INTRODUCTION

Various organs including the heart (1), brain (2), kidney (3), liver (4), skeletal muscle (5) and stomach (6) respond to brief exposure to ischemia with an increased resistance to severe long-lasting ischemia, and this phenomenon is called ischemic preconditioning. Beneficial effect of direct ischemic preconditioning has been also found in the pancreas. Exposure to short-lasting pancreatic ischemia inhibits the development of ischemia/reperfusion- (7) and cerulein- induced pancreatitis (8), as well as accelerates healing in the course of this disease (9, 10). Also, pancreatic ischemic preconditioning improves pancreatic islets recovery after cold preservation of the pancreas (11).

Previous studies have shown that pancreatic ischemic preconditioning or heparin, applied before induction of acute pancreatitis inhibit the development of this disease and accelerate pancreatic recovery. The aim of the study was to determine the influence of treatment with heparin on protective effect of ischemic preconditioning (IP) in ischemia/reperfusion-induced acute pancreatitis. Heparin was administered twice, before and during induction of acute pancreatitis. IP was performed by short-term clamping of celiac artery, 30 min before induction of acute pancreatitis. Acute pancreatitis was induced in rats by clamping of inferior splenic artery for 30 min followed by reperfusion. Rats were sacrificed after 6-h and 24-h reperfusion. Results: IP alone caused a mild pancreatic damage associated with a limited increase in plasma amylase activity, concentration of pro-inflammatory interleukin-1β and plasma level of D-dimer. Pretreatment with heparin or IP applied alone reduced the severity of acute pancreatitis. Both these procedures caused a similar reduction in plasma lipase, amylase and interleukin-1β, as well as in histological signs of pancreatic damage. These changes were associated with partial reversion of the pancreatitis-evoked fall of pancreatic blood flow and DNA synthesis. Combination of heparin plus IP reduced the protective effect of heparin or IP applied alone. It was manifested by an increase in pancreatic damage and plasma level of lipase, amylase and interleukin-1β, as well as by reduction in pancreatic DNA synthesis and plasma concentration of D-dimer and interleukin-10. Conclusions: heparin abolishes the protective effect of ischemic preconditioning in ischemia reperfusion-induced pancreatitis. This observation suggests that initial clot formation is necessary to induce pancreatic protection by IP.

Key words: ischemic preconditioning, heparin, acute pancreatitis, coagulation, D-dimer,
The present study was designed to assess the influence of heparin administration on the protective effect of ischemic preconditioning in ischemia/reperfusion-induced pancreatitis.

MATERIALS AND METHODS

Animals and treatment

Studies were performed on male Wistar rats weighing 240–260 g and were conducted following the experimental protocol approved by the Committee for Research and Animal Ethics of Jagiellonian University and the Local Commission of Ethics for the Care and Use of Laboratory Animals. Rats were housed in cages with wire mesh bottoms, with normal room temperature and a 12-h light-dark cycle. Studies were carried out on 180 rats divided randomly on nine experimental groups: [1] sham-operated control rats without induction of acute pancreatitis; [2] sham-operated rats treated with heparin without induction of acute pancreatitis; [3] rats exposed to ischemic preconditioning without induction of acute pancreatitis; [4] rats treated with heparin and exposed to ischemic preconditioning; [5] sham-operated rats with induction of acute pancreatitis; [6] rats exposed to ischemic preconditioning prior to induction of acute pancreatitis; [7] sham-operated rats treated with heparin combined with induction of acute pancreatitis; [8] rats treated with heparin prior to exposure to ischemic preconditioning and induction of acute pancreatitis; [9] rats exposed to ischemic preconditioning prior to heparin administration and induction of acute pancreatitis.

Unfractionated heparin (Heparinium, Polfa, Warszawa, Poland) was administered subcutaneously twice at the dose of 150 U/kg/injection. The first dose was administered 30 min before sham-operation (group 2 and 7) or exposure to ischemic preconditioning (group 4 and 8), or 30 min after exposure to ischemic preconditioning (group 9). The second dose of heparin was given 3 h after the first dose. The dose of heparin, 150 U/kg was chosen because this dose caused two-fold increase in activated partial thromboplastin time (aPTT) in our previous (25, 26) and current studies, and a fixed therapeutic range for the aPTT of 1.5 to 2.5 times the control value has become widely accepted (32).

