

THE EXPERIMENTAL INVESTIGATION OF FIBRINOLYTIC SYSTEM UNDER THE INFLUENCE OF FLOCALIN IN CONDITIONS OF ACUTE HYPOXIC KIDNEY INJURY

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In the experiments on rats subjected to acute hypoxic histochemical nephropathy, caused by sodium nitrite and 2,4-dinitrophenol, fibrinolytic activities of blood plasma, urine, renal cortex, medulla, and papilla after treatment with flocalin – the activator of K_{ATP} channels, were studied. It was shown that in the conditions of acute kidney hypoxic injury flocalin administration resulted in the increase and essential restoration of fibrinolysis in blood plasma diminished under hypoxia, which was due to the growth of non-enzymatic fibrinolysis, whereas in urine and renal medulla the appreciable increase of enzymatic fibrinolytic activity took place. Moreover, the treatment of hypoxic nephropathy animals by flocalin resulted in the marked restoration of kidney ion regulatory and protein excretory functions that proves the positive influence of K_{ATP} channels activation on the one of the biochemical mechanisms of acute kidney injury as well as the protective effect of flocalin in relation to tubular cells of nephron. The obtained results testify to the beneficial effects of K_{ATP} channels activation in the conditions of acute hypoxic kidneys injury.

Key words: flocalin, fibrinolysis, acute hypoxic kidney injury.

The essential biochemical characteristic of plasma membranes ATP-sensitive potassium (K_{ATP}) channels, which are gated by intracellular nucleotides ATP and ADP, is their sensitivity to disturbances in cellular metabolism. Subsequently, rapid activation and opening of K_{ATP} channels in response to the decrease of ATP level, in particular under conditions of ischemia and hypoxia, results in plasma membrane hyperpolarization and the decrease of voltage dependent inflow of Ca^{2+} ions. The latter event is accompanied by drastic disturbance of energy metabolism compliance with the functional needs of the cell and initiates the pathological cascades leading to the profound cellular pathology.

The regulatory role of K_{ATP} channels in conditions of cellular oxygenation restriction was first studied on cardiomyocytes and smooth muscle

cells of blood vessels [1], that resulted in the use of pharmacological activators of these channel types for treatment of cardiovascular diseases. In recent years the convincing evidence of cardioprotective and vasodilatory effects of the original K_{ATP} channels fluoride-containing activator Flocalin – N-(4-difluoromethoxyphenyl)-N'-1,2,2-trimethylpropyl-N''-cyanoguanidine [2] were obtained. Owing to the presence of fluoride, flocalin is more selective to K_{ATP} channels and less toxic compared to other representatives of potassium channels modulators that makes it perspective and promising pharmacological substance as physiological regulator and protector of metabolic processes disturbed under hypoxic and ischemic conditions.

Participation of K_{ATP} channels in the biochemical processes that control functions of kidney cells

and the overall body homeostasis maintenance executed by kidneys prompted the investigations to study the effects of activators of K_{ATP} channels on the functional properties of kidneys. Our previous research using the models of acute nephropathy demonstrated that flocalin does not disturb homeostatic reactions in kidneys under physiological conditions and gave evidence to nephroprotective properties of flocalin which increases creatinine excretion and decreases hypercreatininemia, proteinuria and sodium ions loss in conditions of experimental pathology [3].

On the other hand, the clinical and experimental data are accumulated which give evidence of kidneys participation in the regulation of procoagulative and fibrinolytic chains in the whole body [4-6]. In particular, in the case of kidney failure of metabolic origin the disturbance of homeostasis which leads to hemocoagulation and thrombosis takes place [6]. Additionally, the progression of kidney damage correlates with elevation in kidney mesangium cells and urine of type IV collagen which is a marker of decreased activity of proteolysis in the system of proteolysis/fibrinolysis [7]. It should also be noted that the inhibition of fibrinolysis is a sign of kidney failure, whereas chronic kidney disease is associated with simultaneous increase of thrombosis risk [8]. Pathophysiological role of disturbed fibrinolysis in the mechanisms of nephropathy was also confirmed by correlation between fibrinolytic activity and the basic markers of kidney failure progression, which are hypercreatininemia, decreased glomerular filtration rate (GFR), elevation of protein excretion [9].

