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Abstract

Cystatin C (CC) is a cysteine protease inhibitor present in cerebrospinal fluid (CSF) at high concentration. The abnormal CSF CC levels have been associated with numerous neurological diseases. It has been proposed as a pain biomarker and as a neuroprotector factor. We had analyzed its effect on sleep and on the recovery from a traumatic brain injury (TBI) in rats. We found that high doses of CC increased rapid eye movement sleep and worsened the TBI recuperation as they increased mortality and bleeding. Because the body temperature has an impact on sleep regulation and on recuperation after a cerebral trauma, we decided to determine if the administration of CC had an effect on body temperature and if it produced a modification in the perception of pain. The intracerebroventricular injection of CC caused a significant decrease in body temperature at higher doses (90, 175, 350, 700 fmoles) and the highest doses also increased the latency of the response to a hot plate, a central pain-perception test.

Introduction

Cystatin C (CC) is a low molecular weight (~13 kDa) protein present in cerebrospinal fluid (CSF) at high concentration [1]. It represents about 0.6% of the total CSF protein [2]. CC has the highest molar concentration of the cysteine protease inhibitors in cerebrospinal fluid [1, 3 - 4]. Cysteine proteases are lysosomal enzymes implicated in antigen processing [5], glioma invasiveness [6] and ischemic neuronal death [7]. CC selectively and reversibly inhibits cathepsins B, H, L, and S, some of the most important cysteine proteases [1, 3]. In neurological inflammatory diseases and leptomeningeal metastasis, low CC levels are accompanied with high activities of cathepsins in the CSF [8]. These findings suggest that the excessive expression of the proteases or a decrease in CC may cause a dysfunction or structural damage of the organ. Moreover, CC can be neuroprotective in cultures exposed to cytotoxic challenges, using a mechanism independent of the

role of CC as a cathepsin inhibitor [9]. Recently, CC also has been described as a pain biomarker [10], and has been extensively studied in recent years [8, 11-12].

We analyzed the effect of CC in sleep regulation and found that it decreases wakefulness and increases rapid eye movement sleep (REMS). We have also found an increase in the expression of CC after REMS deprivation and a tendency to decrease after a 2-h period of REMS rebound. We further showed that REMS deprivation increases the expression of cathepsin H, one of the proteases inhibited by CC [13]. We have also analyzed the role of CC in recovery after a traumatic brain injury (TBI) and have found that intracerebroventricular (icv) injection of CC caused a dual response in the TBI recovery; high doses worsen the recuperation whereas a low dose increases it [14 - 15].

Temperature and sleep are interrelated processes and under normal environmental conditions, the rhythms of the core body temperature and sleep propensity vary inversely across the day and night in healthy young adults [16]. Hypothermia has been shown to decrease mortality and morbidity and improve long-term outcomes by protecting the brain from a secondary brain injury [17]. Thus, we decided to determine the effect of CC on temperature. Moreover, because CC has been described as a pain biomarker [10], we decided to determine the effect of CC use on a central pain-perception test.

Material and methods

Male Wistar rats (250 to 300 g) were maintained under a controlled dark-light cycle (12 h:12 h, lights on at 08:00) with food and water ad libitum. All animal experiments were made according to guidelines and approval of the local ethical committee, in agreement with the Declaration of Helsinki.

Twenty-two male Wistar rats (250 to 350 g) were used for temperature and hot plate recordings. They were implanted, under anesthesia, with a stainless steel cannula aimed at the right lateral ventricle ($P = 0.8$, $L = 1.5$, $V = 3.8$) that was used to administer the drugs. One week after the surgery, rats received an icv injection of saline (4 μ L, 1 μ L/min) in the morning at

10:00 hs with the aid of a Hamilton microsyringe controlled by an infusion pump. Fifteen minutes after the injection, the rectal temperature was recorded and fifteen minutes later the pain perception was determined by using the hot plate test. Rats were awake during these procedures

Rats were divided into five groups and the next day 45 (n=5), 90 (n=4), 175 (n=5), 350 (n=4), or 700 (n=4) fmoles/4 uL of human CC (ABCAM) were injected icv into groups 1 to 5. Fifteen minutes later the rectal temperature was again recorded and then another fifteen minutes later the latency on the hot plate was determined.

