RAPID COMMUNICATION

Protective effect of inducible nitric oxide synthase inhibitor on pancreas transplantation in rats

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Abstract

AIM: To investigate the effect of inducible nitric oxide synthase inhibitor, aminoguanidine, on pancreas transplantation in rats.

METHODS: A model of pancreas transplantation was established in rats. Streptozotocin-induced diabetic male Wistar rats were randomly assigned to sham-operation control group ($n = 6$), transplant control group ($n = 6$), and aminoguanidine (AG) treatment group ($n = 18$). In the AG group, aminoguanidine was added to intravascular infusion as the onset of reperfusion at the dose of 60 mg/kg, 80 mg/kg, 100 mg/kg body weight, respectively. Serum nitric oxide (NO) level, blood sugar and amylase activity were detected. Nitric oxide synthase (NOS) test kit was used to detect the pancreas cNOS and inducible NOS (iNOS) activity. Pancreas sections stained with HE and immunohistochemistry were evaluated under a light microscope.

RESULTS: As compared with the transplant control group, the serum NO level and amylase activity decreased obviously and the evidence for pancreas injury was much less in the AG group. The AG (80 mg/kg body weight) group showed the most significant difference in NO and amylase (NO: 66.0 ± 16.6 vs 192.3 ± 60.0 , $P < 0.01$ and amylase: 1426 ± 177 vs 4477 ± 630 , $P < 0.01$). The expression and activity of tissue iNOS, and blood sugar in the AG (80 mg/kg body weight) group were much lower than those in the transplant control group (iNOS: 2.01 ± 0.23 vs 26.59 ± 5.78 , $P < 0.01$ and blood sugar: 14.2 ± 0.9 vs 16.8 ± 1.1 , $P < 0.01$).

CONCLUSION: Selective iNOS inhibitor, aminoguanidine as a free radical, has a protective effect on pancreas transplantation in rats by inhibiting NO and reducing its toxicity.

INTRODUCTION

Pancreas transplantation is frequently complicated by acute pancreatitis, largely due to ischemia/reperfusion injury secondary to cold preservation^[1,2]. During the reperfusion period, oxygen-derived free radicals can lead to a severe impairment. Nitric oxide (NO) is a free radical with a strong reactivity, and has a fierce cytotoxicity. However, NO can significantly dilate blood vessels and remit vasospasm of grafts. Therefore, NO plays an ambivalent role in ischemia/reperfusion during pancreas transplantation. In this study, we established a model of pancreas transplantation in rats to investigate the expression of nitric oxide synthase (NOS) isoforms, and the effect of inducible nitric oxide synthase (iNOS) inhibitor (aminoguanidine) on pancreas transplantation.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250-300 g (Experimental Animal Center, China Medical University, China) were used as donors and recipients. The animals were kept in standard conditions with free access to water and rodent chow. Diabetes was induced by intravenous injection of streptozotocin at a single dose of 55 mg/kg body weight. Only rats with non-fasting plasma glucose levels of more than 22 mmol/L were used as recipients. We performed recipient transplantation surgery on days 14 and 15 after the injection of streptozotocin. A total of 30 recipient animals were randomly assigned to the sham-operation group ($n = 6$) in which animals underwent midline laparotomy only, transplant control group ($n = 6$) in which animals underwent transplantation and received a bolus injection of saline instead of aminoguanidine, and aminoguanidine treatment group ($n = 18$) in which animals

Table 1 Serum NO level and amylase activity 4 h after transplantation (mean $\pm$ SD)

Group	n	NO (μ mol/L)	Amylase (U/dL)
Sham-operation group	6	30.0 $\pm$ 3.5	342 $\pm$ 73
Transplant control group	6	192.3 $\pm$ 60.0 ^b	4477 $\pm$ 630 ^f
AG-60 mg/kg body weight	6	137.3 $\pm$ 21.1	2848 $\pm$ 354
AG-80 mg/kg body weight	6	67.9 $\pm$ 19.5 ^d	1494 $\pm$ 263 ^h
AG-100 mg/kg body weight	6	66.0 $\pm$ 16.6	1426 $\pm$ 177

^b $P < 0.01$, ^f $P < 0.01$ vs sham-operation group; ^d $P < 0.01$, ^h $P < 0.01$ vs transplant control group.

underwent transplantation. Before reperfusion, a bolus injection of aminoguanidine (60 mg/kg, 80 mg/kg or 100 mg/kg body weight) was given *via* the vena dorsalis penis.

