Effects of Ischemic and Hypoxic Preconditioning on the State of Mitochondria and Function of Ischemic Kidneys

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Laser confocal microscopy showed that fluorescence of tetramethylrhodamine ethyl ether probe specifically accumulating in energized mitochondria significantly decreased in renal tubular epithelium after 40-min thermal ischemia, while fluorescence of dichlorodihydrofluorescein and diaminofluorescein probes in the same structures increased under these conditions, which attests to increased generation of ROS and NO, respectively. These forms were generated predominantly in mitochondria of tubular epitheliocytes. Hypoxic preconditioning (a series of sessions of breathing hypoxic mixture) preserved functional activity of mitochondria and prevented activation of ROS and NO generation. Ischemic preconditioning of the kidney consisting of three preliminary episodes of vascular clamping (5 min with 5 min reperfusion periods) also increased the percentage of functionally active mitochondria and prevented activation of NO synthesis without appreciably modifying ROS production. Both protective methods significantly reduced the severity of postischemic dysfunction of the kidney.

Key Words: *renal ischemia; antiischemic protection; hypoxic preconditioning; ischemic preconditioning, laser confocal microscopy*

Improvement of ischemic tolerance of the kidney is an important problem of modern surgery, urology, transplantology. This can be attained via administration of drugs exhibiting antihypoxic and membranotropic properties, local cooling of the organ, and hypoxic and ischemic preconditioning (HP and IP, respectively). It was shown that several sessions of breathing a mixture containing 10-12% O_2 increased ischemic resistance of the myocardium, kidneys, and other organs [1,2]. Repeated (2-3 times) short-term (3-5 min) clamping of blood vessels feeding the organ alternating with periods of reperfusion directly before the main period of organ ischemia produced a similar effect [6,9,10]. The mechanism of this effect remains not quite clear. It is known that IP not only maintains ATP level in tissue during ischemia [15], but also stimulates NO synthesis [6] and activates adenosine receptors [9,14] and ATP-sensitive potassium channels [4].

We studied the effects of HP and IP on the functional state of mitochondria and generation of ROS and NO during the early reperfusion period in comparison with functional aftereffects of 40-min ischemia of the kidney.

MATERIALS AND METHODS

Experiments were carried out on 57 outbred male rats (250-280 g). During HP the animals placed into

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a sealed box breathed oxygen/nitrogen mixture; the oxygen concentration was initially 12% and gradually decreased to 9%. The duration of each session increased from 30 to 70 min. A total of 10-12 HP sessions were carried out. On the next day after the last HP session 40-min ischemia of the left kidney was induced by clamping of the renal pedicle. IP was carried out by 3-fold clamping of the left kidney vessels for with 5-min reperfusion intervals, after which 40-min ischemia was induced. The animals exposed to 40-min ischemia without preconditioning served as controls. The right kidney was removed in all cases.

The state of mitochondria was evaluated by confocal microscopy on an LSM510 confocal scanning microscope (Karl Zeiss) with proprietary software. Preparations for microscopy were prepared as follows: the ischemic kidney was removed 10 min after bloodflow resumption and thin sections of the cortical matter were prepared and stained with specific fluorescent dyes. Tetramethylrhodamine ethyl ether probe (TMRE, Molecular Probes) accumulating in mitochondria in accordance with the $\Delta\psi$ value was used for studies of the cell mitochondria transmembrane potential ($\Delta\psi$). 2,4-Dichlorofluorescein in the form of membrane-penetrating diacetate ether (2,7-DCF DA, Molecular Probes) served as the probe for ROS. In order to detect NO production in renal cells, the sections were incubated with 4,5-diaminofluorescein in the form of diacetate ether (DAF-2 diethyl ether, Calbiochem). TMRE was visualized by laser at excitation wavelength λ=543 nm and the fluorescence was measured at λ=560-590 nm. 2,7-DCF and DAF-2 dyes were stimulated by laser with excitation wavelength of 488 nm with fluorescence emission at λ=505-530 nm. Confocal images of renal sections at 10-fold magnification and standard parameters of microscopy (stimulation intensity, degree of programmed amplification of the signal, *etc.*) were used for quantitative analysis of fluorescence intensity. The fluorescence intensity was evaluated using a 255-point scale, the area of zones with high fluorescence intensities (>50 U) was estimated, the specific ratio of these areas to total fluorescence area was evaluated.

Functional consequences of previous ischemia of the kidney and effects of HP and IP were evaluated by changes in blood creatinine, sodium, and potassium concentrations, creatinine clearance, and sodium reabsorption on day 2 after ischemia.

