MELATONIN INHIBITS THE PROTEIN C ANTICOAGULANT PATHWAY IN RATS

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ABSTRACT

This study examines the influence of melatonin on the PC anticoagulant pathway in rats. The experiment was performed on 52 male white Wistar rats weighing 200-220 g. Animals were equally divided into 4 groups. They were treated in three consecutive days, every 12 hours, subcutaneously: 1st group – with saline solution (solvent for melatonin and luzindole); 2nd group – with melatonin, daily dose 0.2 mg/kg body weight; 3rd group – with luzindole, nonstop dose of 0.4 mg/kg body weight; 4th group – melatonin, one hour after pretreatment with luzindole. The required amount of blood was taken under urethane narcosis via direct cardiac puncture. After three days of administration of melatonin, a significant decrease in the antigen concentration of protein C, protein C activity, activated protein C and thrombomodulin was observed. The soluble form of the endothelial receptor for protein C, activity of protein S and free protein S were significantly elevated. The competitive melatonin receptor antagonist – luzindole, when administered alone and in pretreatment, effectively removes the observed effects of melatonin by blocking exogenous, as well as endogenous melatonin. In conclusion, our data give us reason to assume that melatonin significantly reduces the activity of the protein C anticoagulant pathway in rats.

Keywords: *melatonin, protein C, protein S, thrombomodulin, sEPCR*

INTRODUCTION

Hemostasis is a process with a strong protective effect and results from the balance between the coagulant, anticoagulant and fibrinolytic systems. One of the important mechanisms of the anticoagulation system is the protein C (PC) anticoagulant pathway, which includes membrane proteins and circulating proteins: PC, thrombomodulin, endothelial protein C receptor (EPCR) and protein S (1). They form a multimolecular complex that provides efficient proteolysis of substrates, anticoagulant and cytoprotective effects (2,3). Like all systems in the body, hemostasis is subject to complex regulatory effects, and more and more evidence is accumulated about the significant role of the endocrine system (4,5).

The effects of melatonin on hemostasis are relatively poorly studied. Literary data suggest that melatonin indubitably affects processes related to hemostasis in both experimental conditions and clinical observations. It should be noted that experimental studies are conducted on animal models with vari-

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ous disabilities, rather than intact animals, which complicates the assessment of results (6). Clinical observations are based on tracking the individual parameters of hemostasis in patients with different primary diseases and comorbidities, which determines the contradictory nature of the conclusions (7,8).

Deriving from the exposition so far, as well as our previous publications (9,10,11) related to studying melatonin's effects on hemostasis, which prove an irrevocable tendency towards heightened coagulation, it is only logical to raise the question of melatonin's place and role in the anticoagulant system. Based upon these thoughts we've set it as our goal to study the effects of melatonin on key parameters of the protein C anticoagulant pathway, which constitutes a primary part of the anticoagulant system.

MATERIAL AND METHODS

The experiment was conducted upon 52 male white Wistar rats, weighing 200-220g, in accordance with the European Convention for protection of experimental animals (Protection of animals used for experimental purposes, Council Directive 86/609/EEc of November 1986, Directive 2010/63/EU of the European Parliament and of the Council of September 2010). Animals were housed under standard conditions, with free access to standard pellet food and water ad libitum, with a 12-hour light/dark cycle.

Animals were divided into four equal groups – one control and three experimental. Treatment was administered subcutaneously over a span of three consecutive days, twice a day, over 12 hour intervals. The first (control) group was injected with saline – a solvent of melatonin and luzindole, with the same schedule and amount per kg body weight, the second group – with melatonin (Merk, Germany) in a daily dose of 0.2mg/kg body weight, the third group – with luzindole (Sigma Chemicals/St. Louis, MO, USA) in a daily dose of 0.4mg/kg body weight, the fourth group – with melatonin one hour after pretreatment with luzindole, using the same procedure and dosages.

