BIOPHYSICS AND BIOCHEMISTRY

Inhibition of GSK-3β Decreases the Ischemia-Induced Death of Renal Cells

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Pharmacological preconditioning with insulin and lithium ions prevented the death of renal cells under conditions of ischemia/reperfusion. Preincubation of cells with insulin or lithium ions decreased production of reactive oxygen species after ischemia/reoxygenation. These agents also prevented the development of mitochondrial dysfunction in renal cells induced by ischemia/reoxygenation. It was hypothesized that the protective effects of these agents are related to inhibition of glycogen synthase kinase-3(. This enzyme is inactivated upon phosphorylation of serine residue in position 9. We found that *in vivo* administration of lithium ions to animals before renal ischemia prevents the development of kidney failure.

Key Words: *preconditioning; ischemia; reoxygenation; oxidative stress*

Ischemia/reperfusion injury of the kidneys is a major cause of acute renal failure, which results from epithelial cell death in the nephron tubules. The death of nephron cells occurs during ischemia and after the restoration of blood flow. One of the major causes of renal cell death and dysfunction of the kidneys after ischemia/reperfusion is oxidative damage due to imbalance in the production and utilization of reactive oxygen species (ROS).

ROS are produced by various enzyme systems, including cyclooxygenase, lipoxygenase, and NADPH oxidase, but mitochondrial enzymes are the main source of ROS [3,5,8,14]. ROS play an important regulatory function [6]. However, an increase in the content of ROS and/or maintenance of ROS at high level for a long time can cause serious damage to DNA, proteins, and lipids. Hypoxia induces some changes in the cells. After the restoration of oxygen supply, these changes cause a significant increase in ROS concentration (primarily, in the mitochondria). Oxidative damage is followed by the induction of intracellular signal pathways, which results in cell death. It is partly related to the cascade reaction of ROS generation in mitochondria [15].

High intracellular concentration of ROS induces the development of nonspecific mitochondrial permeability (NMP), which plays a crucial role in cell function. It is currently accepted that NMP is a partial reaction of the apoptotic cascade [7]. It should be emphasized that NMP can serve as a cause or consequence of ROS generation and can lead to cyclic amplification of the oxidative damage, so-called ROS-induced ROS release [15].

There are several mechanisms for cell protection from ischemia/reperfusion. They are associated with preconditioning (*i.e.*, tissue training due to alternation of short-term ischemia and reoxygenation of the same duration). Protective signal pathways are activated

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during ischemic preconditioning. They can be triggered by some pharmacological substances. One of the major stages in the protective signal cascade during preconditioning is inhibition of glycogen synthase kinase-3β (GSK-3β, serine-threonine kinase) [9]. Activity of this enzyme decreases during serine phosphorylation in position 9. This inhibition of GSK-3β increases the threshold of NMP activation, which results in reduction of cell death. Published data show that GSK-3β inhibition during pharmacological preconditioning decreases the area of infarction and contributes to postischemic functional recovery [11]. Our previous studies showed that GSK-3β inhibition protects the ischemic kidney from injury [12]. Here we studied the mechanisms for death of renal epithelial cells during ischemia/reperfusion (as an approach to cell protection by GSK-3β inhibition).

MATERIALS AND METHODS

In series I, the primary culture of rat kidney cells was prepared. The kidneys were aseptically excised from 3-7-day-old rat pups. Each experiment with culture isolation was conducted on 1 animal. The kidneys were minced and placed in Hanks buffered balanced salt solution (BBSS, pH 7.4). The tissue was washed, put in 0.1% collagenase solution in Hanks BBSS, and incubated at 37°C for 30 min. The remaining large fragments were removed. The supernatant was centrifuged at 200*g* for 3 min. The cell pellet was resuspended in DMEM/F12 medium (1:1). The cell suspension was maintained in a tube for 10-12 min, the pellet containing renal tubules was taken. The cells were cultured in a medium of DMEM/F12 and 10% fetal bovine serum until 60-70% confluence. This sample was used in further experiments. Nitrogen was delivered through Hanks solution (hypoxic conditions) to induce ischemia. The cells were incubated in a hypoxic solution at 37°C for 24 h. ROS generation and membrane potential of the inner mitochondrial membrane were evaluated. The post-hypoxic period of reoxygenation varied from 30 min to 2 h. Cell death was studied after 24 h. This period is required for oxidative stress-induced damage and apoptotic cell death.