Thus, numerous experimental and clinical data give evidence to the profound connection of the whole body fibrinolysis system and biochemical functions of kidneys, especially nephron cells bioenergetics which, in its turn, is intrinsically controlled by K_{ATP} plasmatic membranes channels. Proceeding from this, the aim of the present research was to study the changes of fibrinolytic activity of plasma, urine and kidneys in rats after activation of K_{ATP} channels with flocalin under the conditions of acute hypoxic nephropathy in interrelation with the functional state of kidneys.

Materials and Methods

The experiments were performed using laboratory white mature rats of both sexes weighing 0.15-0.17 kg. Acute kidney damage, i.e. hypoxic histohe-mic nephropathy (HHHN) was reproduced by the

method of sequential administration to experimental animals of sodium nitrite and 2,4-dinitrophenol [10]. Two hours after nephrotoxins injection 7 animals with acute HHHN were subjected to intraventricular administration of flocalin (7 days, 5 mg/kg on 1% starch mucus). Two control groups – 7 healthy rats and 7 animals with HHHN without activation of K_{ATP} channels received starch mucus in the same way. Flocalin concentration in plasma was not determined. Taking into account the changes in pharmacokinetics at the absorption stage of intragastric administration of starch suspension the dose of flocalin 5 mg/kg exceeded the doses used by the other investigators for studying cardiovascular effects [11]. Besides, dimethylacetamide, which decreases vascular tone and potentiates vascular effects of flocalin, was used in these studies as a solvent.

During next 2 h after the last dose of flocalin urine was collected and subjected to the analysis of Na^+ , K^+ and protein concentration with the calculation of their excretion levels [12, 13]. Euthanasia of rats was performed under nembutal anesthesia (30 mg/kg, intraperitoneally), according to the regulations of European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986).

The state of fibrinolysis in plasma, urine and renal cortex, medulla and papilla of kidneys was determined according to azofibrin lysis method (set of reagents Simko, Ltd, Lviv, Ukraine). Total (TFA), non-enzymatic (NFA) and enzymatic (EFA, $EFA=TFA-NFA$) fibrinolytic activities were measured and assessed in $E_{440}/hour$, where E_{440} is an index of extinction. The principle of the original method is formation of plasmin after azofibrin incubation with the standard amount of plasminogen. The activity of plasminogen was estimated by the coloring of solutions in the alkaline environment with the presence of ϵ -aminocaproic acid (NFA) and without it (TFA) [14].

Statistical analysis of the data was performed using SPSS Statistics 17.0 software. The type of distribution was estimated using Kolmogorov–Smirnov criterion. Estimation of the differences between the samples was conducted using parametric Student's *t*-test (for normal distribution) and nonparametric Mann–Whitney *U* test (for distributions not conforming to the normal law). The level of significance was $P \leq 0.05$. All results are represented as a mean \pm standard deviation of a sample ($M \pm m$).

Results and Discussion

It was shown that under acute HHHN TFA of plasma decreased by 42% 7 days after administration of sodium nitrite and 2,4-dinitrophenol. Fibrinolytic activity of urine in the rats with acute hypoxic kidney injury was also inhibited (TFA decreased by 26.8%) predominantly because of the decrease of non-enzymatic component by 34.5% (Table 1).

The activation of K_{ATP} channels in the rats with acute HHHN led to the increase of TFA in plasma by 85.7% due to 1.8 fold elevation of NFA. Fibrinolytic activity of urine after flocalin administration also showed the increase of TFA (by 59.7%) with substantial elevation of EFA. In comparison to the group of rats without pharmacological correction, the enzymatic component of TFA increased by 79%.

Fibrinolytic activity in kidney tissues under the conditions of acute hypoxic injury was characterized by the decreased indexes only in the renal medulla: TFA and NFA diminished by 15.2% and 38.3%, respectively. The opening of K_{ATP} channels led to the increase of NFA by 9.7%. Flocalin also caused the increase of EFA by 9.7% in the rats with acute HHHN and this index was by 54.6% higher than control (Table 2).

