Oxidative stress, advanced glycation end products and residual renal function in the rat model of unilateral ureteral obstruction: effects of phlogenzym and losartan

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Aim. Oxidative stress plays a role in the pathogenesis of ureteral obstruction. Methods. We studied parameters of oxidative status, levels of advanced glycation end products (AGEs), and contralateral (CL) kidney function in the rat model of unilateral ureteral obstruction (UUO). The effect of Phlogenzym (12 mg/day orally); losartan (20 mg/l in drinking water), and their combination was studied. Results. In placebo-administered UUO rats AGEs and malondialdehyde levels were higher than in the sham operated controls. Function of the CL kidney was slightly impaired, its collagen content and protein/deoxyribonucleic acid ratio (P/DNA) in the glomeruli increased. All treatments prevented the rise in collagen content, P/DNA ratio, and improved CL kidney function. Phlogenzym ameliorated lipid peroxidation and AGE levels. Conclusions. In the model of UUO systemically increased oxidative stress may play a role in development of tubulointerstitial fibrosis and in the functional impairment of the CL kidney. Suppression of the oxidative stress and blockade of angiotensin-I receptors might mitigate the progression of obstructive uropathy.

Keywords: ureteral obstruction, advanced glycation end products, oxidative stress, malondialdehyde, collagen.

Introduction. In the model of unilateral ureteral obstruction (UUO) altered hemodynamics, hypoxia, infiltration with macrophages, up-regulated renin-angiotensin-aldosterone system producing vasoactive compounds, result in tubulointerstitial fibrosis of the ligated kidney [1–3]. Angiotensin (Ang) II may promote cell growth and fibrosis via overexpression of growth factors and cytokines, and the induction of oxidative stress [4]. The latter one is in the tubulointerstitium of obstructed kidney reflected by
increased heme oxygenase-1 expression, accumulation of N\(^\text{\textsuperscript{-}}\)(carboxymethyl)lysine (CML), and perturbation of tubular antioxidants; and systemically by the rise in plasma malondialdehyde (MDA) levels \([5, 6]\). The enhanced formation of reactive oxygen species (ROS) may exert toxic effects in other tissues and organs. The contralateral (CL) kidney, due to its compensatory hypertrophy, might be the most susceptible.

Advanced glycation end products (AGEs) are formed on proteins by non-enzymatic glycation and/or glycoxidation. With decreased renal function they accumulate in tissues and circulation, due to retention and enhanced synthesis under exaggerated oxidative- and carbonyl-stress \([7, 8]\). Interaction of AGEs with their specific cell surface receptor RAGE leads to the production of ROS, which accelerate formation of AGEs \([9]\). Experimental studies suggest the interaction between the AGE-RAGE and the renin-angiotensin systems \([10]\). \textit{In vitro}, Ang II receptor 1 blockers (ARBs) lower AGEs formation, and suppress the AGEs-induced enhanced Ang 1 receptor protein \([11, 12]\). They also attenuate the accumulation of AGEs \textit{in vivo} \([13, 14]\).

In studies on pig proximal tubular cells (LLC-PK1) trypsin prevented the AGEs-induced cell hypertrophy and accumulation of AGEs \([15, 16]\). In rodents administration of proteases improved the course of various renal diseases \([17, 18]\). Whether administration of proteases interferes with AGEs and oxidative stress \textit{in vivo} remains unclear.

We investigated the possible involvement of the systemically enhanced oxidative stress in relation to the function and structure of the CL kidney, in the model of UUO. AGE-lowering- and antioxidant-potential of the above mentioned treatment modalities, and their combination was studied.

**Materials and methods.** The trial was conducted according to the guidelines for studies using laboratory animals, after the approval by the local Ethics Board for Experimental Animals (Bratislava).

**Rats.** Male Wistar rats (180–220 g, VELAS Praha, Czech Republic) were caged under controlled humidity, temperature, and light/dark cycle, with free access to drinking water and food (SP1, Top Dovo, Czech Republic). After induction of UUO rats were pair-fed to the UUO placebo administered group.