Temperature: Fifteen minutes after injection of saline solution or CC the temperature was measured using a rectal thermocouple thermometer. The lubricated probe was inserted about 3.5 cm until a stable reading temperature was obtained.

Hot-Plate Test: Analgesic activity was determined using a Hot Plate device (Socrel, model DS35). This method evaluates the reaction time of mice or rat which are dropped on a heated surface and thus confronted with a heat stimulus applied to the plantar surface. When a central analgesic agent is administered to the animals, reaction time is markedly increased [18 - 20].

This apparatus consists of a stainless steel plate (25 X 25 cm) that can be heated and maintained at a constant temperature. The plate was set at 55.8 °C. Rats were placed on the plate and restricted in their movement by a Plexiglas cylinder (20-cm diameter and 20-cm height). The time elapsed from the moment the rat was placed on the plate to the time the rat started to lick its hind paw was recorded.

Statistical analysis: The results are reported as mean values + SEM. For the rectal temperature and time of latency in the hot plate test, significant differences were obtained using one-way analysis of variance and Duncan as post-hoc test, with $P < 0.05$ taken as statistically significant. To correlate temperature and time latency a linear-regression analysis was made.

Results and discussion

CC icv injection caused a significant decrease in the rectal temperature at doses of 90, 175, 350, and 700 fmoles, as shown in illustration 1.

Cystatins may play a defensive role in extracellular fluids by protecting organs from the cysteine proteases

of invading pathogens. CC is widely distributed in almost all tissues of vertebrates and is secreted by many kinds of cell types into a number of body fluids [1, 3 - 4].

In the nervous system CC immunoreactivity has been detected in microglial cells, astrocytes, the choroid plexus, and some neurons [21]. CC may play an important role in inflammatory neurologic diseases [22]. Increased CSF CC concentrations have been found in meningitis, encephalitis, and myelitis [23]. However, the CSF CC levels were significantly reduced in the acute phase of inflammatory neurologic diseases such as Guillain-Barré syndrome, chronic, inflammatory demyelinating polyneuropathy, and multiple sclerosis [24 - 25]. CC is also involved in the development of many degenerative disorders of the CNS such as Alzheimer's disease [26].

In the immune system, cystatins have important immunomodulatory functions; they increase the production of the tumor necrosis factor, interleukin 10, and nitric oxide [22]. The macrophages isolated from CC-knockout mice (*cysC*?) and stimulated by interferon-? had higher basal values of interleukin 10 and a lower tumor necrosis factor and nitric oxide [27]. Conversely, CC physically interacts with the TGF-? receptor II as an antagonist [28] and the central administration of TGF- ? produces fever [29]. Consequently the decrease in rectal temperature caused by CC could be an indirect effect of its immunomodulatory functions.

We also measured a significant increase in the latency time in the hot-plate test, but only with the highest doses used (see illustration 2).

The gene that codes for CC has been described as one of the genes whose expression was upregulated by carrageenan-caused peripheral inflammation in the rat using subtraction cloning and differential hybridization [30]. It was hypothesized that the CSF CC could be a biomarker for pain in humans [31]. Moreover, cathepsin S, one of the proteases inhibited by CC, has been reported as pronociceptive in a model of spinal pain [32]. All these data are in agreement with our study that shows that CC decreased the central perception of pain. Recent studies suggest that CC can be classified as a marker of inflammation or tissue injury, but not necessarily pain [33].