The required amount of blood was taken under urethane anesthesia by cardiac puncture in disposable syringes. Sodium nitrate was used as anticoagulant (0.11 mol/l); in a blood to citrate ratio 9:1. The citrate blood was centrifuged for 10 minutes at 3000 revolutions/minute. The citrated plasma was frozen

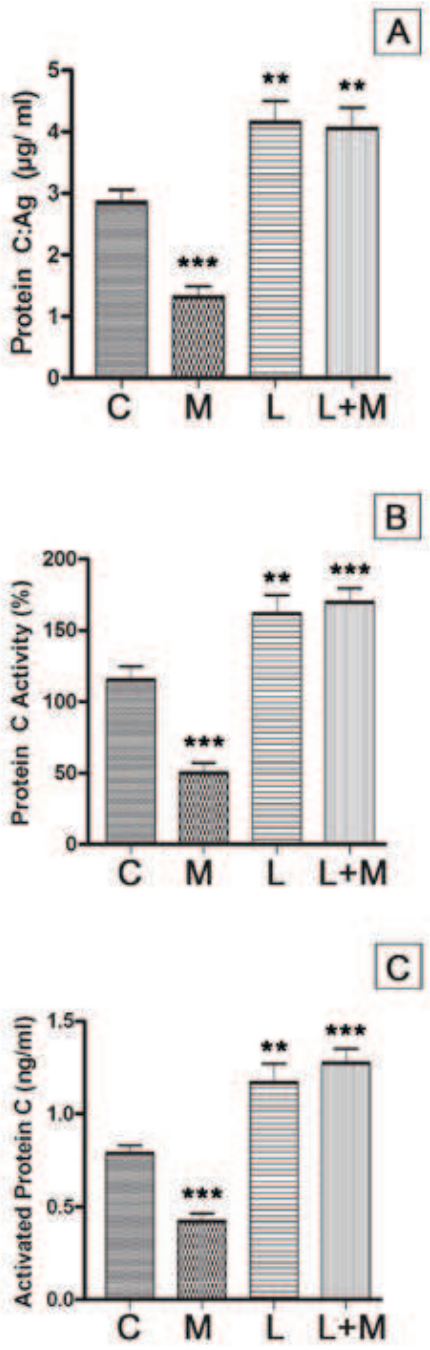

Fig. 1. Effects of melatonin (0.2mg/kg body weight), luzindole (0.4mg/kg body weight) and melatonin 1 hour after pretreatment with luzindole (same dose), all administered s.c. to male Wistar rats, 2 times a day, for three consecutive days on (PC: Ag) (A), (PC: Act) (B) and (APC: Ag) (C). Used abbreviations: C - control group injected saline; M - melatonin; L - luzindole. Data are presented as $\bar{x} \pm S\bar{x}$*, ***- p <0.001; **- p <0.01.*

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at –60°C and the parameters were determined by the tenth day.

The majority of the analyzed parameters were determined by an ELISA method with a kit of Diagnostica Stago (France), with the exception of thrombomodulin, determined by an IMUBIND®ELISA kit, American Diagnostica Inc., USA.

All data were processed via variation analysis utilizing the student-fisher t-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