Thus, normalization of the decreased TFA in blood plasma due to combined action of hemic (sodium nitrite) and histotoxic (2,4-dinitrophenol) hypoxia under the influence of K_{ATP} channels activation was a result of NFA increase. Non-enzymatic fibrinolysis is implemented with the complex compounds of heparin with specific proteins of blood

(fibrinogen, thrombin, and plasminogen). The authors of the study [15] have found that the increase of conductivity of mitochondrial potassium channels of the rat liver and ATP-dependent K^+ inflow in acute hypoxia is an important chain of regulation of energy metabolism. Flocalin (activator of K_{ATP} channels) modulates oxidative phosphorylation system of mitochondria (disrupted by 2,4-dinitrophenol) toward the side of ATP synthesis. It is possible that this modulation inhibits the development of hepatorenal syndrome of hypoxic origin [16]. We assume that the improvement of biochemical and functional state of the liver, where coagulation factors (fibrinogen, prothrombin and proconvertin) and anticoagulants (heparin) are synthesized, manifested an increase of heparin-dependent NFA.

The similar dynamics of fibrinolysis was observed in urine of rats with acute HHHN under the conditions of fibrinolytic activity of plasma increased by flocalin. More substantial increase of TFA in urine than in plasma pointed to the increment of fibrinolytic potential in the kidneys. Due to the intensive blood supply the components of tissue fibrinolysis, particularly from renal medulla were also found in the rat urine. In renal medulla there are S3 segments of the proximal tubules and the distal tubules of kidneys where the transportation of Na^+ and K^+ takes place. The concentration of these ions determines to a large extent blood and urine osmolarity. That is why we studied the state of ion regulatory function which enables the assessment of the ability of kidneys to dilute and concentrate urine, as

Table 1. The changes of fibrinolytic activity of plasma and urine under the influence of K_{ATP} channels activation with flocalin (5 mg/kg, 7 days) in the rats with acute HHHN ($M \pm m$, $n = 7$)

Index	Control	HHHN	HHHN + Flocalin
<i>Blood plasma</i>			
TFA, E_{440} /h/ml	0.12 ± 0.015	$0.07 \pm 0.011^*$	$0.13 \pm 0.024^\#$
NFA, E_{440} /h/ml	0.08 ± 0.016	0.05 ± 0.013	$0.09 \pm 0.012^\#$
EFA, E_{440} /h/ml	0.04 ± 0.004	0.02 ± 0.016	0.04 ± 0.014
<i>Urine</i>			
TFA, E_{440} /h/ml	1.90 ± 0.103	$1.39 \pm 0.128^{**}$	$2.22 \pm 0.315^\#$
NFA, E_{440} /h/ml	0.87 ± 0.035	$0.57 \pm 0.126^*$	0.79 ± 0.143
EFA, E_{440} /h/ml	1.15 ± 0.061	0.81 ± 0.150	$1.45 \pm 0.214^\#$

Here and in Table 2: $*P \leq 0.05$, $**P \leq 0.01$ – significant difference in comparison with control; $^\#P \leq 0.05$ – significant difference in comparison with HHHN; n – the number of rats in every group

Table 2. The changes of fibrinolytic activity of kidney tissues under the influence of K_{ATP} channels activation with flocalin (5 mg/kg, 7 days) in the rats with acute HHHN ($M \pm m$, $n = 7$)

Index	Control	HHHN	HHHN + Flocalin
<i>Renal cortex</i>			
TFA, $E_{440}/h/ml$	4.16 ± 0.568	4.94 ± 1.278	4.91 ± 0.397
NFA, $E_{440}/h/ml$	2.26 ± 0.573	3.50 ± 1.436	3.47 ± 0.547
EFA, $E_{440}/h/ml$	1.90 ± 0.352	1.44 ± 0.455	1.23 ± 0.662
<i>Renal medulla</i>			
TFA, $E_{440}/h/ml$	11.14 ± 0.217	$9.45 \pm 0.516^*$	10.88 ± 0.411
NFA, $E_{440}/h/ml$	6.87 ± 0.311	$4.24 \pm 0.134^{**}$	$4.65 \pm 0.127^{\#}$
EFA, $E_{440}/h/ml$	5.12 ± 0.153	4.19 ± 0.626	$6.48 \pm 0.322^{**\#}$
<i>Renal papilla</i>			
TFA, $E_{440}/h/ml$	9.18 ± 0.125	10.09 ± 0.452	8.85 ± 0.424
NFA, $E_{440}/h/ml$	4.47 ± 0.342	5.10 ± 0.134	3.49 ± 0.753
EFA, $E_{440}/h/ml$	5.44 ± 0.410	4.89 ± 0.315	5.19 ± 0.557

well as the role of K_{ATP} channels activation in condition of violation of water and salt balance caused by hypoxia (Fig. 1).