We also determined if there were a correlation between the temperature decrease and latency in the hot plate test. We did find a discrete but significant correlation between the effect of CC on temperature and the perception of pain (see illustration 3), because the rectal temperature decreased in rats as latency in the hot plate test increased. This behavior has been

reported for the administration of substances as a neurotensin analog [34] that reduces the rectal temperature almost 5°C and causes antinociception in the hot plate test. This behavior is not general because it has been reported that another neurotensin analog did selectively cause hypothermia without effect in nociception [35], and morphine, which causes antinociception, increases the rectal temperature [36]. More studies are needed to understand the role of CC and cathepsins in the regulation of body temperature and in the perception of pain.

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Illustrations

Illustration 1

Effect of CC on temperature. Bars represent the mean \pm SEM of rectal temperature measured 15 minutes after the icv injection of CC at different doses. Means with different letters are statistically different. One way ANOVA and Duncan test. $p < 0.0001$; $F_{5,37} = 40.498$.

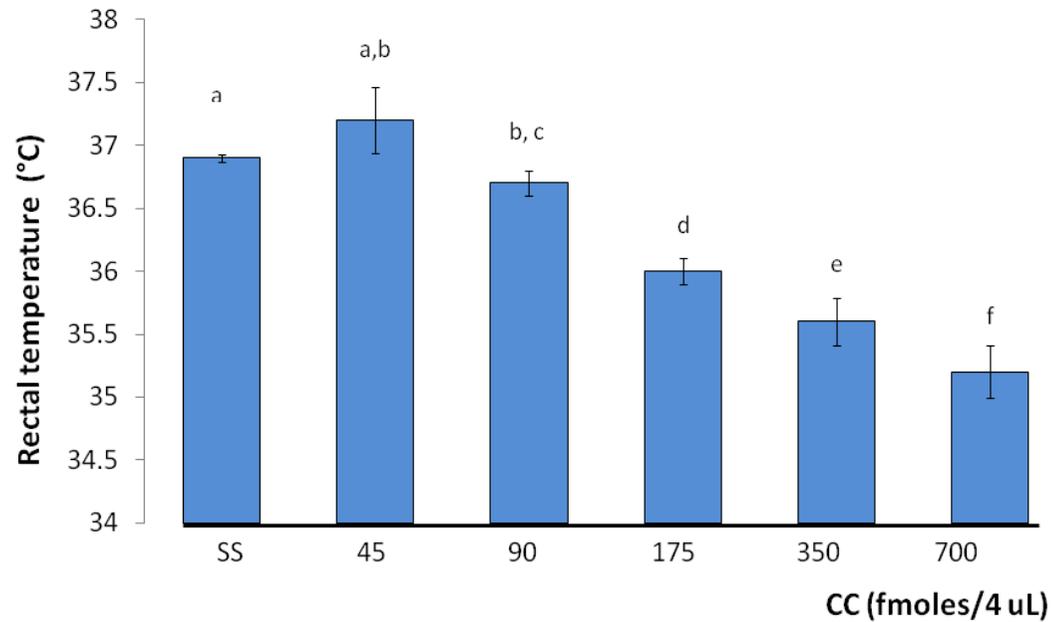


Illustration 2

Effect of CC on pain perception. Bars represent the mean \pm SEM of latency to the first sign of paw licking or jumping measured 30 minutes after the icv injection of CC at different doses. Means with different letters are statistically different. One way ANOVA and Duncan test. $p < 0.0001$; $F_{5,37} = 7.561$