**Induction of unilateral ureteral obstruction.** Forty rats were subjected to UUO in i. p. thiopental narcosis. Briefly, right ureter was liberated, ligated twice with sterile silk, and cut between two ligations. Six sham-operated rats served as controls (CTRL).

**Experimental protocol.** UUO rats were randomized into 4 groups per 10 animals, administered during 14 days: a) placebo (UUO-P); b) a fixed mixture of proteases (UUO-E, Phlogenzym, «MUCOS Pharma», Germany) in a dose of 12 mg/day in 1 ml of tap water (each dose contained 2.42 mg trypsin, 4.54 mg brom elain, and 5.04 mg flavonoid rutosid); c) ARB (UUO-ARB, Losartan, MSD, USA, 20 mg/l in drinking water); d) combined treatment (UUO-COMB, both drugs in the above mentioned dosage). Control and UUO-P rats were gavaged by 1 ml of water.

Body weight and blood pressure (tail plethysmography) was recorded. At sacrifice (thiopental narcosis), blood was sampled from abdominal aorta and urine from bladder. Standard blood chemistry was determined (Vitros 250 analyzer, «J&J», USA). Plasma or whole blood was stored at –70°C for determination of: total antioxidant status (TAS) and glutathione peroxidase activity (GPX), («Randox», UK); plasma MDA \([19]\) and lipofuscin (LF) \([20]\) concentrations; and AGE specific fluorescence \([21]\). CML concentration was quantified with competitive ELISA using monoclonal antibodies according to the method developed by «Roche Diagnostika», Germany. One AGE unit (U) represented 50 % reduction in binding. Proteinuria was determined by a pyrogallol red method.

Kidneys were weighed. Collagen content in renal cortex was determined in formaline fixed paraffin embedded slices, stained with hematoxilyne/eosine and Van Gieson. The contrast red area stained as collagen was expressed in per cent of the cortical tissue area with aids of computerized video camera. In glomeruli isolated by differential sieving method \([22]\) the DNA \([23]\) and protein content \([24]\) was determined.

**Statistics.** The data were tested for normality and equality of variance, and compared either by one-way analysis of variance (ANOVA) with post hoc Scheffe’s test; or by Kruskal-Wallis with Mann-Whitney \(U\)-tests. Results are given as mean ± SD, or as median, mean ± SD (not normally distributed data); \(p < 0.05\) was considered significant.
Results and discussion. Role of accumulation of AGEs in pathogenesis of UUO (Table). At sacrifice the body weight of the UUO-P rats was lower than that of CTRL rats. The weight of the CL kidney and kidney/body weight ratio was comparable. UUO resulted in hypertrophy of the glomeruli in CL kidney, as indicated by rise of protein/DNA ratio (Fig. 1, a). In LLC-PK1 cells, AGE-modified albumin induced cell hypertrophy via stimulation of protein synthesis and inhibition of its degradation [15, 16]. The latter one was, at least partially, caused by the decline in lysosomal cathepsin activity, due to down-regulation of mRNA levels [25]. Plasma AGE-specific fluorescence (Table) and CML concentrations (Fig. 2) were significantly higher in the UUO-P rats than in the CTRL, but not on the account of plasma albumin or glucose concentration. AGEs rose despite only a moderate changes in plasma creatinine levels. The rise in protein content of isolated glomeruli may be causally linked to enhanced circulating AGE levels, as supported by a direct relation between plasma CML and P/DNA content ($r = 0.567$, $p < 0.02$).