Transplantation and collection of specimens

Synergetic pancreaticoduodenal transplantation was performed in diabetic recipients to assess islet cell functions. After overnight fasting with free access to water, the rats were anesthetized and underwent heterotopic pancreaticoduodenal transplantation as previously described^[3] with certain modifications. After shaving and disinfecting the abdomen with 75% alcohol, a midline incision was made. The donor pancreas was isolated on an aortic segment branching off the celiac axis and the superior mesenteric artery. The venous outflow was provided by the portal vein. Pancreas grafts were flushed with and stored in cold (4°C) heparinized lactate Ringer's solution. Heterotopic intra-abdominal transplantation was performed by end-to-side anastomosis of the aortic segment of the graft and the recipient infrarenal aorta. The graft portal vein was anastomosed to the recipient vena cava using the same technique. Enteric diversion of exocrine graft secretion was accomplished by end-to-side duodenojejunostomy. The abdomen was closed in two layers with 2-0 silk suture. After a single intramuscular injection of 5 mg cefamandole post-operation, the rats were kept under warming lamps until they became active. The warming and cooling ischemic time was less than 15 min and 25 min, respectively. The animals were killed after 4 h of reperfusion. The pancreas was harvested and divided into two segments with one fixed in 10% PBS formalin and the other preserved at -70°C. The blood was withdrawn without anticoagulant and centrifuged at 2000 r/min for 10 min. The serum was preserved at -20°C.

Determination of serum NO and NOS levels

Nitrate reductase was used to detect the serum NO level and NOS test kit was used to detect the cNOS and iNOS activity in the pancreas.

Determination of serum blood and amylase levels

Serum glucose concentration was measured with an Exac Tech blood glucose meter in samples collected from the cut tip of the tail. Serum amylase concentration was measured with a multianalyzer (Clinilizer, CL-7150, Nippon Denshi, Tokyo, Japan).

Histopathology examination

One pancreas segment was fixed in 10% PBS formalin, dehydrated through a grade ethanol series, washed in xylene and embedded in paraffin. The segment was cut into 4 μ m-thick sections. The sections were stained with haematoxylin and eosin and evaluated using light microscope.

Immunohistology

Primary antibody and anti-iNOS polyclonal antibody were produced in rabbits. Strept avidin-biotin complex immunoperoxidase staining system was used, and the positive staining was reddish-brown in color.

Statistical analysis

The data were presented as mean $\pm$ SD. All statistical analyses were performed using the SPSS 10.0 software. Differences in groups were tested by analysis of variance (ANVOA). $P < 0.05$ was considered statistically significant.

RESULTS

Serum NO level

The NO level increased significantly in transplant control group and decreased in the sham-operation group ($P < 0.01$) 4 h after reperfusion. After administration of aminoguanidine (AG), a selective iNOS inhibitor, NO level decreased significantly ($P < 0.01$). The effect of AG (80 mg/kg body weight) was obviously better than that of AG (60 mg/kg body weight) ($P < 0.01$). However, the effect of AG (100 mg/kg body weight) was not better than that of AG (80 mg/kg body weight) ($P > 0.05$) (Table 1).

Serum amylase activity

The amylase activity was higher in the transplant control group than in sham-operation group ($P < 0.01$). After administration of AG, the amylase activity decreased markedly, and the effect of AG (80 mg/kg body weight) was better ($P < 0.01$) (Table 1).

Blood sugar level

The blood sugar level decreased after pancreas transplantation, and was the lowest in the AG (80 mg/kg body weight) group ($P < 0.01$) (Table 2).

