

**EFFECTS OF PITUITARY ADENYLATE CYCLASE
ACTIVATING POLYPEPTIDE IN KIDNEY AND
PINEAL GLAND**

PhD thesis

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I. INTRODUCTION

1. PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP)

Pituitary adenylylating polypeptide (PACAP) was first isolated in 1989 from ovine hypothalamus based on its potency to activate adenylylating cyclase. The peptide exists in two forms, with 27 and 38 amino acid residues. In mammalian tissues PACAP38 is the dominant form. It is a member of secretin/glucagon/vasoactive intestinal polypeptide family and shares identity with VIP. PACAP occurs both in the central and peripheral nervous system, but it is present also in non-neural tissues. It acts through G protein-coupled receptors. There are two types of PACAP receptors: (1) the specific PAC1 receptor, which binds PACAP with much higher affinity than VIP and (2) VPAC1 and VPAC2 receptors, which bind them with similar affinities.

Previously, protective effects of PACAP have been studied mainly in different models of neuronal damage. PACAP has also been shown to have protective effects in non-neural tissues, e.g. cardiomyocytes, endothelial cells, pituitary adenoma cells, ovarian follicular cells, T cells. However, the protective effect of PACAP could not be observed in all cell types. Our previous investigations revealed that PACAP failed to protect JAR human choriocarcinoma cells exposed to different stressors. The antiapoptotic and antiinflammatory effects of PACAP may be responsible for its protective effect.

2. RENOPROTECTIVE EFFECT OF PACAP

The effects of PACAP in the kidney are not yet fully known. The presence of all 3 PACAP receptors indicates its physiological importance. The renoprotective effect has been demonstrated earlier in myeloma kidney injury, where PACAP could reduce the TNF- α level in rat kidney. Previously, it has been shown that PACAP is able to prolong the renal ischemic time, decrease mortality and attenuate tubular degeneration in a rat model of ischemia. However, the exact mechanism, by which PACAP acts as a renoprotective agent, is not yet known.

3. FACTORS INFLUENCING THE EFFECTS OF PACAP: PACAP AND THE BIOLOGICAL RHYTHMS

Previously, it has been described that the neuroprotective effect of PACAP shows gender and age-dependence in the animal model of Parkinson's disease. Furthermore, the effects of PACAP on gonadotroph cells depend also on the time-point of treatment and on the day of the estrous cycle in case of female rats. In our present studies, we investigated the role of the daily rhythm as a possible influencing factor.

Role of PACAP in the regulation of circadian rhythmic processes

The major part of the physiological processes is periodically repeated. The most studied rhythm is the circadian, which can be characterized by approximately 24 hours long cycles. In mammals the role of PACAP in the regulation of circadian rhythms has already been proven. The mammalian biological clock is the suprachiasmatic nucleus, which receives photic information from the retina through the retinohypothalamic pathway. In mammals, PACAP can be detected in a population of retinal ganglionic cells and is the main co-transmitter of glutamate in the retinohypothalamic pathway in mammals.

The pineal gland also plays an important role in the regulation of the diurnal rhythms. It affects most circadian processes through its melatonin production. PACAP is present both in the avian and mammalian pineal gland, where it stimulates melatonin synthesis. Furthermore, PACAP enhances the arylalkylamine N-acetyltransferase (AA-NAT), which is the rate-limiting enzyme of melatonin production.

Avian pinealocytes display a persistent rhythm even under *in vitro* circumstances. Although PACAP stimulates melatonin production, it does not cause any phase shift. The molecular mechanism by which PACAP influences the circadian rhythmic processes is only partially known. PACAP may act through complex signal transduction pathways. Accordingly, one aim of our studies was to examine the effect of PACAP on p38 MAPK phosphorylation and on 14-3-3 protein and their daily changes.

