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Insulin Attenuates Gene Expression of Inducible Nitric Oxide Synthase during Hemorrhagic Shock in Rats

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Abstract.
Besides being a metabolic hormone, insulin plays key role in other biological functions such as anti-inflammatory response. However, this property of insulin has not been extensively studied. Mounting evidence from clinical studies indicate that insulin therapy could significantly reduce systemic inflammatory responses under various conditions that could otherwise lead to organ failure or death. Since it is believed that this effect could at least in part be through the regulation of nitric oxide (NO) levels, we investigated the effect of insulin on gene expression of inducible nitric oxide synthase (iNOS) during hemorrhagic shock in rats. Hemorrhagic shock was induced by withdrawal of blood from the femoral vessels and the subjects were resuscitated using fluids with or without insulin. Blood samples were extracted to measure mean arterial pressure, arterial blood gas, glucose levels, and NO levels. In addition,iNOS gene expression and hepatic injury analyses were performed using extracted liver tissue. While arterial pressure and blood glucose levels revealed a significant difference in insulin-treated samples compared to the controls before termination, the gas analysis and plasma NO levels did not have significant differences.However, iNOS gene expression was significant decreased in the insulin-treated population indicating the anti-shock property of insulin. Liver injury scores by hepatic injury severity scoring were also in correlation with the iNOS gene expression results. In conclusion, this study demonstrates that insulin could improve hemodynamic index, suppress iNOSgene expression in liver tissue of rodent hemorrhagic shock, and hence reduce hepatic injury pathologically. This is suggestive of a cost-effective method to prevent progression to irreversible shock and reduce mortality.

Keywords: hemorrhagic shock, insulin, iNOS

Introduction

Nitric Oxide (NO), an important signaling molecule in neuronal cells and the immune system, is produced in cells, andcan penetrate the cellular membrane affecting the adjacent cells.Thoughits half-life*in vivo* is only a few seconds,NO easily diffuses through plasma membrane because of its solubility in both aqueous and lipid media.It can cause smooth muscle relaxation and vasodilation through its reaction with the enzyme guanylyl cyclase, in turn lead to production of intracellular cyclic guanosine monophosphate[1],or tissue damage by formingperoxynitriteon reacting with superoxide anions, resulting in DNA fragmentation and lipid oxidation.

NOis produced by nitric oxide synthase (NOS), the expression of which is regulated by the hypoxic state of the tissue in a species- and organ-dependent manner[2].NOS can be classifiedinto inducible NOS (iNOS) and constitutive NOS, which includes neuronal NOS (nNOS) and endothelial NOS (eNOS). Inducible NOS is expressed in macrophages, leukocytes, Kupffer cells, vascular endothelial cells, hepatic cells, and alveolar cells[3],and is known to besuppressed by glucocorticoid, tumor necrosis factor-α antagonist, interleukin-1 receptor, and tyrosine kinase inhibitor [4].While low concentration of NO generated by eNOSisbeneficial for the endothelium and organ function, high concentration of NO generated upon iNOSinduction results in endothelial dysfunction, excessive vasodilation, extravasation, and tissue injury.

Expression of iNOS is generally remarkable under conditions of various shocks leading to excessive production of NOand in correlation with descent of blood pressure[5].Many studies have shown that iNOS expression in internal organs increased during hemorrhagic shock in rat model[6, 7]. It is suggested that excessNOgenerated through iNOS expression could play a key role in the development of refractory hemorrhagic shock andnon-responsiveness to fluid resuscitation and various vasopressors [8].

Insulin is known to have effects oniNOS expression in addition tothe down regulation of plasma glucose level. A study has shown that macrophage NO production and iNOS mRNA expression were elevated in streptozotocin-induced diabetic rats and insulin administration reduced NO production [9]. Another study revealed that addition of insulin to cytokine and lipopolysaccharide-treated skeletal muscle cells reduced NO production [10].

Hemorrhagic shock results in failure of multiple organs due to iNOS gene expression. Hence, suppression of iNOS expression is a potential treatment to hold progression of organ failure and increase survival during hemorrhagic shock. As insulin shows a promising effect in suppressingiNOS gene expression, weinvestigateits effect under hemorrhagic shock in rat models.