Ischemic preconditioning of the pancreas or sham operation was performed in rats after fasting for 24 h with free access to water. Rats were anesthetized with ketamine (50 mg/kg i.p., Bioketan, Vetoquinol Biowet, Gorzow Wielkopolski, Poland) and after longitudinal laparotomy, the celiac artery was clamped two times for 5 min with 5 min interval. In sham-operated rats, longitudinal laparotomy and mobilization of the pancreas without clamping any artery was performed. Thirty min after sham operation (group 5), exposure to ischemic preconditioning (group 6 and 8) or administration of heparin (group 7 and 9), acute pancreatitis was induced by severe pancreatic ischemia followed by reperfusion as described previously (33). Rats were reanesthetized with ketamine. Ischemia of splenic region of the pancreas was induced by clamping of splenic inferior artery using microvascular clips. Thirty min later, microvascular clips were removed to obtain pancreatic reperfusion and the abdominal cavity was closed by suture. In sham-operated control animals, longitudinal laparotomy and mobilization of pancreas without clamping any arteries was performed. Animals were anesthetized again after pancreatic reperfusion lasting for 6 or 24 h.

Determination of pancreatic blood flow

At the time of experiment cessation the abdominal cavity was opened and the pancreas was exposed for the measurement of the blood flow, using laser Doppler flowmeter PeriFlux 4001 Master monitor (Perimed AB, Jarfalla, Sweden), as described previously (34). The pancreatic blood flow was presented as percent change from control value obtained in sham-operated saline-treated rats.

Biochemical analysis of plasma

After measurement of pancreatic blood flow, arterial blood was taken from the abdominal aorta, anticoagulated with 3.8% sodium citrate. APTT was determined in fresh plasma, using Plastelin LS (Organon Teknika Corporation, Durham, NC, USA). Plasma D-dimer concentration was determined using a latex-enhanced immunoturbidimetric assay (D-dimer test, Roche Diagnostics). Plasma lipase and amylase activity was determined with a Kodak Ectachem DT II System analyzer (Eastman Kodak Company, Rochester, NY, USA) using Lipa and Amyl DT Slides (Vitros DT Chemistry System, Johnson & Johnson Clinical Diagnostic, Inc., Rochester, NY, USA), as described previously (35). Plasma concentration of interleukin-1β (IL-1β) and interleukin-10 (IL-10) were measured using the BioSource Cytoscreen rat IL-1β and IL-10 kits (BioSource International, Camarillo, California, USA) based on ELISA, as described previously (36).

Determination of pancreatic DNA synthesis

After the blood withdrawal, the pancreas was carefully dissected out from its attachment to the stomach, duodenum, and spleen. Fat and peripancreatic tissue were trimmed away. Samples of pancreatic tissue were taken for study of DNA synthesis and morphological examination. Pancreatic DNA synthesis was measured by incubation of minced pancreatic tissue at 37°C for 45 min in 2 ml of medium containing 8 µCi/ml of [3H]thymidine ([6-3H]-thymidine, 20–30 Ci/mmol, Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic), as described previously (37). DNA synthesis was expressed as [3H]thymidine disintegrations per minute per microgram DNA (dpm/µg DNA).

Histological examination of pancreatic damage

Samples of pancreatic tissue excised from the body portion for morphological examination were fixed for 24 h in buffered 10% formalin and embedded in paraffin. Slides were stained with hematoxylin and eosin and examined by two pathologists uninformed about treatment given. The histological grading of edema, leukocytic inflammatory infiltration, vacuolization of acinar cells, hemorrhages and necrosis was made using a scale ranging from 0 (absent) to 3 for maximal alteration as described previously in detail (38). Results of histological examination have been expressed as a predominant histological grading in each experimental group of animals.

Statistical analysis

Statistical analysis of data was carried out by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant when P was less than 0.05. Results have been expressed as means ±S.E.M.

