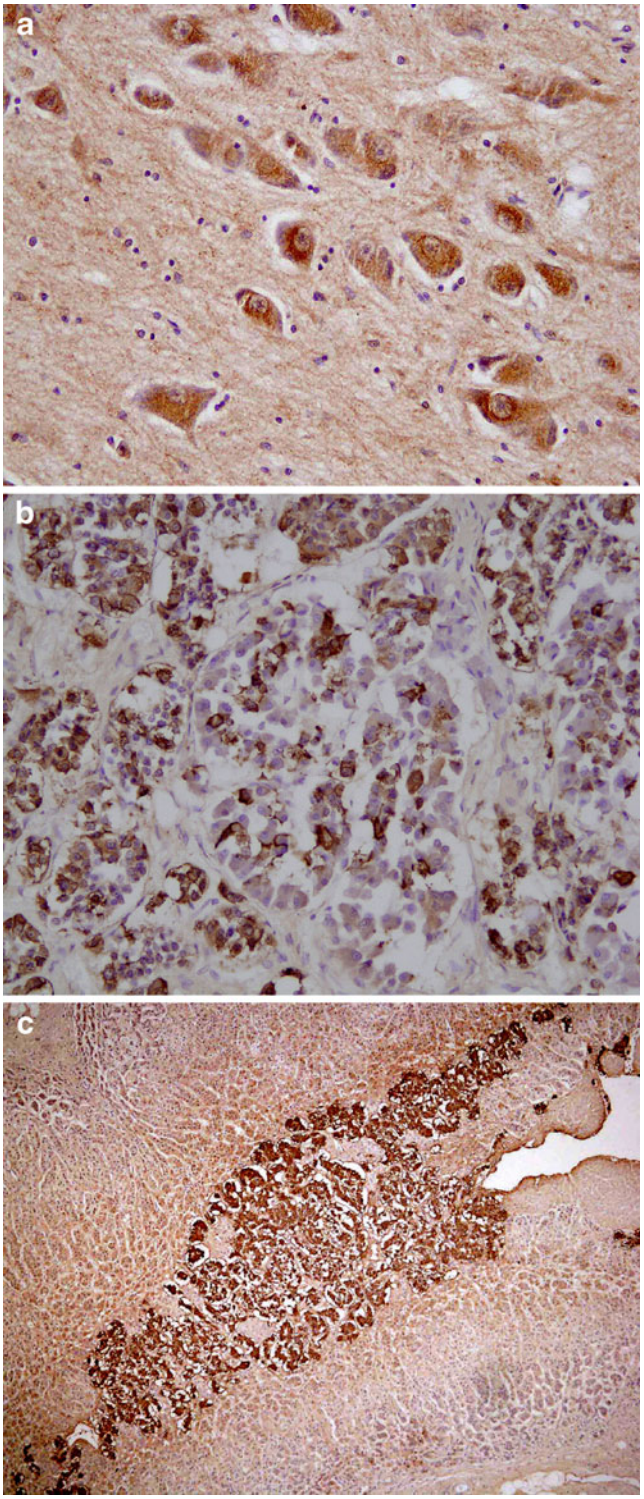

Fig. 3 Micrographs showing immunostaining of chromogranin A in the hypothalamus (**a** original magnification, ×200), adenohypophysis (**b** original magnification, ×100), and adrenal medulla (**c** original magnification, ×40) in a case of sharp instrument injury (41-year-old female, 12 h postmortem)

to 10^{-3}) than serum levels, except for hypothermia cases, although clinical investigation has shown that the CSF CgA concentration is usually higher than the serum value [30]. In most cases, postmortem CSF CgA levels approximated clinical reference values [30]. These observations suggest a delayed increase in CSF CgA during the death process; thus, an elevated CSF CgA level may indicate prolonged agony involving neuroendocrinal activation.

With regard to cause of death, the most characteristic findings were seen for fatal hypothermia (cold exposure), hyperthermia (heat stroke), and intoxication. For hypothermia, the serum CgA level was low, although it was higher than clinical reference values (0.6–2.4 pmol/ml), but the CSF level was increased, showing a moderate negative correlation to the serum level. However, the findings for serum and CSF levels were reversed for hyperthermia, and both levels were low for intoxication, irrespective of the causative substances including MA, psychotropic drugs, and CO. For hypothermia, serum Adr and Nad levels were also lower with postmortem cutoff values as previously shown [2], although they were higher than clinical reference values [2, 31, 32]. However, the difference was clearer for CgA. Thus, simultaneous measurements of serum and CSF CgA concentrations may be useful for investigating these causes of death, for which morphological evidence is often very poor. For this purpose, the postmortem cutoff values for serum and CSF CgA levels were estimated to be 20 and 10 pmol/ml, respectively. The combined use of other biochemical and immunohistochemical markers may be more effective [17, 18, 25, 26, 28, 33–35].