The addition of flocalin in the rats with acute HHHN prevented the loss of Na^+ and K^+ by means

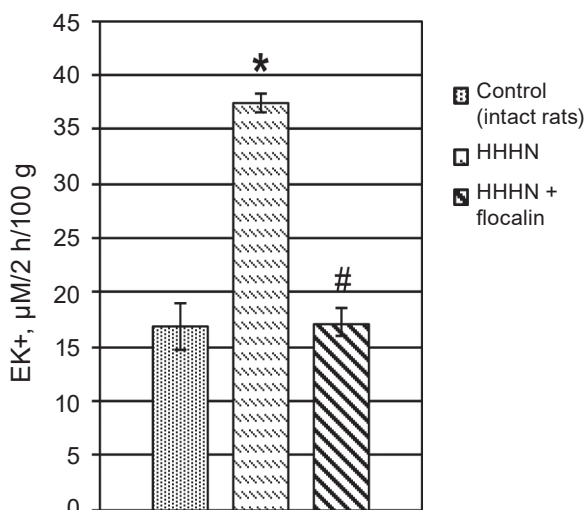


Fig. 1. The changes of ion regulatory function of kidneys under the influence of K_{ATP} channels activation with flocalin (5 mg/kg, 7 days) in rats with acute HHHN, ($M \pm m$, $n = 7$). Notes: * $P < 0.05$ – significant difference in comparison with control; # $P < 0.05$ – significant difference in comparison with HHHN; E – excretion of ions with urine

of intensification of tubular ions reabsorption. It should be noted that K_{ATP} channels opening may be considered as a mechanism of Ca^{2+} -mediated reactions modulation. Calcium ion is a fibrinolysis inhibitor and, as such, plays a particularly important role in fibrin polymerization [17]. The increase of plasma fibrinolysis activity and flocalin induced vasodilation result in microcirculation enhancement and improvement of the functional state of proximal tubules. It leads to the increase of production of urokinase which is plasminogen activator [18]. At the same time the growth of fibrinolytic activity of renal medulla was stipulated by the increase of both urokinase mediated EFA and NFA. We cannot exclude that the opening of K_{ATP} channels of vascular wall in renal medulla leads to the increase of NFA stimulator heparin produced in medulla by heparinocytes and is deposited in tubular epithelium of the nephron.

In our study the reduction of protein excretion standardized by glomerular filtration also pointed to the enhancement of tubulocytes functional ability under the influence of activation of K_{ATP} channels (Fig. 2).

The reduction of tubular proteinuria occurs exceptionally through the increase of reabsorption of filtered low molecular proteins in proximal tubules. In the previous studies we ascertained the increase of alkaline phosphatase (AP, EC 3.1.3.1) activity in

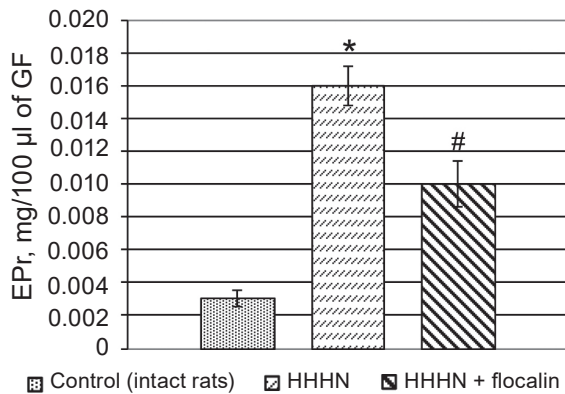


Fig. 2. The influence of K_{ATP} channels activation by flocalin (5 mg/kg, 7 days) on protein excretion in urine of rats with acute HHHN, ($M \pm m$, $n = 7$).

Notes: * $P < 0.05$ – significant difference in comparison with control; # $P < 0.05$ – significant difference in comparison with HHHN; EPr – excretion of protein with urine, GF – glomerular filtrate

the rats with toxic nephropathy after flocalin induced activation of K_{ATP} channels [19]. Like urokinase, AP is a direct activator of fibrinolysis. At the same time AP donates phosphorus for ATP, it is contained in a significant amount in the brush border of epithelial cells of proximal tubules, and it is a marker of energy metabolism and functional state of tubulocytes.