Method

Subjects and study design

Twenty-fiveSprague-Dawley male rats weightingbetween 300 and 400 grams were used as subjects in this study. They were randomly assigned to three groups namely control 1, control 2, and experimental groups and subjected to different resuscitation fluids during the experiment. While Lactated Ringer's solutionwas used for the control 1 group, which included 10 rats, Lactated Ringer's solution with 20% glucose was used for the control 2 group with 5 rats. The resuscitation fluid of the experimental groupof 10 ratsincluded Lactated Ringer's solution, 20% glucose, and 6 units/kg regular porcine insulin.

Procedure

Anesthesia was induced by a gas system using isofluranealong with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (15 mg/kg), and maintained with intravenous sodium pentobarbital (5 mg/kg/hr) and pancuronium (1mg/kg). Subsequently, orotracheal intubation was performed with a 16-gauge cannula and mechanical ventilation was applied using a rodent ventilator. Cannulations to the femoral vein and artery were done through dissection and 2 ml blood per 100 gweight of the subject was withdrawn for hemorrhagic shock induction. After maintenance of hemorrhagic shock for 1 hour, fluid resuscitation was performed with different resuscitation fluids (10 ml/kg)for the three groups as described above. Finally, the subjects were sacrificed after an observation period ofanother 90 minutesand the liver tissuesextracted.

Data collection

Mean arterial pressure was measured continuously with blood pressure monitor (Dynascope DS-5100E, Japan)as hemodynamic parameter, while gas and glucose levels of the arterial blood were measured using a blood gas analyzer (ABL 2000, Copenhagen) and a portable glucometer (Accu-check, Germany), respectively. Plasma NO including nitrate and nitrite was measured with a commercial kit using Griess reagent and were expressed inμmol/L.Band intensity of iNOSgene was measured by an image densitometer (Bio-Rad, USA) after reverse transcriptase-polymerase chain reaction was performed (PCT-200 Peltier Thermal Cycler, USA) using extracted liver tissue samples to determine the expression levels. Furthermore, a pathologist reviewed the hematoxylin-eosin stained tissue after fixation and embedding of liver tissue with hepatic injury severity scoring, which was used in previous study [11] and included steatosis, portal inflammation, spotty necrosis, ballooning degeneration, and capsular inflammation.

Data Analysis

Data were analyzed through Mann-Whitney and Kruskal-Wallis analysis using SPSS package. The statistical significance threshold was set as 0.05.

Results

Mean arterial pressure (MAP)

MAPs during baseline period were 78.00 ± 14.12 mmHg in control 1 group, 77.80 ± 15.11 mmHg in control 2 group, and 83.60 ± 24.60 mmHg in experimental group, showing no significant difference. MAPs for three groups were 47.40 ± 3.20 mmHg, 46.00 ± 2.35 mmHg, and 49.00 ± 7.66 mmHgduring the induction of hemorrhagic shock, and 56.70 ± 15.17 mmHg, 53.60 ±7.16 mmHg, and 53.00 ±7.40 mmHg during the shock period in respective order, with no significant difference. MAPs before termination period were measured at 58.00 ± 25.17 mmHg, 70.20 ± 15.94 mmHg, and 80.44 ± 17.85 mmHg respectively, showing significant difference between control 1 and experimental groups (P=0.043).

Arterial blood gas analysis

There was no significant differencein the values of pH, pCO_2, PO_2, and HCO_3 between control 1, control 2, and experimental groups during baseline and termination periods(Table 1).

Table 1. Comparison of arterial blood gas analysis.

		Groups	
	Control 1	**Control 2**	**Experimental**
Baseline			
pH	7.35± 0.14	7.40± 0.12	7.38± 0.06
pCO_2(mmHg)	36.06± 14.88	35.14± 11.51	37.01± 9.62
PO_2(mmHg)	91.92± 29.72	79.50± 24.04	95.42± 29.58
HCO_3(mEq/L)	18.58± 3.17	20.62± 2.01	20.91± 2.92
Termination			
pH	7.30± 0.11	7.29± 0.14	7.34± 0.09
pCO_2(mmHg)	37.60± 21.77	39.94± 8.76	40.10± 7.14
PO_2(mmHg)	69.68± 31.15	71.72± 19.34	87.59± 24.22
HCO_3(mEq/L)	16.96± 6.34	18.52± 3.30	21.23± 4.05