RESULTS

Morphological examination

Macroscopic and microscopic examination did not show any damage of the pancreas in sham-operated control rats (Tables 1

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and 2). Also, administration of heparin was without effect on pancreatic histology in these rats. In rats exposed to pancreatic ischemic preconditioning, 6 h after the start of reperfusion, morphological examination revealed minimal pancreatic damage or normal pancreatic histology (Table 1). The transient mild interlobular edema, scarce perivascular leukocyte infiltration and 1-2 hemorrhagic foci per slide were found in about half of cases. In the rest of these animals, ischemic preconditioning did not affect pancreatic morphology (Table 1).

Eighteen hours later and 24-h after the start of reperfusion, pancreases of all animals exposed to ischemic preconditioning alone showed normal morphology (Table 1). In rats treated with heparin and exposed to ischemic preconditioning, pancreatic histology was similar to that observed in rats exposed to ischemic preconditioning alone.

Exposure to severe 30 min pancreatic ischemia followed by reperfusion led to the development of acute necrotizing pancreatitis in all rats tested. After 6-h reperfusion, we observed interlobular and moderate intralobular edema, moderate perivascular and scarce diffuse leukocyte infiltration associated with necrosis of less than 15% to 35% of acinar cells and 1 to 5 foci of hemorrhages per slide (Table 1). After 24-h reperfusion pancreatic damage was higher than after 6-h reperfusion. In half of cases, we observed abundant diffuse leukocyte infiltration associated with necrosis of 15-35% of cells, vacuolization of acinar cell. Pancreatic edema and hemorrhages reached the same grade as in after 6-h reperfusion (Table 2).

Ischemic preconditioning, applied prior to induction of pancreatitis, reduced the pancreatitis-evoked pancreatic damage (Tables 1 and 2). After 6-h and 24-h reperfusion it was found as a reduction in pancreatic edema, inflammatory infiltration, necrosis and hemorrhages. Moreover after 24-h reperfusion, we observed reduction in number of acinar cells with intracellular vacuolization (Table 2).

Pretreatment with heparin before induction of acute pancreatitis attenuated the development of morphological signs of pancreatic damage. This effect was observed after 6-h and 24-h reperfusion and it what was found as a reduction in pancreatic edema, inflammatory infiltration, necrosis and hemorrhages (Tables 1 and 2).

Pretreatment with heparin before exposure to ischemic preconditioning and induction of acute pancreatitis almost completely abolished protective effect of heparin or ischemic preconditioning applied alone. After 6-h reperfusion, it was manifested as an increase in pancreatic edema and number of hemorrhages (Table 1); whereas 24 h after the start of reperfusion, we observed additionally an increase in necrosis of pancreatic cells and inflammatory leukocyte infiltration (Table 2).

Similar cancellation of protective effect of heparin or ischemic preconditioning on pancreatic tissue in rats with induction of acute

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Numbers represent the predominant histological grading in each group.

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Numbers represent the predominant histological grading in each group.

Table 1. Effect of heparin (H), ischemic preconditioning (IP) and ischemia/reperfusion; (IR)-induced pancreatitis on morphological signs of pancreatic damage after 6 h reperfusion.

Table 2. Effect of heparin (H), ischemic preconditioning (IP) and ischemia/reperfusion; (IR)-induced pancreatitis on morphological signs of pancreatic damage after 24 h reperfusion.
Pancreatitis was observed while ischemic preconditioning was applied before heparin administration (Tables 1 and 2).