1. Effects of melatonin and luzindole on plasma levels of PC antigen (PC: Ag), PC activity (PC: Act) and activated PC (APC: Ag). From Figure 1A it is evident that melatonin reduces the plasma level of PC antigen from 2.85±0.21μg/ml (control group) to 1.31±0.16μg/ml ($p<0.001$). After administration of luzindole only, as well as after pretreatment with it, an increase in PC: Ag is observed, the registered values were respectively 4.15±0.35μg/ml ($p<0.01$) and 4.05±0.34μg/ml ($p<0.01$). We note a significant reduction in PC activity (%) in the animals treated with melatonin – down to 50.13 ± 6.98 ($p <0.001$), as demonstrated in Fig. 1B. PC activity increases to 161.70±12.91 ($p<0.01$) after the application of luzindole, while pretreatment with luzindole increases it up to 169.70±9.83 ($p<0.01$). The value of PC activity in the control group of animals was 115.20±9.72. The changes in the levels of activated protein C under the effect of melatonin and luzindole are presented in Fig. 1C. It is evident that melatonin significantly decreases APC from 0.79±0.04ng/ml (control group) to 0.42±0.04ng/ml ($p<0.001$). Luzindole, in its turn, increases APC up to 1.17±0.10ng/ml ($p<0.01$). Melatonin, administered after pretreatment with luzindole also increases APC to 1.28±0.07ng/ml ($p<0.001$).
2. Effects of melatonin and luzindole on the levels of soluble protein C receptor (sEPCR) and thrombomodulin. Fig. 2A shows that the three-day administration of melatonin leads to an increase in sEPCR from 118.40±6.36ng/ml (control group) to 169.20±13.86ng/ml ($p <0.01$). After administration of luzindole only, as well as after pretreatment with it, a decrease in sEPCR was observed, with registered values relative to the control group of animals of 50.19±4.00ng/ml ($p<0.001$) and 39.69±5.51ng/ml ($p<0.001$), respectively. Fig. 2B shows the effect of melatonin on the level of thrombomodulin and we can see a reduction from 6.26±0.48ng/ml (control group) down to 2.62±0.16ng/ml ($p<0.001$). The stand-alone application of luzindole increases thrombomodulin to 9.72±0.79 ng/ml ($p<0.01$). Pretreatment with luzindole shows a more pronounced increase in thrombomodulin to 9.67±0.54ng/ml ($p<0.001$).

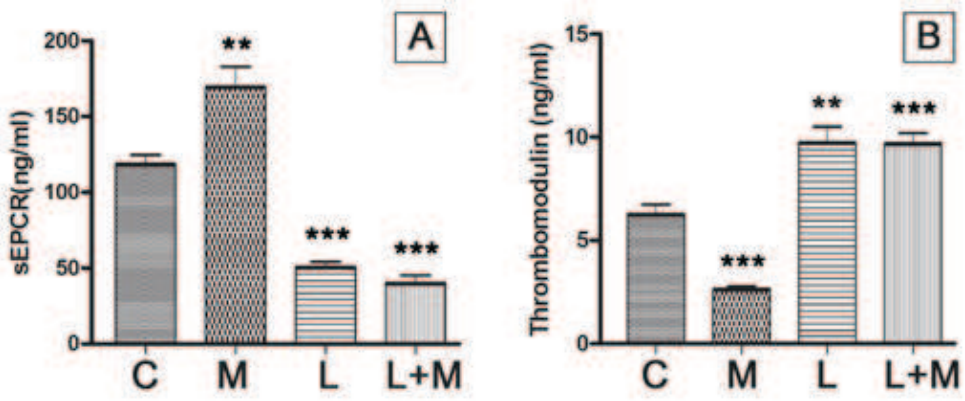

Fig. 2. Effects of melatonin (0.2 mg/kg body weight), luzindole (0.4 mg/kg body weight) and melatonin 1 hour after pretreatment with luzindole (same dose), all administered s.c. to male Wistar rats, 2 times a day, for three consecutive days on sEPCR (A) and thrombomodulin (B). Used abbreviations: C - control group injected saline; M - melatonin; L - luzindole. Data are presented as $\bar{x} \pm S\bar{x}$, ***- $p <0.001$; **- $p <0.01$.

3. Effects of melatonin and luzindole on the levels of free protein S antigen (free PS: Ag) and protein S activity (PS: Act). The results presented in Fig. 3A show that the three-day administration of melatonin increases free protein S levels from 6.81±0.53 μg/ml (control group) to 9.77±0.68μg/ml ($p<0.01$). Luzindole reduces the levels of free protein S to 3.02±0.39μg/ml ($p<0.001$). Pretreatment with luzindole repeats this effect and the decrease in free protein S is down to 3.65±0.25μg/ml ($p<0.001$). Fig. 3B shows the changes in the protein S activity (%). The three-day administration of melatonin increases the protein S activity to 171.50±9.92 ($p<0.001$). Luzindole and pretreatment with it reduce the protein S activity to 42.33±5.44 ($p<0.001$) and 50.31±4.16 ($p<0.001$), respectively. The value of protein S activity in the control group of animals was 105.80±9.73.