Activity of NOS isoforms in pancreas tissue

Four hours after reperfusion, the iNOS activity in pancreas tissue increased significantly ($P < 0.01$), but the cNOS activity had no change ($P > 0.05$). After administration of AG (80 mg/kg body weight), the iNOS activity decreased obviously ($P < 0.01$) while the cNOS activity remained normal (Table 3).

Histology

The pancreas was enlarged and swollen in the transplant control group, and appeared relatively normal in all animals of the sham-operation and AG (80 mg/kg body weight) groups. The microscopic pancreatic injury, as indicated by intracytoplasmic vacuoles, interstitial oedema, polymorphonuclear cell infiltrate, venous congestion, and

Table 2 Blood sugar level 4 h after transplantation (mean $\pm$ SD)

Group	n	Pretransplantation (mmol/L)	Posttransplantation (mmol/L)
Sham-operated control	6	19.6 $\pm$ 1.4 ^a	-
Transplant control group	6	20.1 $\pm$ 2.0 ^a	16.9 $\pm$ 2.0
AG-60 mg/kg body weight	6	19.9 $\pm$ 1.5 ^a	16.8 $\pm$ 1.1
AG-80 mg/kg body weight	6	19.8 $\pm$ 1.7 ^a	14.2 $\pm$ 0.9 ^b
AG-100 mg/kg body weight	6	20.5 $\pm$ 1.6 ^a	15.1 $\pm$ 1.8 ^c

^a $P > 0.05$, ^b $P < 0.01$ vs AG-60 mg/kg body weight post transplantation; ^c $P > 0.05$ vs AG-80 mg/kg body weight post transplantation.

Table 3 Activity of NOS isoforms in pancreatic tissue 4 h after transplantation (mean $\pm$ SD)

Group	n	cNOS (U/mL)	iNOS (U/mL)
Sham-operation group	6	5.35 $\pm$ 1.01 ^a	1.87 $\pm$ 0.19
Transplant control group	6	5.91 $\pm$ 0.71 ^a	26.59 $\pm$ 5.78 ^b
(AG-80 mg/kg body weight)	6	5.64 $\pm$ 0.97 ^a	2.01 $\pm$ 0.23 ^d

^a $P > 0.05$, ^b $P < 0.01$ vs sham-operation group; ^d $P < 0.01$ vs transplant control group.

local tissue hemorrhage and necrosis occurred 4 h after transplantation (Figure 1A and B). However, none of the samples from the AG (80 mg/kg body weight) group revealed histological evidence of pancreatic injury (Figure 1C and D).

Immunohistochemistry

Four hours after reperfusion, heavily stained specimens from transplant control group were positive for anti-iNOS, while iNOS staining was mainly localized on the endothelium, vascular smooth muscle, and islet cells (Figure 1E and F). No stained anti-iNOS antibody was detected in all specimens from the AG (80 mg/kg body weight) group (Figure 1G and H).

DISCUSSION

A model of pancreas transplantation in rats was established. Four hours after pancreas graft reperfusion, the expression and activity of iNOS on pancreas increased significantly, serum NO level and amylase activity, leading to severe pancreatitis, whereas cNOS remained normal. After administration of AG, the iNOS activity and NO concentration decreased, the toxicity of NO as free radicals was reduced, and the amylase activity decreased markedly. The severity of ischemia/reperfusion injury and postgraft pancreatitis was reduced, protecting the pancreas graft against ischemia/reperfusion injury.

Ischemia/reperfusion injury remains a major problem in pancreas transplantation. During the reperfusion period, endothelial dysfunction, activation of endogenous enzymes, leucocyte recruitment and activation all lead to generation of oxygen-derived free radicals, promote lipid peroxidation and deplete glutathione and other antioxidation compounds, leading to pancreatitis^[4]. Contradictory results about the role of NO in pancreatic ischemia/reperfusion have been reported^[5]. NO may lose an electron to form nitrosonium cation (NO⁺), which can combine with the