4. AIMS

We planned to investigate the followings:

1. the presence of PACAP and distribution of PAC1 receptor in rat kidney
2. the renoprotective effects of PACAP and its possible molecular mechanism under *in vivo* and *in vitro* circumstances
3. the influence of daily rhythm on the effects of PACAP on cell survival and signal transduction pathways

II. INVESTIGATION OF THE RENOPROTECTIVE EFFECT OF PACAP

MATERIALS AND METHODS

I. Investigation of the role of PACAP in PACAP knockout (KO) mice

I/1. *In vitro* experiments

A. Effect of *in vitro* oxidative stress on renal cells of wild-type and PACAP KO mice

Primary culture of neonatal mouse kidney cells was obtained from 2 to 7-day-old wild-type and PACAP KO mice (n=7 in each experiment, repeated 4 times). Cells were incubated in DMEM/F12 medium for 48 hours. Renal cells of wild-type and PACAP KO mice were either left untreated (control group) or were exposed to 0.5, 1.5 or 3 mM H₂O₂ for 2 or 4 hours.

B. Effect of exogenous PACAP against oxidative stress in PACAP KO renal cells

In the second set of our experiments, we tested whether exogenously given PACAP can counteract the increased susceptibility of PACAP KO kidney cells to H₂O₂. Isolated cells were randomly assigned in the following experimental groups: control cells with no treatment; treatment with 10 or 100 nM PACAP alone, 0.5 mM (4h) or 1 mM (2h) H₂O₂ alone; cells treated with 0.5 mM (4h) or 1 mM (2h) H₂O₂ and 10 or 100 nM PACAP together.

C. Effect of *in vitro* hypoxia on renal cells of wild-type and PACAP KO mice

Experiments were performed under the above-described circumstances. *In vitro* hypoxia was evoked by CoCl₂-treatment. The applied experimental groups were: control group without treatment; 500 µM CoCl₂ for 24 or 48 hours.

Measurement of cell viability

Viability of the renal cells was determined by colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), based on the reduction of MTT into a blue formazan dye by viable mitochondria and absorbance was measured by an ELISA reader at 570 nm.

I/2. Histological analysis of kidney from PACAP KO mice

Kidneys from wild-type and PACAP KO animals (n=7 in each group) were investigated. Animals were anesthetized using isofluran, renal vessels were isolated after median laparotomy. Renal vessels of one side of the kidneys were clamped for 45 or 60 minutes (the kidney of the other side serve as a control). After 2 weeks survival, under pentobarbital anesthesia kidneys were removed and placed in 4% paraformaldehyde for fixation. Serial 10 µm thick sections from paraffin-embedded kidneys were made and stained with routine haematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) staining. Histological sections were evaluated 0-1-2 grading scale based on the following morphological signs: tubular dilation, disappearance of PAS+ glycocalyx layer in the proximal tubule, presence of lymphocytes and macrophages, PAS+ cylinders in the tubules.

I/3. Identification of PACAP in mouse kidney

Identification of PACAP38 in mouse kidney (n=3) was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Spectrum was compared to PACAP38 standard.

II. Investigation of the effect of PACAP in rat kidney

II/1. Effect of exogenous PACAP on cell survival *in vitro*

Primary kidney cell cultures were obtained from 2 to 7-day-old newborn Wistar rats. Cells were assigned to one of the following experimental groups: control group of cells with no treatment or treated with 10 or 100 nM PACAP1-38 alone; cells treated with 1, 3 or 6 mM

H₂O₂; cells exposed to different concentrations of H₂O₂ together with 10 or 100 nM PACAP. Cells were treated with the above-mentioned concentrations of chemicals for 2 or 4 hours. After proving the protective effect of PACAP in this set of experiments, we repeated the cell culture treatments to study the lowest concentration of PACAP still effective under these experimental conditions. Cells were left untreated (control), exposed to 1 mM H₂O₂; or treated with increasing concentrations of PACAP1-38 (10 pM, 100 pM, 1 nM and 10 nM) together with the oxidative stress. Cells were exposed to these treatments for 2 hours.

II/2. Effects of PACAP on Bcl-2 level in rats subjected to renal ischemia/reperfusion

Male Wistar rats (300–350 g) were anesthetized with isoflurane. Heparin (100 IU/kg bodyweight), and PACAP (100 µg dissolved in 100 µl saline) or vehicle were injected into the jugular vein before laparotomy. Renal vessels were cross-clamped after median laparotomy for 45 or 60 min (n=5/group). Animals were sacrificed immediately after warm ischemic time or 10 min after reperfusion. Kidneys were removed and further processed for Western blot analysis of Bcl-2.