The effects that were obtained on the 7th day of flocalin use show that flocalin action was not accompanied by desensitization of response to K_{ATP} channels agonist. The direct evidence of potassium channels opening with flocalin (which is a fluorine containing analogue of pinacidil [20]) is the result of the studies with the use of specific inhibitors of K_{ATP} channels [21, 22]. Owing to thorough experimental investigations cardioprotector flocalin is positioned primarily as a selective activator of K_{ATP} channels [2, 11, 20-23]. It has also been stated that flocalin inhibits sodium and Ca^{2+} channels of L-type in cardiomyocytes [23]. The representatives of pharmacological blockers of calcium channels are characterized with antiproteinuric effect [24, 25]. The synergism with different attachment points in the mechanisms is a benefit of pharmacological compounds and provides the efficacy of flocalin at repeated use.

Thus, flocalin induced K_{ATP} channels activation in the rats with acute hypoxic kidney injury leads to the increase of fibrinolytic activity of plasma, urine, and renal medulla. Activation of fibrinolysis inhibited by hemic and histotoxic hypoxia is followed by

the improvement of ion regulatory kidney function and decrease of proteinuria. The combination of positive dynamics of fibrinolysis and the functional state of kidneys gives evidence of corrective influence of K_{ATP} channels activation on one of the biochemical processes of acute hypoxic kidney injury and protective action of flocalin in the tubular part of nephron.

ЕКСПЕРИМЕНТАЛЬНЕ ДОСЛІДЖЕННЯ ФІБРИНОЛІТИЧНОЇ СИСТЕМИ ПІД ВПЛИВОМ ФЛОКАЛІНУ ЗА ГОСТРОГО ГІПОКСИЧНОГО ПОШКОДЖЕННЯ НИРОК

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В експериментах на щурах із гострою гіпоксичною гістогемічною нефропатією (ГГН), зумовленою нітритом натрію та 2,4-динітрофенолом, вивчали стан фібринолітичної активності плазми крові, сечі, коркової, мозкової речовини та сосочку нирок під впливом активації K_{ATP}^+ каналів флокаліном (5 мг/кг, внутрішньошлунково, 7 днів). Показано, що за розвитку ГГН після введення флокаліну відбувалося підвищення та відновлення пригніченої гіпоксією фібринолітичної активності завдяки активації неензиматичного фібринолізу в плазмі крові, тоді як у сечі та в мозковій речовині нирок істотно збільшувалась ензиматична фібринолітична активність. Встановлена і статистично підтверджена позитивна динаміка показників іонорегулювальної функції нирок і екскреції протеїну вказують на захисний вплив активації K_{ATP}^+ каналів на один із біохімічних механізмів патогенезу гострого гіпоксичного пошкодження нирок і протекторної дії флокаліну в каналцевому відділі нефрону.

Ключові слова: флокалін, фібриноліз, гостре гіпоксичне пошкодження нирок.

ЭКСПЕРИМЕНТАЛЬНОЕ ИССЛЕДОВАНИЕ ФИБРИНОЛИТИЧЕСКОЙ СИСТЕМЫ ПОД ВЛИЯНИЕМ ФЛОКАЛИНА ПРИ ОСТРОМ ГИПОКСИЧЕСКОМ ПОВРЕЖДЕНИИ ПОЧЕК

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В экспериментах на крысах с острой гипоксической гистогемической нефропатией (ОГН), вызванной нитритом натрия и 2,4-динитрофенолом, изучали состояние фибринолитической активности плазмы крови, мочи, коркового, мозгового вещества и сосочка почек под влиянием активации K^+_{ATP} каналов флокалином (5 мг/кг, внутривенно, 7 дней). Показано, что в условиях развития ОГН после введения флокалина повышение и восстановление угнетенной гипоксией фибринолитической активности происходило благодаря активации неэнзиматического фибринолиза в плазме крови, тогда как в моче и в мозговом веществе почек существенно увеличивалась энзиматическая фибринолитическая активность. Установленная и статистически подтвержденная положительная динамика показателей ионорегулирующей функции почек и экскреции протеина указывают на защитное влияние активации K^+_{ATP} каналов на один из биохимических механизмов патогенеза острого гипоксического повреждения почек и протекторного действия флокалина в канальцевом отделе нефрона.

Ключевые слова: флокалин, фибринолиз, острое гипоксическое повреждение почек.

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