Expression of ceruloplasmin in cavernosal tissue of paradoxical sleep deprived rats

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ABSTRACT

Objective: Ceruloplasmin (Cp) is a copper-binding protein expressed in several tissues that plays distinct functions depending on the physiological context. Recently, increased ceruloplasmin expression in cavernosal tissue has been related to diabetes-induced erectile dysfunction. Additionally, several ceruloplasmin activities are to modulate the bioavailability of nitric oxide, which is required for penile erection, suggesting that ceruloplasmin may be a candidate gene that regulates the physiology of penile erection. In previous studies, our group consistently demonstrated that paradoxical sleep deprivation (PSD) increases penile erection in male rats, but the underlying molecular mechanism is still unknown. Methods: We investigated the involvement of ceruloplasmin in paradoxical sleep deprivation-induced penile erection, determining the expression of ceruloplasmin isoforms in cavernosal tissue of rats subjected to paradoxical sleep deprivation, with or without a sleep recovery period. Results: Our findings demonstrated that paradoxical deprivation significantly increased the mRNA level of secreted ceruloplasmin by 1.3-fold compared to the controls, while the expression of glycosylphosphatidylinositol-anchored ceruloplasmin statistically differed only between the paradoxical sleep deprivation and sleep-recovery groups, with ~20% less in the rebound group. However, this subtle modulation of mRNA level was not observed at the protein level. Conclusion: These data indicate that ceruloplasmin is not a factor that directly coordinates erectile function.

Keywords: ceruloplasmin, diabetes mellitus, erectile dysfunction/chemically induced, gene expression, penile erection, sleep deprivation.

INTRODUCTION

Ceruloplasmin (Cp) is a copper-binding protein that is expressed in several tissues, including liver, brain, retina and lung(1,5). Although Cp is generally considered not a factor that directly coordinates erectile function. Cp is known as a multifunctional protein and its expression is regulated in diverse physiological and pathological circumstances. During development, Cp expression is rapidly increased in the liver and lungs, and Cp becomes the major copper-binding protein in plasma after birth(3,7). The induction of inflammation transiently increases Cp expression, suggesting that Cp is an acute-phase protein(8). Moreover, copper bound at a specific site on Cp can induce oxidation of low-density lipoprotein, and a high plasma level of Cp has been associated with hypercholesterolemia(9,10). On the other hand, ferroxidase activity of Cp appears to be essential for iron metabolism and is protective against iron-mediated oxidative damage(11,12). Together, these findings indicate that Cp can be either protective or harmful to the organism, depending on the context.

Recently, increased Cp expression in cavernosal tissue has been associated with erectile dysfunction (ED) in streptozotocin-induced diabetic rats(13). Moreover, Cp seems to participate in the homeostasis of nitric oxide.
in the cage was changed daily and food and water were available ad libitum throughout the PSD period by placing pellets and water bottles on a grid located on top of the tank. TSD was achieved by the gentle-handling method as described elsewhere. Four independent experiments were performed including C, PSD and R groups and two experiments including C and TSD groups. The rats used in this study were maintained and treated according to ethical and practical guidelines for the use of laboratory animals.

Tissue collection and total RNA extraction

The animals were decapitated immediately after sleep deprivation and sleep recovery procedures. The brain and cavernosal tissues were rapidly dissected, flash frozen in liquid nitrogen, and then stored at -80°C until RNA extraction. Total RNA was extracted from whole tissues using Trizol (Invitrogen) according to the manufacturer’s instructions.

Reverse transcription and quantitative Real time PCR (RTqPCR)

Total RNA was reverse transcribed into cDNA using SuperScript™ III Platinum® Two-Step qRT-PCR kit with SYBER® Green (Invitrogen). Reverse transcription was performed at 25°C for 10 min, 42°C for 50 min and then 85°C for 10 min. Each cDNA sample was then used as a template for real-time PCR amplification using the same kit (Invitrogen). Amplification and detection was performed using an Applied Biosystems 7500 Real-Time PCR system. Data was analyzed using ImageQuant software (GE). Total protein staining was performed using SYPRO Ruby protein blot stain (Invitrogen) and used as a loading control.