II/3. Cytokine array after *in vivo* ischemia/reperfusion

Male Wistar rats (300–350 g) were anesthetized with isoflurane. Heparin (100 IU/kg bw) and PACAP (100 µg dissolved in 100 µl saline) or vehicle were injected into the jugular vein before laparotomy. Renal vessels were cross-clamped after median laparotomy for 45 or 60 minutes. Animals were sacrificed, and kidneys were removed 24 h after ischemia/reperfusion. Kidneys from control, vehicle-, and PACAP-treated ischemia/reperfusion group were processed for cytokine array studies (n=4 in each group). Cytokine array from tissue homogenates was performed using Rat Cytokine Array Panel A Array kit from R&D Systems.

II/4. Investigation of presence of PACAP and PAC1 receptor

Identification of PACAP38 in rat kidney (n=3) was performed by using matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry. Spectrum was compared to PACAP38 standard. Presence and distribution of PAC1 receptor were detected using immunohistochemical staining. Rat kidneys were fixed (n=4) in 4% paraformaldehyde and 10 µm thick sections from paraffin-embedded kidney were made.

Immunohistochemistry was performed using polyclonal PAC1 receptor antibody (1.100, Sigma). All the next steps were performed using ABC kit following the manufacturer's instructions (Vectastain, Hungary).

II/5. Changes of PACAP following renal ischemia/reperfusion in vivo

Male Wistar rats weighing 300-350 g were anesthetized with isoflurane, and renal vessels were freed and warm ischemic damage was induced by unilaterally cross-clamping both renal pedicles for 60 minutes. Animals were sacrificed, and kidneys from both the ischemic and the intact sides were removed 1, 6 and 24 hrs after reperfusion (n=5 in each group). Control kidneys were not operated (n=5). Kidneys were processed for radioimmunoassay (RIA) measurements.

RESULTS

I. Investigation of the role of PACAP in PACAP knockout (KO) mice

I/1. In vitro experiments

Results of cell viability experiments show that both 2 and 4 hours of H₂O₂ exposure resulted in a significant decrease in cell viability in renal cell cultures from both wild and PACAP KO mice. In all groups, cells from PACAP KO mice displayed increased sensitivity to oxidative stress: cell viability was significantly lower compared to wild type cells with all concentrations and time intervals studied. In the second set of the experiments, we found that exogenously administered PACAP38 counteracted the decreased percentage of living cells upon H₂O₂ exposure in PACAP KO renal cells. In case of CoCl₂-induced *in vitro* hypoxia similar increased sensitivity of PACAP KO kidney cells could be observed both in case of 24 and 48 hours long treatments.

I/2. Histological analysis of kidney from PACAP KO mice

The structure of the kidney showed the same appearance by H&E and PAS staining in the wild type and PACAP knockout animals. The ratio of the cortex and medulla appeared normal in both groups.

In control kidneys (not clamped side) significant decrease could be observed in the tubular dilation.

In contrary to the previous findings, investigating the kidneys exposed to ischemia/reperfusion, we found a marked difference between wild-type and PACAP KO kidneys both after 45 and 60 minutes renal ischemia. The changes characteristic for the ischemic renal damage (tubular degeneration, lymphocyte infiltration) were more pronounced in kidneys from PACAP KO mice. However, in kidneys from PACAP KO mice, renal ischemia/reperfusion resulted in significantly worse histological outcome.

I/3. Identification of PACAP in mouse kidney

Mass spectrometry analysis revealed that PACAP38 is present in mouse kidney. A definite peak at 4535.6 Da was found in the kidney homogenates, as seen in the PACAP38 standard.

II. Investigation of PACAP in rat kidney

II/1. Effects of exogenous PACAP on cell survival *in vitro*

Viability of renal cells after H₂O₂ treatment was measured by MTT assay. Treatment with PACAP1-38 alone did not alter cell viability. In contrast, both 2 and 4 hours of H₂O₂ exposure resulted in a significant decrease in cell viability. Co-treatment with 10 or 100 nM PACAP led to significant increase in the ratio of living cells both in the 2- and 4-hour groups, with no significant difference between the 10 and 100 nM concentrations of PACAP. A separate set of experiments revealed that the lowest effective concentration was 100 pM PACAP1-38, which still significantly increased cell survival. Higher concentrations all led to increased cell survival, with best effects with 10 nM PACAP1-38.