Serum CgA levels were usually higher for other causes of death including injury, fire fatality, asphyxiation, and ACD (about 20–500 pmol/ml; ×10–500 of clinical reference value), showing a large interindividual difference, although most drowning cases had higher levels (>100 pmol/ml). These findings suggest huge stress involving neuroendocrinal activation in the above-mentioned acute deaths. The relationship between serum CgA and catecholamine levels was not clear for all cases; however, a marked positive correlation was detected for hyperthermia. Previous investigations showed that plasma CgA and Nad concentrations were highly correlated when the sympathochromaffin system was activated markedly, but the correlation was otherwise relatively weak [5]. These results suggest that the activation of the S/A system is mainly involved in the hyperthermic stress response [36, 37]. However, CgA and catecholamines in circulating blood might be mainly derived from sources other than the adrenal medulla [5, 30], because no relationship was detected between the serum CgA level and immunopositivity in the adrenal medulla for all cases or individual causes of death. For other fatalities, the absence of a significant correlation between serum levels of CgA and catecholamines (Adr, Nad, and DA) suggests the activation

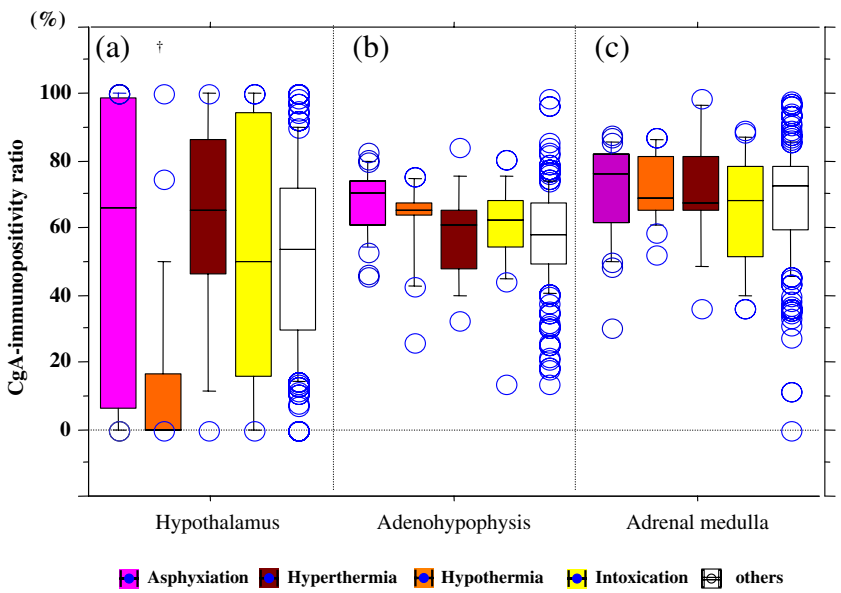

Fig. 4 Cellular chromogranin A (*CgA*) immunopositivity ratio in the hypothalamus (**a**), adenohypophysis (**b**), and adrenal medulla (**c**) with regard to cause of death. Hypothalamus: significantly lower, †$p<0.0001$, hypothermia vs. other causes of death

of multiple endocrine and neuroendocrine systems during the death process, including the S/A and HPA systems [38–40]. These observations suggest the serum CgA level to represent the magnitude of individual physical stress responses during the death process, independent of catecholamine levels in cases other than hyperthermia. A low serum CgA level, especially in cases of intoxication and hypothermia, may indicate physical inactivity. However, in some blunt head injury cases with a longer survival time, high serum CgA levels may indicate persistent neuroendocrinal activation due to posttraumatic influence involving increased CSF pressure even under disturbed consciousness; neuroendocrinal activation may not simply depend on the status of consciousness [41].

The CSF CgA level was low in most cases other than hypothermia (<10 pmol/ml), remaining close to clinical reference values (2.5–3.5 pmol/ml); however, a large case difference, with a substantial increase in about half of cases, was seen for blunt injury and ACD, possibly depending on the survival time accompanied by prolonged agony involving neuroendocrinal activation, as described above. Thus, a lower CSF CgA level in cases of sharp instrument injury and asphyxiation as well as drowning and fire fatality irrespective of blood CO-Hb levels may be due to a shorter survival time. However, the CSF CgA level was independent of catecholamine levels and CgA immunopositivity in the hypothalamus and adenohypophysis, except that a correlation to hypothalamus neuronal CgA immunopositivity was detected for hypothermia. Therefore, the CSF CgA level may reflect the magnitude and duration of the central nervous system's responses to stress in the death process, independent of catecholamine levels [42, 43], especially in cases of blunt injury and ACD. CSF CgA levels were rarely low in head injury deaths, suggesting incomplete damage to neuroendocrine system [44–46]. In hypothermia cases, a low hypothalamus neuronal CgA immunopositivity with a positive correlation to the CSF CgA level may represent a terminal state of hypothalamus dysfunction involving the depletion of CgA-containing secretory glanules in a prolonged death due to cold exposure [47]. However, there was no correlation of the serum or CSF CgA level to catecholamine levels. In contrast, CSF CgA levels were low in hyperthermia cases, despite a prolonged death accompanied by a significant elevation in the postmortem serum CgA level. This may be a consequence of brain dysfunction during systemic multiple organ failure due to hyperthermia. These observations suggest CgA to be an independent marker of lasting stress due to environmental thermal hazards involving hypo- and hyperthermia and also intoxication.

Regarding the evaluation of CgA immunopositivity in the hypothalamus, adenohypophysis, and adrenal medulla, a careful consideration is needed due to an extensive interindividual difference, especially for the hypothalamus neurons. At least, however, a low CgA immunopositivity of the hypothalamus neurons was characteristic to hypothermia. In other cases, CgA immunopositivity may represent the magnitude of cumulative stress responses involving the S/A and HPA systems at the time of death independent of the cause of death [48], although further invetigation is needed using other immunohistochemical markers [49]. With respect to this, CgA immunohistochemistry of other endocrine and neuroendocrine cells may provide further information regarding stress responses of individual tissue structures.

In conclusion, these observations suggest a characterictic neuroendocrinal activation in fatal hypo- and hyperthermia and also intoxication. CgA may be a useful biochemical and immunohistochemical marker for investigating these causes of death, for which morphological evidence is often

poor. Postmortem serum and CSF CgA levels may represent the magnitude of physical responses and the central nervous system's responses to stress in the death process, respectively.

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