Biochemical and functional findings

Treatment with heparin or ischemic preconditioning applied alone or the combination of heparin with ischemic preconditioning was without effect on plasma lipase activity (Fig. 1), pancreatic DNA synthesis (Fig. 5) and pancreatic blood flow (Fig. 8) in rats without induction of acute pancreatitis. On the other hand, ischemic preconditioning applied alone slightly, but significantly increased plasma amylase activity (Fig. 2), plasma concentration of L-1β (Fig. 3) and D-dimer concentration (Fig. 7) after 6-h reperfusion. In this group of animals, plasma concentration of IL-10 was also increased, reaching about 140 and 300% of control value after 6-h and 24-h reperfusion, respectively (Fig. 4). Heparin given alone, increased aPTT by about 105 and 43% after 6-h and 24-h reperfusion, respectively (Fig. 6). Heparin applied before ischemic preconditioning abolished the ischemic preconditioning-induced increase in plasma IL-1β concentration (Fig. 3) and reduced the ischemic preconditioning-induced increase in plasma IL-10 level (Fig. 4).
Induction of acute pancreatitis caused around 15-fold increase in plasma activity of lipase (Fig. 1) and 10-fold increase in plasma activity of amylase (Fig. 2). These effects were observed after 6-h and 24-h reperfusion. After 6-h reperfusion plasma concentration of proinflammatory IL-1β was increased by around 300% when compared to control (Fig. 3); whereas pancreatic DNA synthesis and pancreatic blood flow were reduced by 57 and 70%, respectively (Figs. 5 and 8). Ischemia/reperfusion-induced pancreatitis also strongly increased aPTT (Fig. 6) and plasma D-dimer concentration (Fig. 7). Plasma concentration of anti-inflammatory IL-10 was not affected by pancreatic ischemia followed by 6-h reperfusion (Fig. 4); whereas 18 h later, we observed around 5-fold increase in plasma IL-10 concentration in this group of animals (Fig. 4).

Exposure to ischemia preconditioning before induction of acute pancreatitis reduced the development of this disease. Biochemical markers of acute pancreatitis such as plasma activity of lipase (Fig. 1) and amylase (Fig. 2) and plasma concentration of proinflammatory IL-1β (Fig. 3) were reduced; whereas pancreatic DNA synthesis, an index of cell vitality, was increased when compared to acute pancreatitis alone (Fig. 3). These effects were associated with an improvement of pancreatic blood flow.
and reduction in plasma IL-10 concentration (Fig. 4), aPTT (Fig. 6) and plasma D-Dimer concentration (Fig. 7). Reduction in plasma D-dimer concentration was especially manifested after 24-h reperfusion (Fig. 7). In rats with induction of acute pancreatitis, also pretreatment with heparin attenuated the development of ischemia/reperfusion-induced pancreatitis. In biochemical examination, it was found as a reduction of the pancreatitis-evoked increase in plasma lipase (Fig. 1) and amylase (Fig. 2) activity, and plasma concentration of proinflammatory IL-1β (Fig. 3). Also, heparin administration partly reversed the pancreatitis-evoked fall in pancreatic DNA synthesis (Fig. 5) and pancreatic blood flow (Fig. 8). Moreover, pretreatment with heparin partly reversed the pancreatitis-evoked increase in aPTT and reduced plasma level of D-Dimer (Fig. 5). Plasma concentration of anti-inflammatory IL-10 was not affected by pretreatment with heparin in rats with ischemia/reperfusion-induced pancreatitis after 6-h reperfusion; whereas after 24-h reperfusion this parameter was significantly reduced (Fig. 4).
Either administration of heparin prior to ischemic preconditioning or exposure to ischemic preconditioning prior to heparin administration abolished pancreatoprotective effect of heparin or ischemic preconditioning applied alone before induction of acute pancreatitis. In biochemical examination, it was manifested as an increase in plasma level of lipase (Fig. 1), amylase (Fig. 2) and interleukin-1β (Fig. 3), and as a reduction in pancreatic DNA synthesis (Fig. 5) and pancreatic blood flow (Fig. 8). Plasma concentration of IL-10 and D-dimer reached similar value to that observed in animals pretreated with heparin alone before induction of acute pancreatitis.

**DISCUSSION**

Our present study has confirmed and extended previous observations that pancreatic ischemic preconditioning (7-10) or heparin (22-26) applied before induction of acute pancreatitis...
inhibits the development of pancreatitis and accelerates the recovery in this disease. In our present study, it has been found as a reduction in pancreatic damage associated with a decrease in plasma level of pro-inflammatory IL-1β and plasma activity of pancreatic digestive enzymes, lipase and amylase. Reduction in plasma concentration of IL-1β has been in harmony with a decrease in leukocyte infiltration of pancreatic tissue in morphological features. Beneficial effect of pancreatic ischemic preconditioning or heparin has been also manifested by attenuation of the pancreatitis-induced fall in pancreatic DNA synthesis and pancreatic blood flow.