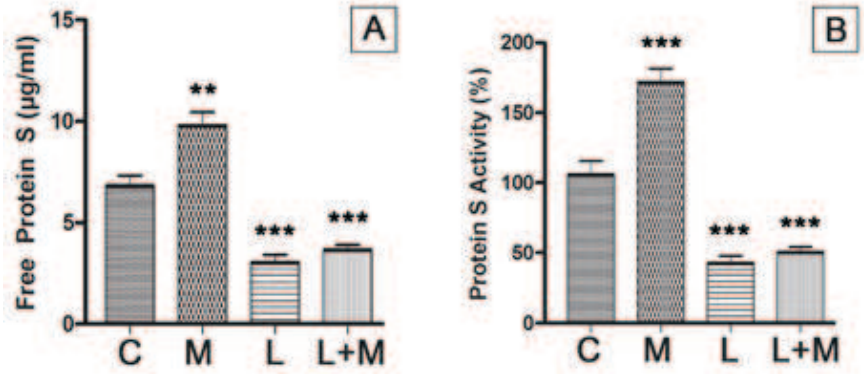

Fig. 3. Effects of melatonin (0.2 mg/kg body weight), luzindole (0.4 mg/kg body weight) and melatonin 1 hour after pretreatment with luzindole (same dose), all administered s.c. to male Wistar rats, 2 times a day, for three consecutive days on the plasma levels of (free PS: Ag) (A) and (PS: Act) (B). Used abbreviations: C - control group injected saline; M - melatonin; L - luzindole. Data are presented as $\bar{x} \pm S\bar{x}$, ****- $p<0.001$; **- $p<0.01$.*

DISCUSSION

Protein C – a vitamin K-dependent glycoprotein plays a key role in the protein C anticoagulant pathway, synthesized in hepatocytes and circulating in plasma as an inactive zymogen. It is activated on the surface of endothelial cells – a process catalyzed by thrombin-thrombomodulin complex. EPCR further stimulates the PC activation (12). Our results show that the level of PC: Ag in the plasma of rats injected with melatonin was significantly reduced (Fig. 1A). This could be a manifestation of suppressed PC biosynthesis (13). This statement contradicts data from other sources, which claim that the liver holds primary significance in regard to the synthesis of plasma coagulation factors. Furthermore, melatonin membrane receptors MT_1/MT_2 have been identified in various mammalian tissues, including the liver in rats (14). Extrapolating from the currently available data, at this stage it is difficult to determine the exact mechanism of manifestation of melatonin's suppressive effect. It is very likely that the process is mediated by melatonin MT_1/MT_2 receptors, a fact that is supported by the observed increase in PC: Ag after administration of the non-selective melatonin receptor antagonist – luzindole. Given the proliferation of MT_1/MT_2 receptors in various tissues and organs (15), it is appropriate to assume the intervention of other regulatory relationships and mechanisms which mediate the effects of melatonin on PC: Ag. The three-day injection of melatonin in rats induces a decrease of both the PC: Act, as well as APC (Fig. 1B, 1C). Although the concentration and activity of PC and APC are different indicators, pertaining not only to synthesis but to the stages of PC activation, the observed changes in the three parameters are in the same direction and testify for a suppression of the activity of the anticoagulant PC pathway and a tendency to hypercoagulability.

EPCR is isolated and cloned as an endothelial cell-specific, highly selective and high-affinity bind-

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ing protein for PC and APC (16). EPCR binds PC of the endothelial surface and provides it to the thrombin-thrombomodulin complex for activation. The soluble form sEPCR, which occurs in the proteolytic cleavage of the membrane-bound form can bind APC and deprive him of its anticoagulant function (17). In our study we found a significant increase of sEPCR (Fig. 2A). It is known, that sEPCR circulates in plasma and inactivates the anticoagulant activity of APC (17). The increased level of sEPCR in plasma correlates with the observed decrease in APC.