In series II, ischemia/reperfusion was induced *in vivo*. Experiments were performed on 24 male albino rats weighing 200-250 g and under conditions of free access to food and water. For modeling postischemic renal failure, the renal vascular bundle was isolated under ether anesthesia and clumped with a microvascular forceps for 90 min. Blood flow was resumed after removal of the forceps. Right-sided nephrectomy was performed in all experiments. The surgical wound was sutured. The animals recovered from narcosis. The rat received an intraperitoneal injection of LiCl in a dose of 20 mg/kg to induce pharmacological preconditioning (1 h and 30 min before the surgery). The concentrations of creatinine and urea in blood plasma were measured on day 3 after ischemia using a CellTac blood biochemical analyzer (Nihon Kohden).

Cell viability was evaluated in the standard MTT test with modifications [4] using 96-well culture microplates. Cell apoptosis was studied by staining with annexin V-FITC and further analysis on a CyFlow flow cytometer (Partec GmbH). The degree of apoptotic death was estimated by comparing the count of annexin V-positive cells and total number of cells.

The mitochondrial transmembrane potential was measured in living cells using a potential-sensitive probe, ethyl ester, and tetramethylrhodamine ethyl ester (TMRE). Microscopic analysis of renal cells was performed under a LSM510 laser scanning confocal microscope (Carl Zeiss) with special software. Fluorescence was emitted with a HeNe laser at 543 nm. Fluorescence was measured at 560-590 nm. Cell images were obtained using an oil immersion lens (×10, ×63) at different levels of digital magnification.

ROS generation was detected with 2,7-dichlorodihydrofluorescein (Calbiochem) in the form of a membrane-permeable diacetate ester (2,7-DCFH_2-DA). DCF fluorescence was measured at the excitation and emission wavelengths of 488 and 505-530 nm.

Protein electrophoresis was performed in 12.5% PAAG under denaturing conditions [10]. After electrophoresis, the proteins were transferred to a PVDF membrane (Amersham Pharmacia Biotech). The membrane was blocked in Tris-buffer medium (TBS) with 5% defatted dry milk at 4°C for 12 h. This membrane was successively incubated with rabbit primary antibodies to pGSK-3β and horseradish peroxidase-labeled goat anti-rabbit antibodies. The signal was recorded with a chemiluminescent substrate (ECL Enhanced chemiluminescence system, Amersham Pharmacia Biotech). Chemiluminescence was detected using a Kodak film.

RESULTS

The study of renal cell viability in the MTT test showed that cell death after 24-h ischemia followed by 24-h reoxygenation surpassed 80% (compared to the control; Table 1). Addition of agents inducing pharmacological preconditioning insulin (60 nM) and LiCl (6 mM) to the incubation medium during ischemia increased the number of viable cells after ischemia/reoxygenation to 37 and 38%, respectively. Therefore, lithium ions and insulin improved cell survival during ischemia/reoxygenation (by more than 2 times).

Evaluation of apoptotic death showed that the number of annexin V-positive cells increases by 4 times

(83%) after ischemia/reoxygenation. Incubation of cells with LiCl and insulin during ischemia significantly decreased in the number of apoptotic cells (54.5 and 57.93% of the total cell number, respectively; Table 1). Hence, ischemia/reoxygenation is accompanied by induction of apoptosis in the majority of renal epithelial cells; insulin and lithium ions suppress the apoptotic program.

The data on cell survival after ischemia/reoxygenation indicate that preincubation with LiCl and insulin prevents cell death. The antiapoptotic effect of these agents can be mediated by inhibition of GSK-3β [2] playing a role in the regulation of NMP [9] and development of oxidative stress. Taking these data into account, we measured membrane potential and ROS concentration in cells after ischemia/reoxygenation. Mitochondrial dysfunction is a rapid response of the cell to stress. The state of mitochondria was evaluated over the first minutes of reoxygenation. TMRE fluorescence in mitochondria by the 30th minute of reoxygenation was by 40% lower than in control cells (Fig. 1). This attests to a significant decrease in the membrane mitochondrial potential, *i.e.* mitochondrial dysfunction probably related to the induction of NMP in some mitochondria.