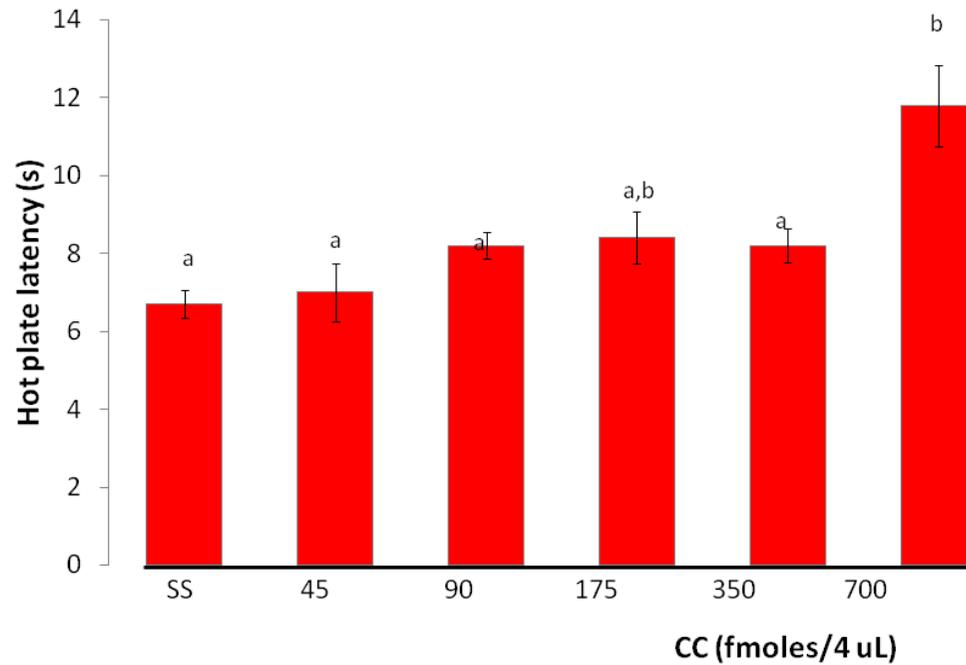
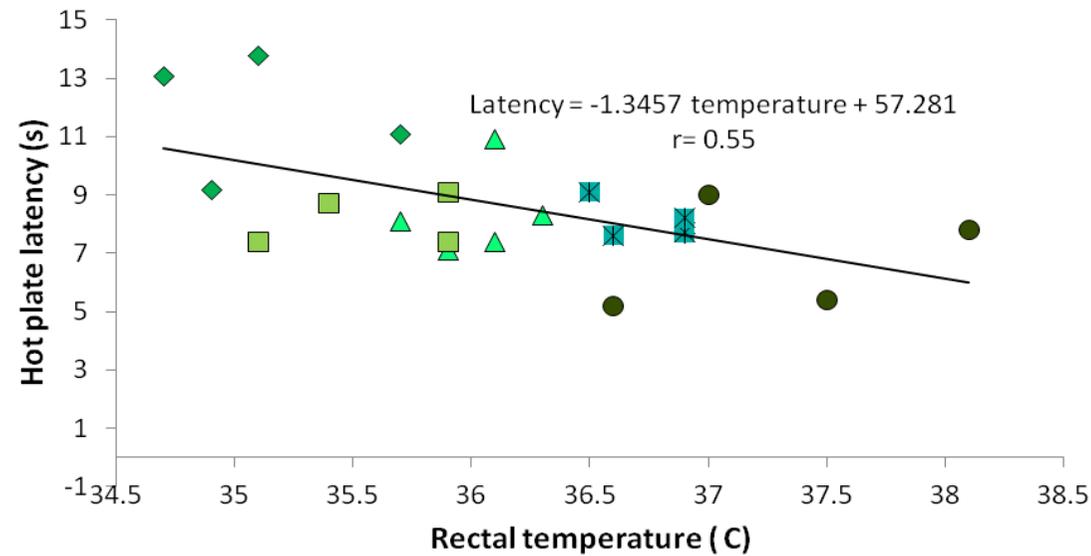


Illustration 3

Temperature vs. hot plate latency. Linear regression analysis of individual data obtained for rectal temperature and hot plate latency after the icv administration of different CC doses: Omicron, 45; $\tilde{\Delta}$, 90; Delta; 175, 350; or loz;, 700 fmoles/4 uL. Slope statistically different from 0, $p < 0.007$; $F_{1,20} = 8.867$; $r = 0.554$



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