II/2. Effects of PACAP on Bcl-2 level in rats subjected to renal ischemia/reperfusion

Western blot analysis showed that Bcl-2 expression did not change after 45 min of ischemia, but it significantly decreased 10 min after reperfusion following 45 min of ischemia. This was significantly elevated in the PACAP-treated group. In animals that underwent 60 min ischemia, Bcl-2 expression was decreased already after terminating the renal ischemia, similarly to the decreased level observed 10 minutes after reperfusion. In both cases, PACAP treatment was able to counteract the ischemia/reperfusion-induced

decrease in Bcl-2 expression: it significantly elevated the activation of this anti-apoptotic molecule.

II/3. Cytokine array after *in vivo* ischemia/reperfusion

Among several cytokines, the expression of the chemokine (C-X3-C motif) ligand 1 (fractalkine, CX3CL1), soluble intercellular adhesion molecule-1 (sICAM-1, CD54), L-selectin (CD62L/LECAM-1), regulated upon activation, normal T cell expressed and secreted (RANTES, CCL5), tissue inhibitor of metalloproteinase-1 (TIMP-1), ciliary neurotrophic factor (CNTF) and macrophage inflammatory protein (MIP)-3 alpha was increased in the kidneys that underwent ischemia-reperfusion compared with the control groups. Thymus chemokine (CXCL7) showed only slight elevation. PACAP treatment in both groups (45 and 60 min of ischemia followed by reperfusion) caused reduction in all of the above-mentioned cytokine activation, as measured by a cytokine array system. The expression of other cytokines analyzed by the array did not show any significant changes.

II/4. Investigation of presence of PACAP and PAC1 receptor

Mass spectrometry analysis revealed that PACAP38 is present in rat kidney. A definite peak at 4535.6 Da was found in the rat kidney homogenates, just as seen in the PACAP38 standard.

Immunohistochemistry revealed PAC1 receptor-like immunoreactivity in kidney sections. Strongest immunoreactivity was found in the renal cortex, especially in the convoluted tubules. Less expressed immunopositivity was found in the renal medulla.

II/5. Changes of PACAP following renal ischemia/reperfusion *in vivo*

RIA measurements showed the presence of PACAP38 and PACAP27-like immunoreactivities (LI) in both the cortex and the medulla of control kidneys. The level of PACAP27-LI was significantly lower than that of PACAP38 in all measurements. Both peptides had lower levels in the medulla than in the cortex. In the cortex, a marked reduction in the immunoreactivity to both peptides was observed 1 hr after reperfusion following 60 min ischemia. This was followed by a significant increase 6 hrs later in both PACAP38-LI and PACAP27-LI on the intact side. At 24 hrs, levels were no longer significantly different from the original ones. On the clamped side, only an acute decrease in PACAP38-LI was measured without subsequent upregulation, levels returned to control

levels 6 and 24 hrs after reperfusion. PACAP27 showed no significant changes on the ischemic side cortex. In the medulla, no significant changes were observed on the intact side in either immunoreactivity. Marked changes could be observed on the ischemic side: a significant elevation in PACAP38-LI was observed 1 hr after reperfusion, and levels stayed high for the next 24 hrs. PACAP27-LI showed no changes in the medulla at any time-point.

DISCUSSION

I. Investigation of the role of PACAP in PACAP knockout (KO) mice

Investigating the renal cells exposed to oxidative stress, we found that there is a significant difference between wild-type and PACAP KO animals in their response to H₂O₂. PACAP KO renal cells displayed increased sensitivity to oxidative stress and CoCl₂-induced *in vitro* hypoxia. In the second part of the experiment we investigated whether the reason of this increased sensitivity was the lack of the endogenous PACAP. Therefore, we applied exogenous PACAP treatment on the PACAP KO renal cells exposed to oxidative stress. We found that PACAP was able to counteract the toxic effect of H₂O₂.

The present findings are in accordance with previous studies examining PACAP KO mice. It has been described that various morphological and functional alterations can be detected in animals lacking endogenous PACAP. These results show, for the first time, that endogenous PACAP protects against oxidative stress in the kidney. The applied *in vitro* oxidative stress model is also important from the point of view of pathomechanism of renal pathologies since oxidative stress can be present as etiological or aggravating factor in case of different renal diseases.