superoxide radicals to form peroxynitrite (ONOO⁻), a highly active free radical with fierce cytotoxicity^[5]. In pathological conditions, significant activation of iNOS by the release of inflammatory cytokines, such as tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β), can increase NO concentration^[6]. It was reported that endogenous NO is involved in formation of pancreatic edema in L-arginine-induced acute pancreatitis by increasing the vascular permeability and protein extravasation^[7]. Treatment with L-NAME significantly reduces amylase activity and edema formation in the pancreas. A study about severe acute pancreatitis has shown a positive correlation between serum NO level and the number of adherent leucocytes^[8]. The expression of iNOS is correlated to changes in the pancreatic histomorphology^[9-12]. The expression of iNOS during reperfusion following pancreatic ischaemia contributes significantly to the development of acute pancreatitis^[13]. Vasoactive mediators, such as bradykinin, platelet activating factor, endothelin and NO, participate in the development of pancreatic microcirculatory failure. Recently, in drug-induced pancreatitis models, some researchers found that there is a correlation among NF-kappaB activation, serum amylase, reactive oxygen species level and tissue damage, suggesting that NF-kappaB and iNOS play a key role in the pathogenesis of acute pancreatitis^[14-16]. After treatment with antioxidants or NOS inhibitors, the levels of myeloperoxidase, serum amylase and NO, as well as iNOS activities are decreased significantly, and the pancreatic inflammation is improved^[14,15]. Ma *et al*^[16] found that the expression of NF-kappaB and iNOS in peritoneal macrophages is significantly higher in rats with severe acute pancreatitis, and anti-inflammatory agents decrease the expression of TNF-alpha, IL-1 and NO in peritoneal macrophages, reducing the severity of pancreatitis. In Folch-Puy's experiment, infusion of a contrast medium into the pancreatic duct could result in an inflammatory process characterized by increased lipase levels in plasma and edema as well as increased myeloperoxidase activity in pancreas, suggesting that activation of NF-kappaB is correlated with iNOS expression in pancreatic cells^[17]. It was reported that ischemia/reperfusion provokes severe acute necrotizing pancreatitis with a high mortality rate and leads to systemic inflammatory reaction due to the activation of cytokine cascade and iNOS, indicating that NO overproduction by iNOS corresponds with the apoptotic process in the pancreas and the lung^[18,19]. In a study on ischemia/reperfusion injury, Duchon found that calcium overload is associated with NO generation, and their combination leads

Figure 1 Histology displaying pancreatic injury (A, B) and no pancreatic injury (C, D), while immunohistochemistry showing positive anti-iNOS (E, F) and no stained anti-iNOS (G, H) in different groups.

to collapse of mitochondrial membrane potential followed by cell death^[20]. It was reported that after administration of selective iNOS inhibitors, the iNOS activity and NO concentration are decreased significantly and the severity of pancreatitis is reduced^[21]. However, some experiments indicate that NO could activate guanylate cyclase, reduce the activity of platelets and inflammatory cells, relax smooth muscle, and dilate blood vessels^[22,23]. Therefore, NO can remit the vasospasm of grafts and decrease the occurrence of vascular crisis. Supplement of NO for donors during reperfusion of pancreatic isografts seems to prevent organ injury because NO attenuates leukocyte-dependent tissue injury^[20]. Thus, it remains debatable whether the increased production of NO due to pancreas transplantation is beneficial or detrimental to the tissue.

Based on the findings of this study and present reports, it is very likely that NO plays a dual role in ischemia/reperfusion injury of pancreas. During the early reperfusion period, NO, under the charge of cNOS, can improve postischemic reperfusion. With the prolongation of reperfusion time, NO depletion results in failure of microcirculation, during which supplement of NO or NOS substrate could protect microcirculation against failure^[24]. When reperfusion is prolonged (more than 4 h),

activation and excessive expression of iNOS due to the release of inflammatory agents such as TNF- α , IL1- β , result in a considerable increase in NO concentration, and the toxic effect of NO as a free radical leads to the development of graft pancreatitis^[25]. Therefore, administration of selective iNOS inhibitors can not only reduce the toxicity of NO as a free radical, but also retain vasodilatation effect, and protect the graft against pancreatitis. Aminoguanidine (AG) is a mechanism-based inactivator of NOS isoforms and exhibits a marked specificity for the inactivation of its inducible isoform, which proceeds through multiple pathways of covalent modification of the iNOS protein and heme residue at the active site^[26].