Western blot

Cavernosal tissue homogenates were analyzed by SDS-PAGE and western blot using anti-ceruloplasmin antibody (Sigma) and secondary antibody conjugated to Alexa Fluor 647 (Invitrogen). The membrane was scanned on Typhoon 9200 (GE) and the bands were quantified using ImageQuant software (GE). Total protein staining was performed using SYPRO Ruby protein blot stain (Invitrogen) and used as a loading control.

Data analysis

Gene stability was evaluated using the GeNorm algorithm, which is freely available for download. The GeNorm algorithm relies on the principle that the expression ratio of two ideal reference genes must be constant between samples. This software calculates the variation of this ratio for all two-by-two combinations of reference genes. Lower M values indicate higher expression stability, with 0.5 being a suggested cut-off for stable genes. The Cp expression fold change was calculated by the $2^{-\Delta\Delta C_T}$ method using the geometric mean expression of selected HK genes as the normalization factor. The Analysis of variance (ANOVA) followed by the Bonferroni post hoc test was conducted on the relative expression values using SPSS software (v 15.0).

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RESULTS

Validation of housekeeping genes

The gene expression study using reverse transcription and real time quantitative PCR (RTqPCR) requires a proper validation of housekeeping (HK) genes in order to produce unambiguous results(30). Therefore, we first examined the effect of sleep deprivation on the gene expression stability of the following HK genes: beta-actin (b-actin), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine guanine phosphoribosyl transferase (HPRT).

We performed RTqPCR of the HK genes using RNA extracted from the cavernosal tissue of controls (C), rats submitted to paradoxical sleep deprivation for 96 hours (PSD) and rats given a recovery period for 24 hours after the PSD (R). The effect of total sleep deprivation for 6 hours (TSD) was also investigated in separate experiments. The RTqPCR data were analyzed using GeNorm software, which scores the gene stability (M) based on the variation of the gene expression ratio between HK genes(39). Lower M values mean higher stability and M=0.5 has been suggested as cut-off for stable genes(29).

Table 1 shows the M values of all replicate experiments. Although there was some variation of the absolute M values between the experiments, in most cases, the 4 genes had M values below the suggested cut-off. Thus, the geometric mean of the four HK genes was used as the normalization factor for Cp expression.

Table 1. Gene expression stability of housekeeping genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exp_1</th>
<th>Exp_2</th>
<th>Exp_3</th>
<th>Exp_4</th>
<th>Exp_1</th>
<th>Exp_2</th>
</tr>
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<tbody>
<tr>
<td>Actin</td>
<td>0.362</td>
<td>0.315</td>
<td>0.139</td>
<td>0.249</td>
<td>0.102</td>
<td>0.331</td>
</tr>
<tr>
<td>B2M</td>
<td>0.362</td>
<td>0.274</td>
<td>0.191</td>
<td>0.249</td>
<td>0.102</td>
<td>0.331</td>
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<tr>
<td>GAPDH</td>
<td>0.537</td>
<td>0.207</td>
<td>0.378</td>
<td>0.417</td>
<td>0.227</td>
<td>0.509</td>
</tr>
<tr>
<td>HPRT</td>
<td>0.589</td>
<td>0.207</td>
<td>0.139</td>
<td>0.299</td>
<td>0.123</td>
<td>0.340</td>
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</tbody>
</table>

The expression of four housekeeping genes was measured in 4 experiments (Exp) containing 3 groups (C/PSD/R) or in 2 experiments containing 2 groups (C/TSD). The gene expression stability was calculated using GeNorm software and the data of each experiment were presented for comparison. CTRL: Control, PSD: paradoxical sleep deprivation, R: PSD+ sleep recovery period, TSD: total sleep deprivation.