Blood glucose level

While blood glucose levels during baseline period were 345.30 ± 117.29 mg/dL in control 1 group, 374.80 ± 124.82 mg/dL in control 2 group, and 361.20 ± 50.74 mg/dL in experimental group, measurements during resuscitation period read 464.22 ± 136.98 mg/dL, 423.80 ± 172.59 mg/dL, and 443.38 ± 88.88 mg/dL in respective groups, thus showing no significant difference. However, blood glucose levels before termination period were found to be 237.50 ± 97.88 mg/dL, 432.00 ± 162.05 mg/dL, and 97.13 ± 51.42 mg/dL in control 1, control 2 and experimental groups respectively, showing significant difference (P=0.002).

Plasma NO level

Plasma NO levels measured before termination period were 28.21 ± 5.37 μmol/L in control 1 group, 32.48 ± 5.22 μmol/L in control 2 group, 26.70 ± 6.52 μmol/L in experimental group, showing no significant difference (P=0.210).

iNOS gene expression of liver tissue

Band densities of iNOS gene expression of liver tissuewere 18350.53 ± 6610.26 l in control 1 group, 9148.33 ± 4081.75 in control 2 group, 3874.07±2313.40 in experimental group, showing significant difference (P=0.017)(Figure 1).

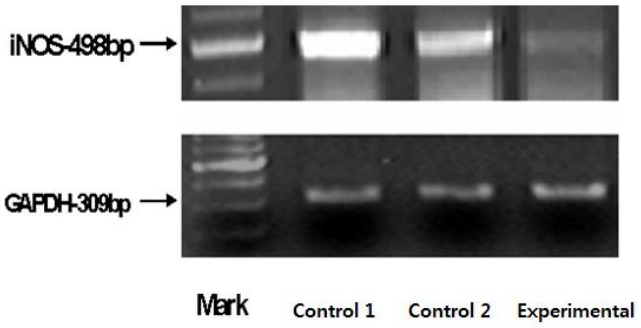

Figure1.band densities of iNOS gene expression in rat liver tissues during hemorrhagic shock.

Pathologic score of liver tissue

Liver injury scores by hepatic injury severity scoring were 5.50 ± 1.84 in control 1 group, 4.00 ± 1.00 in control 2 group, 2.70 ± 1.89 in experimental group, showing significant difference (P=0.009).

Discussion

Insulin has simply been known as a metabolic hormone that controls blood glucose levels. However, its other roles including anti-inflammatory action are still under the investigation. Clinical studies have shown that intensive insulin treatment reduced mortality and systemic inflammatory responses during cardiac surgeries[12],in burn victims[13],and medical intensive care units[14].In critically ill patients, it is suggested that such a therapy could protect the endothelium, likely in part via inhibition of iNOS-induced excessive release of NO and hence contribute to the prevention of organ failure and death [15]. In addition, insulin is known to have an antioxidant effect, inhibiting expression of NADPH oxidase thatproduces superoxide radicals [16].

As insulin has the potential to inhibit the progression of shock to an irreversible stage, we investigated its effect on iNOS expression and in turn NO production during hemorrhagic shock. As expected, the gene expression analysis reveals a reduction of iNOS gene expression[17]. This is further verified by the hepatic injury scores of liver samples from insulin-treated rats compared to the control groups.

However, there are few limitations to this study. Firstly, we did not measure insulin levels directly from the subjects. Dextrose infusion in the resuscitation fluid mighthave increased endogenous insulin release, resulting in an additional anti-shock effect of insulin in control 2 group. Secondly, insulin infusion in the resuscitation fluid of the experimental group resulted in a rapid decrease of blood glucose, although dextrose infusion was performed simultaneously. This abrupt variation in glucose level could have given another form of shock such as hypoglycemia. Lastly, we did not measure potassium levels, which could influence the experimental conditions since insulin infusion can typically result in hypokalemia.

In conclusion, this study has demonstrated that insulin could improve hemodynamic index, suppress iNOSgene expression in liver tissue of rodent, and reduce hepatic injury pathologically under hemorrhagic shock, suggesting that this in turn could inhibit the progression to irreversible shock. As insulin is inexpensive and easily used in clinical settings, anti-shock effect of insulin can be expected to improve survival rate.

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