In the cortex of CL kidney collagen content increased 5-fold (Fig. 1, b). Incubation of LLC-PK1 or immortalized human kidney epithelial cells (IHKECs) with AGE-modified BSA results in intracellular accumulation of AGEs, associated with the induction of pro-fibrotic factors (overexpression of TGF-β1 mRNA, rise in TGF-β1 protein, enhanced activation of protein kinase C, and fibronectin synthesis) [12, 15, 16]. Thus, elevated circulating AGEs may contribute to rise in renal cortex collagen content, as supported by a direct relation between plasma CML and renal cortex collagen content ($r = 0.918$, $p < 0.001$). However, in the UUO model plasma CML accumulates in spite of its enhanced renal excretion (Fig. 2). UUO represents a nonproteinuric model of...
interstitial fibrosis. Thus, a predominant excretion of AGE-modified peptides is anticipated. Their enhanced filtration load might also contribute to the damage of tubule cells with subsequent development of tubulo-interstitial fibrosis.

**Role of enhanced oxidative stress in pathogenesis of UUO** (Table). Fourteen days after the induction of UUO oxidative stress was systemically enhanced, as reflected by increased MDA and lipofuscin levels. CML is considered as integrative biomarker of the cumulative protein damage induced by glycoxidation [26]. Much faster accumulation of CML than that of fluorescent AGEs, and high correlation between CML and MDA ($r = 0.599$, $p < 0.05$), or CML and LF concentrations ($r = 0.556$, $p < 0.05$), support the role of the oxidative stress in CML formation. Since the total antioxidant status and GPX activity were not altered, the enhanced oxidative stress seemed to result from overproduction of ROS, not caused by a compromised antioxidant defense. Shortly (12 h) after the onset of UUO heme oxygenase-1 activity increases, with a time dependent decline within the next 7 days [5]. It might not be excluded that during the early phase of UUO, enhanced production of ROS induces antioxidant enzyme activity, which returns to normal values, or even decreases, later.

Although the function of the CL kidney was only slightly altered, both plasma AGE-specific fluorescent, as well as CML levels correlated highly with those of serum creatinine ($r = 0.560$, $p < 0.05$, and $r = 0.760$, $p < 0.001$, respectively), indicating the important role of the kidney in their removal, and the role of oxidative stress in the impairment of renal function.

**Effects of the treatment.** We compared the established beneficial effects of the administration of losartan with the potential beneficial action of Phlogenzym and their combination on the function of the CL kidney, from the point of interference with AGEs and oxidative status, since: 1) inhibitors of converting enzyme and ARBs ameliorate the alterations induced by UUO [27]; 2) the experimental and clinical data suggest that ARBs may attenuate oxidative stress and formation of AGEs [13, 28, 29]; 3) proteolytic enzymes antagonize the AGE-induced toxicity in various renal cell cultures [15,

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**Fig. 1.** Effects of UUO, and treatment with Phlogenzym, losartan, and their combination on contralateral kidney: $a$ – protein to DNA ratio in isolated glomeruli; $b$ – renal cortex collagen content. **CTRL** – sham operated controls; **UUO** – unilateral ureteral obstruction; **P** – placebo; **E** – Phlogenzym; **ARB** – losartan; **COMB** – combined treatment with $E + ABR$; *p* $< 0.05$ vs. **CTRL**; **p** $< 0.01$ vs. **CTRL**; + – $p < 0.05$ vs. UUO-P; ++ – $p < 0.01$ vs. UUO-P

**Fig. 2.** Plasma levels and urinary excretion of $N’$-(carboxymethyl)lysine (CML): $a$ – S-CML; $b$ – U-CML. **CTRL** – sham operated controls; **UUO** – unilateral ureteral obstruction; **P** – placebo; **E** – Phlogenzym; **ARB** – losartan; **COMB** – combined treatment with $E + ABR$; **Alb** – albumin; **crea** – creatinine
Thus, partial persistence of enhanced oxidative stress under treatment with ARB was further reflected by elevated CML levels, despite of only a mild rise in plasma creatinine, and substantial increase in urinary CML excretion. At first glance this data are contradictory to our previous observation, in which the 12-weeks-long administration of losartan to subtotaly nephrectomized rats completely prevented the rise in plasma AGE levels [13]. However, in both studies losartan significantly increased urinary AGE excretion. We suppose that in spite of enhanced renal excretion longer time is needed to normalize the elevated circulating AGE levels. Moreover, it is equivocal whether the sub-antihypertensive dose of losartan administered in our study is sufficient to block the oxidative stress induced by Ang II. In the rat model of congestive heart failure, an increase in antioxidant defense and a decline in oxidative stress was achieved after administration of a 100-fold higher dose of losartan [29]. Losartan effectively prevented glomerular hypertrophy and collagen accumulation, despite persistent oxidative stress and elevated AGE levels. ARBs were capable to reduce the expression of RAGE in human endothelial cells, and to modify the AGE-RAGE interaction by suppression of RAGE expression in the type 2 diabetic KK/Ta mice [11, 30]. We suppose that suppression of RAGE under ARB treatment may to measurable extent prevent progressive renal damage even under the persisting oxidative stress.