Cp expression in sleep deprived rats

The expression of secreted and GPI-anchored Cp was determined by RTqPCR using the same material described above. First, we observed that cavernosal tissue expresses more of the GPI-anchored isoform compared to the secreted form (p < 0.0001, Figure 1 A). When the expression of each isoform was examined, a 1.3-fold increase of secreted Cp was observed in the PSD group compared to the control group. This alteration was subtle but statistically significant (p = 0.01, Figure 1 B). The expression of Cp-GPI did not differ between the control and PSD groups, but it was significantly decreased in the R group compared to the PSD group (p = 0.04, Figure 1 C). When total Cp expression (sum of both isoforms) was analyzed, a significant difference was found between the control and PSD groups, with a 23% increase in the PSD group (p = 0.04, data not shown). On the other hand, short term total sleep deprivation did not affect the expression of any isoforms in cavernosal tissue (p > 0.05; Figure 2).

Figure 1. Effect of paradoxical sleep deprivation on the expression of ceruloplasmin in cavernosal tissue. A. The expression of secreted Cp was compared to Cp-GPI in control rats. * p < 0.0001. B and C. Secreted Cp and Cp-GPI were evaluated in cavernosal tissue of controls (C), of rats subjected to paradoxical sleep deprivation for 96 hours (PSD), and of rats subjected to PSD and sleep recovery for 24 hours (R). Error bar represents standard error of the mean (SEM). * p < 0.01 compared to control, # p < 0.05 compared to PSD (ANOVA followed by Tukey’s post hoc test).

Figure 2. Effect of total sleep deprivation on the expression of ceruloplasmin in cavernosal tissue. A and B. Secreted Cp and Cp-GPI were evaluated in cavernosal tissue of controls (C) and of rats subjected to total sleep deprivation for 6 hours (TSD). Error bar represents standard error of the mean (SEM).

To verify the specificity of the subtle modulation of expression observed after PSD, we examined Cp expression in brain, a tissue that is not directly involved in the erectile event. As observed in a previous study, Cp-GPI was the predominant form expressed in brain (p < 0.05, Figure 3 A)(26). Both isoforms were constitutively expressed throughout the experimental groups (Figure 3 B and C, p > 0.05).

Confirmation of Cp expression by western blot

In order to confirm the modulation of Cp at the protein level, western blotting was performed using cavernosal tissue homogenate. Anti-ceruloplasmin antibody recognized a single band at ~140 kDa without distinguishing the 2 isoforms. When we omitted the primary antibody, only unspecific bands of secondary antibody were observed (Figure 4 A). The Cp bands were quantified and normalized by the intensity of the bands generated by total protein staining. Although there is a subtle increase of

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the mean Cp level in the PSD and R groups as compared to the control group, no statistical significance was found between the three groups (Figure 4 B).

**Figure 3.** Effect of paradoxical sleep deprivation on the expression of ceruloplasmin in the brain. A. The expression of secreted Cp was compared to Cp-GPI in control rats. *p < 0.05. B and C. Secreted Cp and Cp-GPI were evaluated in brain of controls (C), of rats subjected to paradoxical sleep deprivation for 96 hours (PSD), and of rats subjected to PSD and sleep recovery for 24 hours (R). Error bar represents standard error of the mean (SEM).

**Figure 4.** Protein expression of ceruloplasmin in cavernosal tissue. A. Cavernosal tissue homogenates from controls (C), rats subjected to paradoxical sleep deprivation for 96 hours (PSD), and of rats subjected to PSD and sleep recovery for 24 hours (R) were subjected to western blot analysis using anti-ceruloplasmin (Cp) antibody. B. The band intensity of ceruloplasmin was normalized by the bands of total protein staining. The mean relative expression (Cp/total protein) of all groups was normalized by the mean of control group and presented with respective standard error of the mean (SEM).

**DISCUSSION**

The current findings showed that, although PSD significantly increased the mRNA level of secreted Cp by 1.3-fold compared to the control rats, this subtle modulation was not observed at the protein level, indicating that Cp is not a factor that directly coordinates erectile function.