Published data show that induction of NMP is followed by oxidative stress [15]. Therefore we studied ROS generation in renal epithelial cells after ischemia/reoxygenation. Fluorescence of a ROS-specific probe DCF in cells exposed to ischemia/reoxygenation increased about 20-fold by the 30th minute of reoxygenation (Fig. 1), which confirmed enhanced production of ROS.

Taking into account the effect of pharmacological preconditioning on cell viability after ischemia/reoxygenation, we studied the effect of these agents on the membrane potential and ROS generation in cells. Addition of lithium salts to the incubation medium was followed by a 2-fold decrease in DCF fluorescence in cells after ischemia/reperfusion (Fig. 1). It reflects the decrease in ROS generation. Similar changes were observed after incubation of renal cells with insulin under ischemic conditions. Thus, insulin and lithium ions suppress the signal pathways for oxidative stress in cells under conditions of ischemia/reoxygenation.

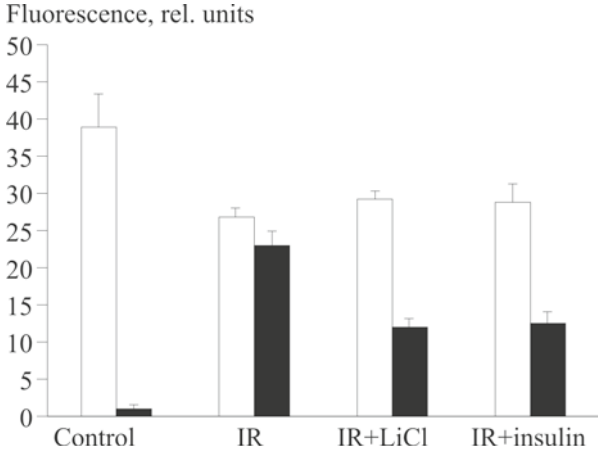

Fig. 1. Effects of LiCl and insulin on the membrane potential (light bars) and ROS generation (dark bars) in the cells exposed to ischemia/reoxygenation (IR). Insulin, 60 nM; LiCl, 6 mM.

TABLE 1. Renal Cell Viability after Ischemia/Reoxygenation (IR)

Group	Cell survival, %	Apoptotic cell death, %
Control	100±3	12.5
IR	16±4	83.0
IR+6 mM LiCl	38±4	54.5
IR+60 nM insulin	37±5	57.9

Our previous studies showed that ischemia/reoxygenation can be accompanied by strong fragmentation of the mitochondrial reticulum in renal cells. These changes can be prevented by lithium ions and insulin [13]. Ischemia/reoxygenation-induced cell death is related to or accompanied by oxidative stress and dysfunction of the mitochondrial apparatus. It manifested in a decrease in the membrane potential and fragmentation of mitochondrial reticulum. A relationship exists between the development of oxidative stress and decrease in the mitochondrial membrane potential [15]. Oxidative stress contributes to induction of NMP, which amplifies ROS generation in mitochondria. These changes are mediated by the positive feedback mechanism. The significant decrease in ROS production and recovery of the mitochondrial membrane potential after preincubation of renal cells with LiCl and insulin indicate that these cells become more resistant to the opening of a nonspecific pore (*i.e.*, to oxidative stress). The increase in cell resistance to oxidative stress improves survival of renal epithelial cells.

For evaluation of the role of GSK-3β in realization of the effects of LiCl and insulin, we studied the possibility of GSK-3β serine phosphorylation in position 9. The intracellular localization of this enzyme was evaluated by an immunocytochemical assay and immunoblotting. Studying the cells after incubation with lithium ions showed that phosphorylated kinase is not present in untreated cells (Fig. 2). Similar results were obtained for cells treated with lithium chloride for 3 h. It should be emphasized that the amount of phosphorylated GSK-3β increases significantly after treatment for 6 h and, particularly, for 24 h (Fig. 2). After incubation with lithium chloride for 24 h, lithium ions have a strong stimulatory effect on phos-