Previous studies have shown in different organs that the ischemic preconditioning-induced protective effect involves numerous mechanisms such as: the preservation of cellular ATP (39), activation of sensory nerves and release of CGRP (7, 40), stimulation of receptors for adenosine or bradykinin (41), reduction in oxidative stress (42) or increase in expression of heat shock protein-70 (8). Moreover, study performed by Williams-Pritchard et al. (43) has shown that cardioprotective effect of ischemic preconditioning involves activity of epidermal growth factor receptor (EGFR). Inhibitors of EGFR tyrosine kinase blocked the protective effect of heparin-binding EGF like growth factor (HB-EGF) on ischemia reperfusion injury (44). This observation indicates that protective effect of ischemic preconditioning involves some mechanisms related to signaling pathways. Also protective effect of heparin is related, among others to growth factors. Growth factors form tight complexes with heparin. This effect has been found e.g. in the case of members of the fibroblast growth factor (FGF) family (44, 45), vascular endothelial growth factor (VEGF) (46), hepatocyte growth factor (HGF) (47) and mentioned above HB-EGF (48, 49). Numerous studies have indicated that these growth factors, especially FGF-1 and FGF-2, cannot bind their receptor or activate signal transduction without the presence of heparin or other glycosaminoglycans, heparan sulfate (50-52). Also, heparin and heparan sulfate protect growth factors from enzymatic degradation (53). On the other hand, experimental and clinical studies have shown pancreatic overexpression of growth factors in the course of acute pancreatitis (54-56) and administration of growth factors such as EGF (57, 58), FGF-2 (59), HGF (60), insulin-like growth factor (IGF-1) (61), growth hormone (62) or ghrelin (35, 63, 64) exhibits protective and therapeutic effect in this disease. Also HB-EGF exhibits protective effect in the gut. Administration of HB-EGF decreases production of reactive oxygen species (65) and reduces the ischemia/reperfusion induced intestinal damage (66). HB-EGF plays an important role in preservation of gut barrier function after hemorrhagic shock (67) and deletion of HB-EGF gene increases susceptibility to necrotizing enterocolitis (68). These data indicate that protective effect of ischemic preconditioning and heparin may involve some common for both factors mechanisms and for this reason combination of heparin with ischemic preconditioning produces weaker protective effect in the pancreas than heparin or ischemic preconditioning applied alone.

Previous studies have shown that exposure to ischemic preconditioning induces mild tissue injury, including release of proinflammatory cytokines, which participate in the induction of protective mechanisms (69). Our present study is in agreement with these findings. We have found that ischemic preconditioning applied alone without induction of acute pancreatitis, causes mild pancreatic damage and significantly increases plasma amylase activity and plasma concentration of proinflammatory IL-1β. These findings suggest that initial tissue damage evoked by mild noxious factors such as ischemic preconditioning is necessary to activate pancreatic and/or systemic self-defensive mechanisms, which lead to an increase in pancreatic resistance against subsequent exposure to severe damaging factors. This conclusion is additionally supported by observations that pretreatment with different mild damaging factors such as bacterial lipopolysaccharides (70) grapefruit-seed extract (71), or low doses of capsaicin (72) inhibits the development of acute pancreatitis.

It should be pointed out that ischemic preconditioning is evoked by short-term clamping of arterial vessels and this procedure must activate coagulation. D-dimer is a product of proteolytic action of plasmin on fibrin polymer and for this reason an increase in plasma concentration of D-dimer is a marker of activated fibrinolysis (73). In our present study, we have found that pancreatic ischemic preconditioning applied alone increases plasma level of D-dimer. This finding indicates that ischemic preconditioning induces initial clot formation with subsequent activation of fibrinolysis. This concept is additionally supported by our previous observation that ischemic preconditioning applied alone reduces euglobulin clot lysis time, indicating acceleration of fibrinolysis (10). Also present findings observed in animals exposed to ischemic preconditioning before induction of acute pancreatitis are in harmony with this concept. Ischemic preconditioning applied before induction of acute pancreatitis has reduced the pancreatitis-induced increase in aPTT and D-dimer concentration. Reduction in aPTT indicates decrease in consumption of factors involved in coagulation; whereas reduction in D-dimer concentration indicates a decrease in level of fibrin degradation products and this effect is most likely a result of a reduction in amount of fibrin.