A crucial cofactor of the thrombin-mediated PC activation is thrombomodulin – a superficially expressed glycoprotein, synthesized in the vascular endothelium (18). Data describing the level of thrombomodulin in plasma are often contradictory. According to some authors, the reduced level is an expression of hypocoagulability, and increased level is a marker of endothelial dysfunction and hypercoagulability (19,20). The three-day administration of melatonin in our study (Fig. 2B) caused a reliable reduction in thrombomodulin, a result which correlates with the established changes in the levels of PC: Ag, PC: Act and APC and points towards suppression of the activity of the anticoagulant PC pathway.

The activated PC, together with its cofactor protein S, inhibits coagulation by irreversible proteolytic inactivation of factor VIIIa and factor Va on the surface of the negatively charged phospholipid membranes (21). After application of melatonin, a significant increase in the free protein S (Fig. 3A) as well as protein S (Fig. 3B) was established. These changes could be an expression of stimulated biosynthesis of protein S in hepatocytes after treatment with melatonin (13).

Luzindole is the first, but not the sole ligand, described as a receptor antagonist of melatonin (22). Standalone administration of luzindole in this study was followed by a significant increase in the levels of PC: Ag, PC: Act and APC (Fig. 1A, B, C). This result could be a manifestation of inhibition of melatonin effects mediated by MT_1/MT_2 receptors. A similar effect was also observed with respect to the level of thrombomodulin – luzindole significantly increased its level (Fig. 2B). Thus, the present stimulation of these key elements of the PC anticoagulant pathway is consistent with the suppression of the level of sEPCR (Fig. 2A) under the influence of the melatonin receptor antagonist. Luzindole causes a significant inhibition of free protein S (Fig. 3A) and its activity (Fig. 3B). From the presented results it is evident that the changes in all parameters after administration of luzindole are in opposite direction to the changes under the same parameters after melatonin application. The described changes in the key elements of the PC anticoagulant pathway under the influence of luzindole could be interpreted as evidence for the involvement of MT_1/MT_2 receptors in accomplishing the effects of melatonin on coagulation.

Treatment of rats with melatonin, one hour after pretreatment with luzindole at the same dose and administration scheme, resulted in a statistically significant increase ($p<0.001$) of APC and PC activity (Fig. 1B, C). In the same direction is the change in the PC antigen concentration (Fig. 1A), while the observed increase was less pronounced ($p <0.01$). Pretreatment with luzindole causes also significant changes in endothelial receptors, which play a key role in the PC anticoagulant pathway, namely: sEPCR is reduced (Fig. 2A), while the thrombomodulin level is increased (Fig. 2B). The effect on the levels of free protein S and protein S activity results in a significant reduction (Fig. 3A, B). The presented results show that pretreatment with luzindole effectively eliminates the observed effects of melatonin. Furthermore, treatment with melatonin after pretreatment with luzindole almost entirely repeates the changes in the studied parameters obtained after administration of luzindole only. This fact allows us to assume that luzindole removes the effect of exogenous melatonin. Especially noteworthy are the obtained results for the following parameters: sEPCR, the levels of free protein S and protein S activity. The observed values after administration of luzindole only, as well as in pretreatment with it, are lower than those of the control group of animals – a fact that gives us the right to assume that the non-selective antagonist of MT_1 and MT_2 significantly blocks endogenous melatonin as well. Based on the described results we could assume a considerably more complicated role of MT_1 and MT_2 in realizing the effects of melatonin.

CONCLUSIONS

The results presented above clearly indicate that melatonin significantly reduces the activity of the PC anticoagulation pathway in rats. The increased activity of this pathway, observed after administration of luzindole independently and prior to treatment with melatonin, allows us to suggest that the blockage of the MT_1 and MT_2 membrane receptors removes the effect of both endogenous and exogenous melatonin. The significantly reduced activity of the PC anticoagulant pathway can be regarded as one of the mechanisms by which this hormone causes an evident tendency to hypercoagulability established in our previous studies (10, 11). Despite the experimental nature of the derived results, it is logical to raise the question of the necessity of preventive hemostasis control in both patients undergoing melatonin therapy(23-26), and patients receiving food supplements containing melatonin (27).

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