We could not detect any dramatic difference between histological appearances of kidneys originating from wild-type and PACAP KO mice. These results indicate that lack of endogenous PACAP does not result in morphological alterations under physiological circumstances. These findings could be due to some compensatory mechanisms or PACAP is not essential for the normal development, and these may account for the lack of developmental abnormality. It can not be excluded, however, that more subtle changes occur in the ultrastructure, function or survival of the kidneys.

In spite of the fact, that presence of PACAP has been described in numerous organs, no data were available on its presence in the kidney. We provided evidence that PACAP38 was present in the kidney by MALDI TOF mass spectrometry.

II. Investigation of PACAP in rat kidney

Survival-promoting actions of PACAP can be detected both in neuronal and non-neuronal tissues. Its protective effect can be observed also in kidney. The goal of our experiments was to investigate whether PACAP was able to influence the survival of renal cells exposed oxidative stress under *in vitro* circumstances and what was the lowest concentration, in which it was still effective under these experimental conditions. Our present results confirm the renoprotective effects of PACAP in a wide range of concentration (10pM→100nM) by showing that kidney cultures are protected against oxidative stress by PACAP administration.

Western blot analysis of kidneys subjected to renal ischemia/reperfusion revealed that preoperative PACAP treatment effectively counteracted the ischemia-induced decrease of anti-apoptotic Bcl-2 expression.

Changes in the levels of Bcl-2 were already observed 10 minutes after reperfusion when ischemia lasted 45 minutes and even earlier, at the time of reperfusion when ischemia lasted longer (60 minutes). The observed increase in Bcl-2 expression may contribute to maintaining the mitochondrial integrity.

PACAP is also known for its anti-inflammatory functions. The inhibiting effect of PACAP on pro-inflammatory cytokine production has been shown to be an important factor in its protective action in inflammatory conditions.

In our present investigation we found that ischemia/reperfusion induced the expression of fractalkine, soluble intercellular adhesion molecule-1 (sICAM-1, CD54), L-selectin, RANTES, CNTF, MIP-3 alpha and TIMP-1, measured 24 hours after the injury. PACAP could counteract these changes. We did not find any alterations in the other examined cytokines under this experimental paradigm. However, we can not exclude other cytokines playing a role in the renoprotective effect of PACAP.

Keeping in mind that the main part of our experiments was performed in rats, we also examined the presence of PACAP in rat kidney using MALDI TOF mass spectrometry. In addition, we found intensive PAC1 receptor-like immunoreactivity in the renal tubuli.

Our present results show that PACAP is present in the rat kidney and the receptors are localized mainly on the tubular epithelial cells, providing the morphological basis for the tubuloprotective effect of the peptide.

Examining the changes of PACAP following renal ischemia/reperfusion, we observed that PACAP38- and PACAP27-like immunoreactivities sensitively react to renal ischemia/reperfusion: marked changes were observed within 24 hours following renal vessel clamping. However, the exact mechanism and functional importance of these changes are not yet known, the increased endogenous PACAP level could be due to the renoprotective role of the peptide. Our present findings indicate that the upregulation of PACAP may be an important factor in renoprotection. The more pronounced and longer-lasting elevation in PACAP-LI in the medulla may be one of the endogenous protective mechanisms that lead to a higher resistance of renal medulla to ischemic damage.

III. INVESTIGATION OF THE EFFECTS OF PACAP IN PINEAL GLAND

MATERIALS AND METHODS

Primary pineal cell cultures were isolated from newly hatched chicks.

1. Effects of PACAP on cell survival against H₂O₂-induced oxidative stress

To determine the effect of PACAP against oxidative stress-induced cell death, cells were assigned to groups of two experimental time-points (9 am and 9 pm). All of these groups underwent 5 types of treatments (5 subgroups each time-point): (1) control group of cells (no treatment); (2) cells treated with 100nM PACAP1-38 for one hour; (3) cells exposed to 1 mM H₂O₂ for one hour; (4) groups treated with 100 nM PACAP and 1 mM H₂O₂ for one

hour and (5) to antagonize the effect of PACAP1-38, 500 nM PACAP6-38 was added simultaneously with 1 mM H₂O₂.