Phlogenzym attenuated accumulation of AGEs and lipid peroxidation products. CML adducts are one of the most relevant ligands for RAGE and mediate NF-kB pathways, resulting in intracellular generation of ROS [9, 31]. In in vitro studies proteases could inactivate the extracellular domain of the RAGE, thus interfere directly with production of ROS, decreasing formation of AGEs and lipid peroxidation products [15, 16]. However, in Phlogenzym, rutinosid is added as antioxidant to stabilize trypsin and bromelain. In vitro, antioxidants ameliorate the toxic effects induced by AGEs [28]. According to our knowledge no data are available on the antioxidant/anti-AGE effects of rutinosid. However, water-soluble rutin derivative suppressed in vitro glycation in tissue proteins [32]. Thus, the observed mechanisms of beneficial effects of Phlogenzym are to be interpreted with caution.
Combined treatment showed additive effects and partially prevented the changes under ARB treatment, suggesting different mechanisms of the beneficial effects on CL kidney function of enzymes or ARBs.

Conclusions. Our study confirms the role of systemically enhanced oxidative stress in UUO, as reflected by increased plasma malondialdehyde, lipofuscin, CML and fluorescent AGE levels. Enhanced oxidative stress may be involved not only in the development of tubulointerstitial fibrosis of the ligated kidney, but also in impairment of the CL kidney. Suppression of oxidative stress and glycoxidation might therefore be of clinical relevance in retardation of the progression of renal disease.

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Оксидативный стресс, конечные продукты гликозии и залишковое функционирование нирки на модели щурев с унілатеральною обструкцією сечоводу: ефект флегоензму та лосартана

Резюме

Мета. Оксидативный стресс відіграє значну роль у патогенезі обструкції сечоводу. Мета роботи полягає у вивченні параметрів оксидативного статусу, оцінюванні рівня кінцевих продуктів глюкози і функціонування контралатеральної нирки на моделі щурів з унілатеральною обструкцією сечоводу (УОС).

Методи. На моделі УОС досліджували ефекти флегоензу (12 мг в день орально) і лосартану (20 мг/л у питьй воді), а також їхньої комбінації. Результати. У щурів з УОС, які отримували плацебо, рівень накопичення кінцевих продуктів глюкози та мальдонілідсу виявився нижчим, ніж у неприємнооперованих контрольних щурів. Функціонування контралатеральної нирки незначно погіршилося, концентрація колагену і співвідношення вмісту білків/дезоксикоспиронеклінової кислоти (Р/DNA) у клубочку нирки підвищено. Обробка досліджуваними лікарськими засобами забезпечила збільшення вмісту колагену, зростання показника співвідношення Р/DNA та покращувала функціонування колагеназної нирки. Флегоенз сприяв підвищенню рівня пероксидного окислення ліпідів та кінцевих продуктів глюкози. Висновки. У моделі УОС систематичне збільшення оксидативного стресу може відрізнятися важливу роль у розвитку тубулінтрерстіційного фіброзу і порушеній функціонування контралатеральної нирки. Універсальна характеристика стресу та блокування рецептора ангіотензину-1 можуть посміювати прогресію обструктивної уропатії.

Ключові слова: обструкція сечоводу, оксидативний стрес, мальдонілідгідрід, колаген.

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