RESULTS

Confocal microscopy showed high fluorescence of TMRE probe in mitochondria of the renal tubules in intact kidneys (Table 1), which indicates high percentage of functionally active mitochondria with high transmembrane potential. The mitochondria were rod- or thread-shaped. The fluorescence intensity in the glomeruli was markedly lower (Fig. 1). Generation of ROS was observed in renal tubules, ROS forming mainly in the mitochondria, which was seen from coincidence of TMRE and DCF fluorescence zones. The glomeruli were characterized by weak DCF fluorescence (Fig. 1). NO generation in structures of intact kidney (primarily in mitochondria) was low (Table 1).

The percentage of tubules containing highly energized mitochondria sharply decreased after ischemia and reperfusion in the kidneys of untrained animals, while the production of ROS and NO in the renal tubular epithelium increased significantly (Table 1). Cells with weak TMRE fluorescence or tubular cells not accumulating this probe, with active fluorescence of DCF and DAF-2, predominated in the majority of cases. This was paralleled by swelling of mitochondria, which filled virtually the entire cytoplasm. In addition, ischemia often led to fragmentation of tubular epithelial mitochondria into small "granules" (Fig. 2). The intensity of TMRE fluorescence in the glomeruli did not change appreciably after ischemia/reperfusion.

TABLE 1. Areas of Active Fluorescence (>50 Units) in Renal Tubules (%; *M*±*m*)

Experimental series	TMRE	DCF	DAF-2
Intact kidneys (*n*=5)	49.1±3.9	33.4±2.2	6.5±0.8
HP			
ischemia/reperfusion (control; *n*=6)	14.0±1.1***	57.2±3.1**	25.0±2.2***
HP+ischemia/reperfusion (*n*=8)	39.2±2.5++	37.9±1.9+	16.7±2.0*+
IP			
ischemia/reperfusion (control; *n*=6)	29.9±2.5**	65.0±5.1**	64.3±3.8***
IP+ischemia/reperfusion (*n*=8)	43.5±2.9+	57.0±5.3*	36.8±2.0***++

Note. *p<0.05, **p<0.01, ***p<0.001 compared to intact kidneys; +p<0.05, ++p<0.001 compared to ischemia/reperfusion.

TABLE 2. Effects of HP and IP on the Function of Ischemic Kidneys (n=6; $M\pm m$)

Parameter	Intact rats	Ischemia (40 min)	HP+40-min ischemia	IP+40-min ischemia
Diuresis, ml/day	15.6±3.2	15.3±2.6	12.8±2.2	7.3±0.7
Blood creatinine, μmol/liter	59±3	62±4	64±3	65±2
Blood sodium, mmol/liter	137±2	141±2	136±2	138±1
Blood potassium, mmol/liter	4.6±0.1	4.4±0.2	5.3±0.2*+	5.1±0.1*+
Creatinine clearance, ml/min/kg	2.92±0.19	2.09±0.21*	2.37±0.23	2.71±0.18*
Sodium reabsorption, %	99.3±0.1	97.7±0.2*	98.9±0.2+	99.9±0.1+

Note. n: number of animals in group. *p<0.05 compared to intact kidneys, +p<0.05 compared to ischemia without preconditioning.

HP significantly reduced changes in the parameters of mitochondrial status caused by ischemia. TMRE fluorescence decreased negligibly, and the difference in the fluorescence intensity in comparison with intact kidneys was statistically negligible. ROS generation also virtually did not in-