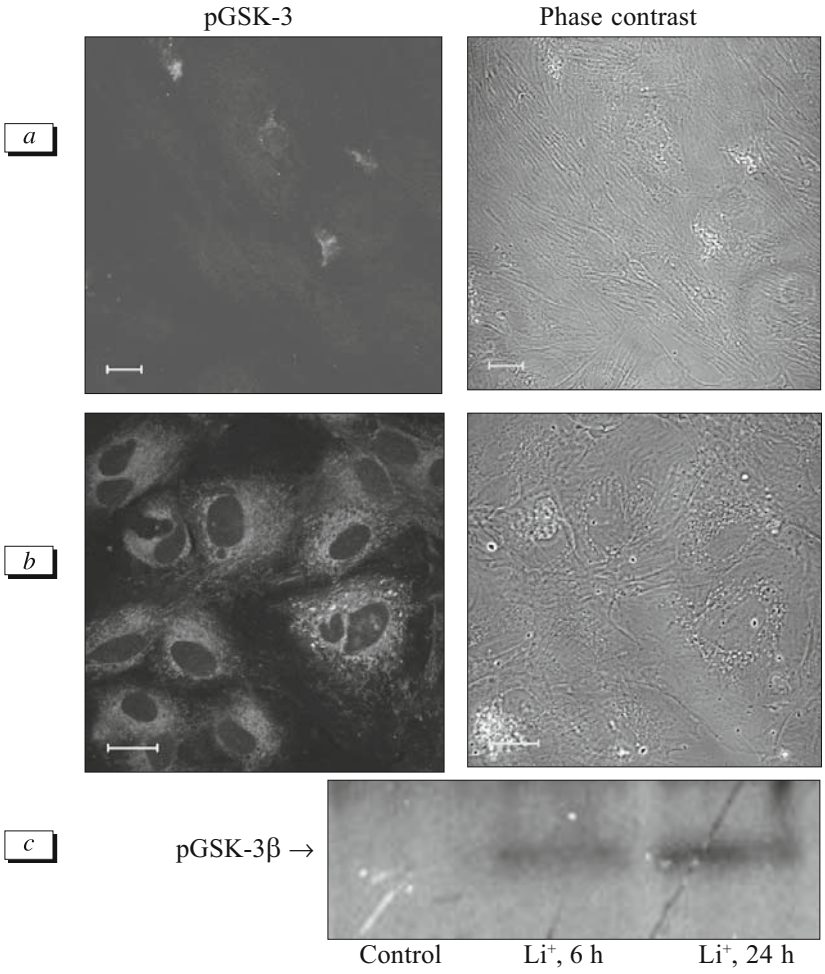

Fig. 2. Increase in the number of pGSK-3β in renal cells during incubation with LiCl. Control cells (*a*); cells after incubation with 6 mM LiCl (*b*); immunoblot of cells after incubation with 6 mM LiCl for 6 and 24 h (*c*).

phorylation of GSK-3β. The observed changes should be accompanied by the inhibition of this enzyme.

The intracellular localization of GSK-3β was studied by means of immunocytochemical staining and confocal microscopy (Fig. 2). The amount of phosphorylated GSK-3β was small in intact cells. However, 24-h incubation with LicCl was followed by a significant increase in pGSK-3β content in renal cells. pGSK-3β was mainly localized in mitochondria. The degree of phosphorylation of cytoplasmic GSK-3β was also higher compared to the control. The amount and localization of total GSK-3β remained practically unchanged after treatment with LiCl (data not shown).

Experiments on the cell culture suggest that protection of renal epithelium from death under the influence of GSK-3β inhibitors *in vivo* improves renal function. This hypothesis was confirmed by the results of studies on animals with renal ischemia. Therefore, these protective agents hold much promise for the treatment of renal deficiency under conditions of ischemia.

One of the major functions of the kidney is excretion of the end-products of body metabolism from the circulation. We measured the concentrations of urea and creatinine in the blood to evaluate renal function. Under normal conditions, the formation and excretion of creatinine from circulation maintains the minimal concentration of creatinine in the blood. The rate of glomerular filtration decreases significantly during renal deficiency and dysfunction of the kidneys. The concentrations of creatinine and urea in the blood increase in the blood under these conditions. By the 3rd day, blood creatinine level in rats with renal ischemia/reperfusion was 6-fold higher than in control animals (Fig. 3). Urea concentration in rats was significantly elevated after renal ischemia (Fig. 3). The increase in creatinine concentration precedes the elevation of urea content, which is consistent with the mechanisms of nitrogen metabolism in animals [1].