The most important finding of our present study is observation that pretreatment with heparin abolishes protective effect of ischemic preconditioning in acute pancreatitis evoked by severe ischemia followed by reperfusion. It has been found as an increase in morphological signs of pancreatic damage and raise in plasma level of lipase, amylase and proinflammatory IL-1β. These changes have been associated by decrease in pancreatic blood flow and pancreatic DNA synthesis, an index of pancreatic cells vitality. As discussed above, pancreatoprotective effect of ischemic preconditioning is probably mainly dependent on early activation of fibrinolysis. Pretreatment with heparin has inhibited the ischemic preconditioning-induced early activation of coagulation and for this reason abolished early activation of fibrinolysis. This concept is documented by our present finding that heparin applied before ischemic preconditioning reduces plasma D-Dimer concentration and prolongs aPTT in rats without induction of acute pancreatitis.

Kinins are a set of proteins involved in inflammation, vascular tone and tissue repair (74). The best studied and the main member of this family is bradykinin. Kinins are synthesized as kininogens, of either high molecular weight or low molecular weight, and inactive until proteolytic cleavage by variety of enzymes, the most important of which are plasma and tissue kallikreins (75). Generation of kinins occurs thought plasma pathway, a tissue pathway and a plasma/tissue-independent pathway. Plasma pathway of kinins production is associated with intrinsic pathway of the coagulation cascade. Production of kinins by the plasma pathway is initiated by interaction of activated factor XII with prekallikrein and high molecular weight kininogen (75). Activated factor XII (factor XIIa) initiates the conversion of prekallikreinogen to kallikrein, which furthers the conversion of factor XII to XIIa. This positive feedback loop is additionally augmented by high molecular weight kininogen releases bradykinin (75). Previous studies have shown that administration of bradykinin reduces heart infarct size in ischemia/reperfusion-induced injury (76) and bradykinin is involved in the cardioprotective effects of ischemic preconditioning (77). Griol-Charhbili et al. have shown that the
ischemic preconditioning-induced cardioprotective effect is reduced in tissue kallikrein-deficient mice, as well as in wild-type mice pretreated with bradykinin receptor antagonist (77). Also heparin affects kallikrein/kinogen system. Study in vitro has shown that heparin abolishes the binding of high molecular weight kininogen to the surface or extracellular matrix of endothelial cell lines (ECV304 and RAEC) in the presence of Zn²⁺ (78). On the other hand, heparin is without effect on the binding of plasma prekallikrein to cell- or extracellular matrix-bound-high molecular weight kininogen and activation of plasma prekallikrein. Moreover, heparin augments hydrolysis of high molecular weight kininogen by plasma kallikrein and release of bradykinin (78). Influence of heparin on bradykinin formation has been also shown by Oschatz et al. (79). They have found that the mast cell-released heparin increases vascular permeability in vivo. This effect has been associated with heparin-induced activation of factor XII and formation of bradykinin (79). Ablation of factor XII or kinin B2 receptors abolished heparin-induced skin edema (79). These data suggest that heparin itself might lead to bradykinin formation, which ought to be taken into consideration as one possible explanation of the heparin induced protection.

There are numerous data showing protective and therapeutic effects of ischemic preconditioning in clinical practice. Direct or remote ischemic preconditioning have been successfully used e.g. in liver (80, 81), esophageal (82), cardiac (83, 84) or brain surgery (85). Our observation that pretreatment with heparin abolishes protective effect of ischemic preconditioning in acute pancreatitis, suggests that pretreatment with heparin may also reduce or abolish protective effect of ischemic preconditioning in other organs. For this reason administration of heparin should be avoid in the case of clinical application of ischemic preconditioning.

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Conflict of interests: None declared.

REFERENCES