At present, some experiments demonstrated that in the transplanted islets, iNOS and toxic NO are produced due to infiltration of inflammatory cells into islets and production of proinflammatory cytokines (such as TNF- α , IL1- β), and an excessive production of NO is deleterious to pancreas β -cells^[27-29].

In conclusion, selective iNOS inhibitor, aminoguanidine as a free radical, has a protective effect on pancreas transplantation in rats by inhibiting NO and reducing toxicity.

COMMENTS

Background

Pancreas transplantation is frequently complicated by acute pancreatitis, largely due to ischemia/reperfusion injury. During the reperfusion period, nitric oxide (NO) may form peroxynitrite (ONOO⁻), a highly active free radical, and has a fierce cytotoxicity. However, NO can significantly dilate blood vessels and remit the vasospasm of grafts, protecting pancreas graft from thrombosis due to transplantation. Therefore, NO plays an ambivalent role in ischemia/reperfusion injury during pancreas transplantation. However, contradictory results about the role of NO in pancreatic ischemia/reperfusion injury have been reported. It remains debatable whether the increased production of NO due to pancreas transplantation is beneficial or detrimental to the tissue.

Research frontiers

Based on the findings of this study and recent reports, it is very likely that NO plays a dual role in ischemia/reperfusion injury of pancreas. During the early reperfusion period, NO under the charge of cNOS, could improve pancreas perfusion. With the prolongation of reperfusion time, NO depletion could result in failure of microcirculation, during which supplement of NO or NOS substrate can protect microcirculation against failure. When reperfusion is prolonged, activation of iNOS due to the release of inflammatory agents, such as tumor necrosis factor α and interleukin-1 β , can increase NO concentration. The toxic effect of NO as a free radical can lead to graft pancreatitis. Hence, administration of selective iNOS inhibitors can reduce the toxicity of NO, and protect graft against pancreatitis.

Innovations and breakthroughs

We established a model of pancreas transplantation in rats. Administration of selective iNOS inhibitors could not only reduce the toxicity of NO as a free radical, but also retain vasodilatation effect, and protect graft against pancreatitis. Aminoguanidine (AG) is a mechanism-based inactivator of NOS isoforms and exhibits a marked specificity for the inactivation of its inducible isoform, which proceeds through multiple pathways of the iNOS protein and heme residue at the active site. Our data also suggest that blood sugar level in AG group was much lower than that in transplant control group, indicating that the selective iNOS inhibitor, AG, has a protective effect on pancreas transplantation.

Applications

Pancreas transplantation can give IDDM additional pancreas to take the place of its own, which has lost the function of insulin secreting. Pancreas regulates insulin secretion, and maintains the blood glucose level. At present, nothing else could achieve this object. Factors influencing pancreas functions following transplantation include graft pancreatitis and rejection which are difficult to treat with a poor prognosis. In this study, after administration of selective iNOS inhibitor AG, the iNOS and amylase activity and NO concentration were decreased, the toxicity of NO as a free radical was reduced. The severity of ischemia/reperfusion injury and postgraft pancreatitis was reduced, protecting the graft against pancreatitis.

Terminology

Inducible nitric oxide synthase (iNOS): NO is synthesized from L-arginine by nitric oxide synthase (NOS). iNOS does not express at normal conditions, and produces NO several orders greater than cNOS and may have a more important pathological role. Aminoguanidine (AG) is a mechanism-based inactivator of NOS isoforms and exhibits a marked specificity for the inactivation of its inducible isoform, which proceeds through multiple pathways of the iNOS protein and heme residue at the active site.

Peer review

This study investigated the effect of inducible nitric oxide synthase inhibitor, aminoguanidine, on pancreas transplantation, showing its scientific and clinical values.

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