Detection of apoptosis and necrosis using annexin V/propidium iodide staining

This method is convenient for distinction between different types of cell death. Annexin V (A5) positive apoptotic cells can be distinguished from the propidium iodide (PI) necrotic cells. Quadrant dot plot shows the percentage of living, necrotic (PI+), early apoptotic (A5+) and late apoptotic (A5+/PI+).

2. Effects of PACAP on signal transduction pathways

Effects of PACAP on the changes of p38 MAPK and 14-3-3 protein were investigated using primary pineal cell cultures. To determine the 24-hour pattern of changes in the p38 MAPK and 14-3-3 protein phosphorylation, cells were assigned to groups of six experimental time-points (8 am, 12 am, 4 pm, 8 pm, 12 pm, 4 am). All of these groups underwent 4 types of treatments (4 subgroups each time-point): control group of cells (no treatment), or groups with PACAP38 treatment (1nM, 10 nM, 100 nM) for one hour. Phospho-p38 MAPK and 14-3-3 proteins were detected by fluorescent staining measured by flow cytometry.

RESULTS

1. Effects of PACAP on cell survival against H₂O₂-induced oxidative stress

Our results showed that H₂O₂ treatment caused significant decrease in the amount of living cells with simultaneous increase in the ratio of necrotic cells at each time-point. Co-incubation with PACAP in the dark phase (9 pm) was able to attenuate the toxic effect of H₂O₂. The survival promoting effect could be counteracted by simultaneously applied PACAP6-38. However, cotreatment with PACAP during the light phase (9 am) did not result in significant differences in the percentage of living cells.

2. Effects of PACAP on signal transduction pathways

In the control group, phosphorylation of p38 MAPK showed significant changes during the 24 hours. Highest levels were observed at 4pm. The phosphorylation of p38 MAPK was continuously increasing from 8 am to 4 pm and continuously decreasing until 12 pm.

Treatment with 1 nM PACAP did not cause any alteration in this pattern: no significant difference was found between PACAP-treated and control group at any time-point.

In turn, using 10 nM PACAP led to a significant difference at all time-points. Levels of the phospho-p38 MAPK after 10 nM PACAP treatment changed similarly to those of the control group, only with a 4 hour-long delay. Under incubation with 100 nM PACAP, no significant difference was found in the levels of phospho-p38 MAPK between the different time-points: the treatment resulted in a constant level of phospho-p38 MAPK independently of the time-point of the exposure within the observed 24-hour time. The disappearance of the daily changes in the levels of p38 MAPK phosphorylation was in marked contrast to the control group. Compared to the control levels, the 100 nM PACAP dose led to a decrease of the phosphorylation level at 12 am and 4 pm, and to an increase at 12 pm. No significant differences were detected at the other time-points.

The level of 14-3-3 protein did not show obvious changes during the 24 hours. PACAP treatment with the lowest dose (1 nM) led to significant changes only at 4 pm and 8 pm. Similarly, 10 nM PACAP had no effect on the 14-3-3 protein during the early daytime and late night hours. This dose caused an elevation in 14-3-3 only at 4 pm. The highest dose of PACAP (100 nM) led to an increase in the levels of 14-3-3 protein at 12 am, 8 pm and 12 pm. At other time-points, no differences were observed between the control and PACAP-treated groups. Comparing all 3 PACAP doses used in the present study, our results show that PACAP was not effective at the early morning hours, and only the highest dose could evoke a change in the appearance of 14-3-3 between midday and midnight hours.

DISCUSSION

1. Effects of PACAP on cell survival against H₂O₂-induced oxidative stress

Viability of pinealocytes exposed to H₂O₂-induced oxidative stress was investigated using annexin V/propidium iodide staining. According to the characteristic features of circadian rhythms, we examined the effects of PACAP at different time-points of a daily cycle. Our results showed that oxidative stress led to a significant decrease in cell viability at both examined time-points. PACAP1-38 alone did not influence the ratio of living cells. In case

of cells co-incubated with H₂O₂ and PACAP1-38 together, no significant effect of PACAP could be observed, if the treatment was carried out at 9 am. In contrast to the aforementioned findings, PACAP1-38 was able to attenuate the toxic effect of H₂O₂ at 9 pm. This protective effect could be counteracted by simultaneously added PACAP receptor antagonist, PACAP6-38.