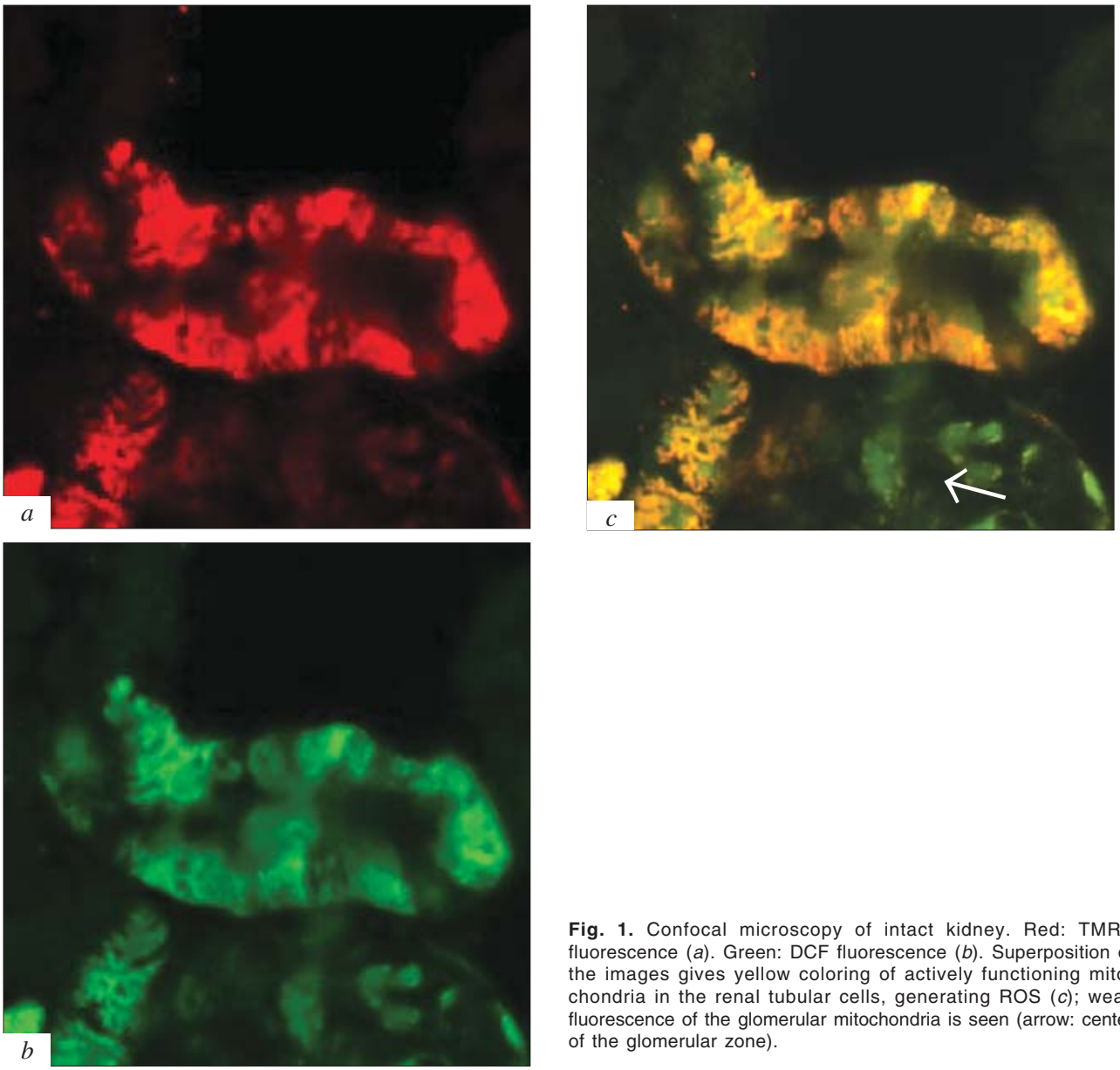

Fig. 1. Confocal microscopy of intact kidney. Red: TMRE fluorescence (*a*). Green: DCF fluorescence (*b*). Superposition of the images gives yellow coloring of actively functioning mitochondria in the renal tubular cells, generating ROS (*c*); weak fluorescence of the glomerular mitochondria is seen (arrow: center of the glomerular zone).