Our group has consistently demonstrated that 96 hours of PSD facilitates erection (for review see27). In humans, augmented sexual fantasy27, increased penile tumescence and masturbation28 have also been reported after sleep deprivation. All of these behaviors have a motivational condition, i.e., related to appetitive aspects of copulatory behavior. In order to better understand the relationship between erectile function and sleep, we have extensively studied the effect of PSD on the erectile function using an animal model (26,29 for review). Interestingly, PSD, as used in this study, resulted in decreased number of intromissions in male rats26, suggesting that PSD had a more pronounced effect on the initial triggering mechanisms for sexual motivation, than on the actual intensity of sexual activity, after a period of sleep deprivation. As pointed out by Alvarenga et al.26, the presence of a female, in addition to the repertoire of behaviors intrinsic to copulatory activity and fatigue, could compromise appetitive behaviors.

Several factors can converge to reduce the time that is available for sleep, including social life, artificial light, shift-work, and sleep disturbances. This leads to sleep curtailment, which has become a trend of modern society that causes several health problems including ED30. Indeed, among the many consequences common with sleep disorders, sexual dysfunction remains the least studied. However, the incidence of sexual dysfunction is significant. Although the relationship between cause (pathology) and effect (ED) seems quite evident, many factors that contribute to sexual dysfunction have not yet been fully elucidated. In this sense, sleep loss in an animal model can be an interesting approach for investigating a possible molecular mechanism involved in the physiology of penile erection. The manipulations of such variables have been conceived and performed in an attempt to expand the comprehension of the complex interactions underlying the regulation of mammalian sexual behavior.

Recently, an epidemiology study with the purpose of estimates the prevalence of ED complaints associated with sleep disturbances as well as with other medical conditions reported that factors such as diabetes and sleep apnea were also significantly associated with a higher risk of ED complaints31. Moreover, the results show that reduced amounts of REM sleep and increased number of arousals, which reflect a non-consolidated sleep pattern, are also risk factors for ED complaints. It is well documented that experimental fragmentation of sleep in young adults disrupts the usual rise of blood testosterone levels during the night32, while REM sleep deprivation decreases testosterone concentrations in male rats33. Collectively, these studies indicate that sleep alterations have marked on hormonal profile, and consequently can affect erectile function.

Given that diabetes induces ED and that both mRNA and protein levels of Cp are enhanced in the cavernosal tissue of diabetic rats, it is plausible to presume that an experimental condition that stimulates penile erection, such as PSD, would decrease Cp expression. However, our results demonstrated that PSD did not alter Cp expression at the protein level, suggesting that Cp expression may not correlate with the erectile function. As a novel finding, further studies are warranted to clarify the complete mechanisms involved in ED and the role of Cp.

We acknowledge that although the PSD effect was small and limited to the mRNA level, the increase of Cp expression selectively occurred in the cavernosal tissue of rats submitted to longer sleep deprivation. Prolonged sleep deprivation is known to be more detrimental than acute sleep deprivation and is considered a risk factor for several immunological alterations and chronic diseases such as obesity, diabetes and cardiovascular diseases34-36. Moreover, Cp expression is often increased in chronic diseases, suggesting that the subtle increase of Cp transcription observed in the PSD group can be indicative of health damage incurred due to prolonged sleep loss.

It is important to note that Cp has NO oxidase activity, which can decrease the bioavailability of NO. NO is an important vasodilator that contributes to vascular smooth muscle relaxation and penile erection12,37. Thus, a drastic in-
crease of Cp levels in pathological conditions can instigate a secondary effect on erectile function, which may be the case for diabetic patients\(^6\). Likewise, smokers with obstructive sleep apnea present higher plasma levels of Cp compared to smokers without apnea. Obstructive sleep apnea has been strongly associated with ED\(^6,10,36\). In summary, our findings indicate that the subtle modulation of mRNA level was not observed at the protein level, suggesting that Cp is not a factor that directly coordinates erectile function.

DECLARATIONS OF INTEREST

All authors have declared that there is no conflict of interest that could be perceived as prejudicing the impartiality of the present study.

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