Previous studies revealed the survival-promoting effects of PACAP in various neuronal cells. PACAP shows similar protective effect in non-neuronal cells exposed to different stressors e.g. endothelial cells, cardiomyocytes, lymphocytes.

Chicken pineal gland, in contrast to mammalian, possesses not only effector function, but it contains a self-sustained circadian oscillator.

Our data show that PACAP enhances the survival of pinealocytes exposed to oxidative stress, but this protective effect depends on the phase of the biological clock. We described, for the first time, that the survival-promoting effect of PACAP can depend on the timing of the administration and on the phase of the biological clock.

Furthermore, we investigated the effects of PACAP on p38 MAPK phosphorylation and 14-3-3 protein in primary pineal cell culture during a daily cycle. Our results indicated that low concentration (1nM) of PACAP did not lead to significant alteration in the circadian changes of p38 MAPK phosphorylation, but higher concentration (10 nM) caused phase-delay and the the highest tested concentration (100 nM) abolish the daily changes of the phosphor-p38 MAPK. The exact functional significance of these dose-dependent changes is not known.

14-3-3 protein plays important roles in various biological processes, including apoptosis and enzyme activity. In the pineal gland, the phosphorylated form of the rate-limiting enzyme of melatonin synthesis, AA-NAT, binds to 14-3-3 complex, thereby stabilizing the enzyme and protecting it against proteolytic destruction. The present results show that PACAP influences the activity of 14-3-3 protein in the pineal cells. This effect of PACAP on 14-3-3 protein was phase-dependent, suggesting that the function of the 14-3-3 protein and its relation to AA-NAT enzyme may be regulated also by the molecular clock in chicken pinealocytes.

In summary, our present results show that PACAP protects the pinealocytes against oxidative stress and modulates phosphorylation of p38 MAPK and the appearance of 14-3-3 protein in the chicken pineal cells, but these effects of PACAP are dose-dependent and also depend on the actual phase of the molecular clock.

IV. SUMMARY OF NEW FINDINGS

I. Investigation of PACAP in PACAP KO mice

We showed that the lack of endogenous PACAP in the kidney resulted in increased sensitivity to *in vitro* hypoxia and oxidative stress and to *in vivo* ischemia/reperfusion. With this finding we have proven that not only the exogenously given PACAP possesses renoprotective effect but the endogenous PACAP also shows survival-enhancing actions.

II. Investigation of the effects of PACAP in rat kidney

Investigating the mechanism of the well-known renoprotective effect of PACAP we demonstrated effects of PACAP in rats subjected to renal ischemia/reperfusion: it is able to attenuate the decrease of Bcl-2 expression; it shows upregulation following ischemia/reperfusion injury; it decreases the increased levels of pro-inflammatory cytokines/chemokines. Furthermore, we detected the presence of PACAP1-38 in rat kidney using MALDI TOF mass spectrometry. In addition, investigating the distribution of PACAP-specific PAC1 receptor, we found that the receptors are localized mainly on the tubular epithelial cells, providing the morphological basis for the tubuloprotective effect of the peptide.

III. Effects of PACAP in pineal gland

We demonstrated that the cytoprotective effect observed in other cell types can also be detected in pinealocytes. But in case of pinealocytes this effect depends on the phase of the biological clock. We obtained similar results investigating two components of the signal transduction pathways. The effects of PACAP both on cell survival and signal transduction pathways are dose- and phase-dependent.

These present findings may have clinical relevance, given that the effects of certain drugs can be different depending on the timing of administration.

Publications related to the thesis:

1. Horváth G, Márk L, Brubel R, Szakály P, Rácz B, Kiss P, Tamás A, Helyes Z, Lubics A, Hashimoto H, Baba A, Shintani N, Fürjes G, Németh J, Reglődi D. Mice deficient in pituitary adenylate cyclase activating polypeptide display increased sensitivity to renal oxidative stress in vitro. *Neurosci Lett.* 2010; 469:70–74. (IF: 1,925)

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Impact factor of publications related to the thesis: **13,339**

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