In the next series, pharmacological preconditioning was produced by *in vivo* administration of LiCl to rats before ischemia to protect the kidneys. Insulin treatment cannot be considered as an adequate model of pharmacological preconditioning, since insulin has a variety of physiological effects in the body. The action of insulin may be followed by various artifacts. In our experiments, LiCl was administered to rats before ischemia. The concentration of creatinine and urea in

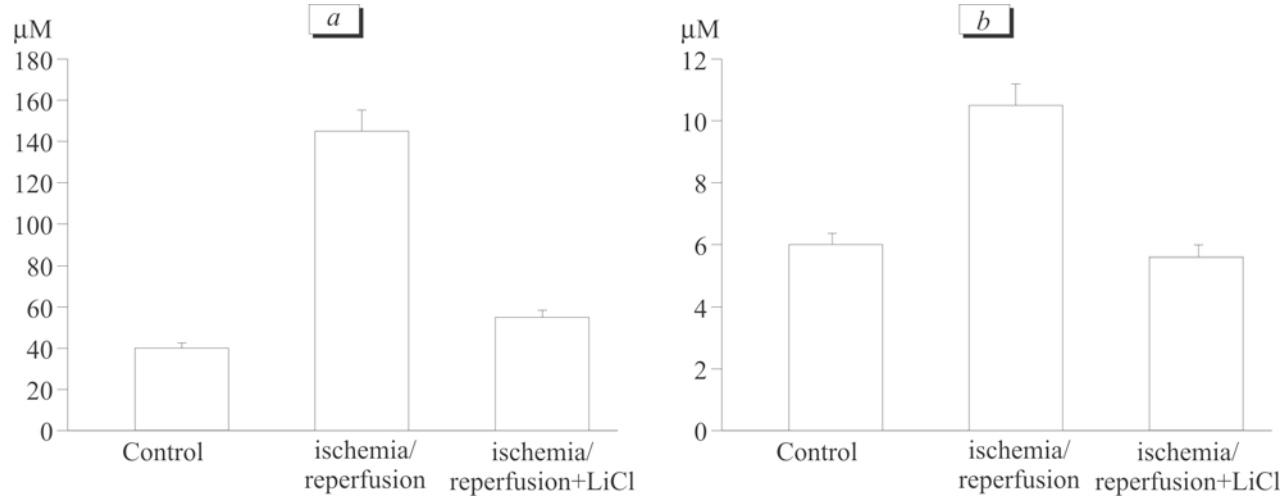

Fig. 3. Effect of pretreatment with LiCl (20 mg/kg) on the development of postischemic renal insufficiency. Concentrations of creatinine (*a*) and urea (*b*).

the blood of LiCl-treated animals was 2-fold lower than in rats exposed to renal ischemia/reperfusion and untreated with protective agents. These data are consistent with the results of our previous studies. We showed that lithium ions decrease the degree of oxidative stress in renal tissue [12]. Therefore, ischemia/reperfusion is accompanied by the development of acute renal failure, impairment of glomerular filtration, and dysfunction of renal tubules. It is manifested in an increase in the concentrations of urea and creatinine in the blood. The development of renal deficiency is probably associated with the death of nephron cells due to oxidative stress and mitochondrial dysfunction. LiCl protects the mitochondria from these negative consequences. Administration of LiCl is followed by partial recovery of renal function. This conclusion is derived from the decrease in urea concentration (up to the control level) and 2-fold reduction of creatinine content after ischemia/reperfusion. Functional recovery of the ischemic kidney is probably related to normalization of mitochondrial function.

We conclude that the death of renal cells under conditions of ischemia/reperfusion is associated with oxidative stress and mitochondrial dysfunction. These changes can be reduced by pharmacological agents LiCl and insulin that have an inhibitory effect on GSK-3β. Pharmacological preconditioning with lithium salts prevents the development of renal dysfunction after ischemia/reperfusion. Our results extend the knowledge of pathological mechanisms for the loss of renal functional reserves during ischemia/reperfusion. These data should be taken into account in the development of pharmacological agents for renal protection.

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