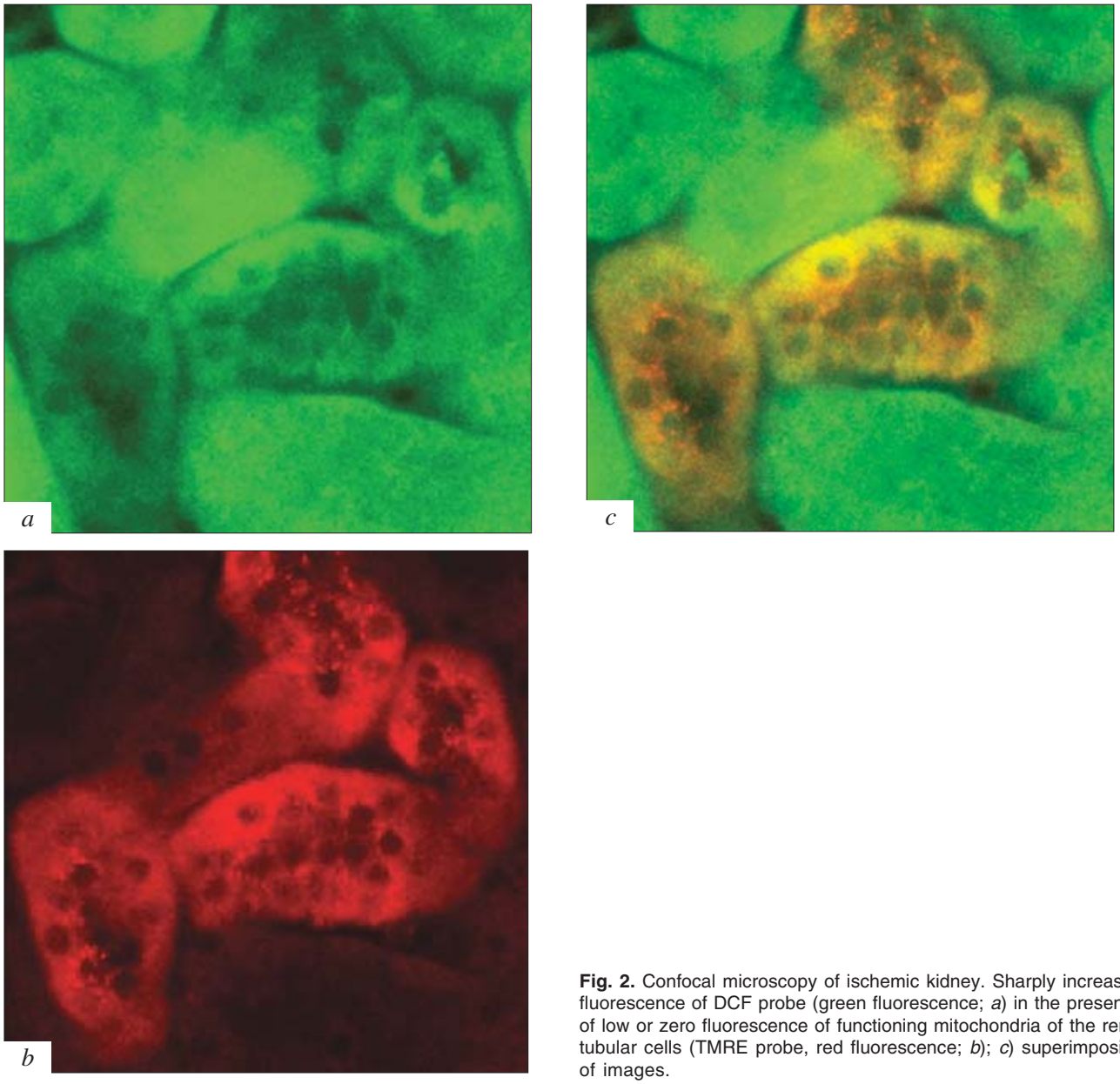

Fig. 2. Confocal microscopy of ischemic kidney. Sharply increased fluorescence of DCF probe (green fluorescence; *a*) in the presence of low or zero fluorescence of functioning mitochondria of the renal tubular cells (TMRE probe, red fluorescence; *b*); *c*) superimposing of images.

crease, NO production by tubular mitochondria increased to a significantly lesser degree than in experiments without preconditioning (Table 1).

IP also prevented the decrease in the percentage of tubules with actively functioning mitochondria, but ROS generation increased almost similarly as in the control (Table 1).

In control experiments 40-min ischemia caused no appreciable changes in blood concentrations of creatinine, sodium, and potassium, while creatinine clearance and sodium reabsorption in the renal tubules decreased significantly (Table 2). In experiments with HP, creatinine and sodium concentrations in the blood did not change, while blood concentration of potassium increased and creatinine clearance tended to increase. Sodium reabsorption during the postischemic period remained within the normal range and significantly surpassed that in the control group. IP significantly improved the function of ischemic kidney, which manifested in higher creatinine clearance and sodium reabsorption during the postischemic period (at the normal level).

Hence, the mechanism of protective effects of HP and IP seems to be associated with suppression of excessive production of NO and oxygen radicals (for HP) during the early postischemic period. The protective effect of HP under conditions of moderately expressed postischemic functional disorders is realized mainly at the level of the renal tubular epithelium, which manifests in prevention of a de-

crease in the tubular reabsorption of sodium without appreciable increase in the glomerular filtration. IP promoted normalization of both tubular and glomerular filtration.

The protective effect of antiischemic protection preconditioning methods was paralleled by inhibition of ROS and NO production. The harmful effect of excessive generation of oxygen radicals on cells causes no doubts, while the data on the biological significance of increased NO production during the postischemic period are contradictory. It seems that the degree of increase in NO concentration is essential for the biological effect. The increase in NO concentration as a result of activation of endothelial isoenzyme (NO synthase) is usually paralleled by improvement of kidney resistance to ischemia [11-13], while activation of the inducible isoform of this enzyme is associated with a decrease in ischemic resistance of the kidney [5,8]. The combination of intense production of ROS and NO is particularly unfavorable, as the formation of highly toxic peroxynitrite is probable in this case [3].

Our experiments showed that ROS and NO are generated predominantly in the renal tubular mitochondria. Activation of the synthesis of these radicals can be associated with the formation of peroxynitrite because of common location of the reaction components, and the protective effect of HP and IT can be mediated by suppression of this compound formation, which promotes the maintenance of mitochondrial function and of energy provision of the regeneration processes during the postischemic period.

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