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VASOACTIVE DRUGS ENHANCE PANCREATIC ISLET BLOOD FLOW, AUGMENT INSULIN SECRETION AND IMPROVE GLUCOSE TOLERANCE IN FEMALE RATS

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Running title: Islet blood flow regulation

Abbreviations used in this paper: ABF, adrenal blood flow; ACE, angiotensin-converting enzyme; Ang II, angiotensin II; ELISA, enzyme-linked immunosorbent assay; IBF, islet blood flow; IPGTT, intraperitoneal glucose tolerance test; KBF, kidney blood flow; NO, nitric oxide; PBF, pancreatic blood flow; RAS, renin-angiotensin system.

Abstract

Pravastatin, irbesartan and captopril are frequently used in the treatment of patients with type 2 diabetes. These drugs are also exerting beneficial metabolic effects, causing an improved glucose tolerance in the patients but the precise nature of the mechanisms by which this is achieved remains elusive. To this end, we have now studied whether these drugs influence insulin secretion *in vivo* through effects on islet blood perfusion. Captopril (3 mg/kg), irbesartan (3 mg/kg), pravastatin (0.5 mg/kg) were injected intravenously to anesthetized female Wistar rats. Blood flow rates were determined by a microsphere technique. Blood glucose concentrations were measured with test reagent strips and serum insulin concentrations with ELISA. Pancreatic blood flow was markedly increased by pravastatin ($p<0.001$), captopril ($p<0.05$) and irbesartan ($p<0.01$). Pancreatic islet blood flow was significantly and preferentially enhanced after the administration of captopril ($p<0.01$), irbesartan ($p<0.01$) and pravastatin ($p<0.001$). Renal blood flow was enhanced significantly, pravastatin ($p<0.01$), irbesartan ($p<0.05$), captopril ($p<0.01$). Captopril and pravastatin also enhanced late phase insulin secretion and positively influenced glycemia upon intraperitoneal glucose tolerance tests. In conclusion, the present study suggests that a local pancreatic RAS and pravastatin treatment may be selectively controlling pancreatic islet blood flow, augmenting insulin secretion and thereby improving glucose tolerance. Our findings indicate significant gender-related differences in the vascular response to these agents. Since statins and RAS inhibitors are frequently used by diabetic patients, the antidiabetic actions of these drugs previously reported might in part occur through the beneficial direct islet effects shown here.

Introduction

Type 2 diabetes is increasing in the Western world and is seen in ever-younger age groups. The disease is characterized not only by hyperglycemia, but also insulin resistance with attendant dyslipidemia, hypertension and endothelial dysfunction. These aberrations may result in cardiovascular and renal disease, and adversely impact the prognosis of diabetic patients in terms of longevity and quality of life. Gender differences also exist, with more pronounced negative effects of diabetes on the lipid profile and blood pressure in women compared with men [1-2]. The diabetic state may also impede the cardioprotective actions normally conferred by estrogen [3]. To improve prognosis of diabetic patients, several risk factors other than glycemia (*e.g.* hypertension, albuminuria, dyslipidemia etc) need to be treated as well. Diabetic patients whose risk factor profile is well controlled are thus commonly being treated with one or more antidiabetic drugs, a lipid lowering statin and an ACE inhibitor or angiotensin receptor antagonist against hypertension and albuminuria.

The systemic renin-angiotensin system (RAS) plays a crucial role in the regulation of arterial blood pressure. In the past few years, it has become increasingly clear that local RAS also exist in various tissues, implying that high local levels of angiotensin II (Ang II) might exert paracrine influences on neighboring cells [4]. In the pancreas of several species, mRNA encoding angiotensinogen and renin, as well as substantial levels of angiotensin II, have been detected [4]. Ang II has been shown to adversely influence pancreatic and islet blood flow through vasoconstrictive effects [4].

Interestingly, both ACE inhibitors or angiotensin receptor antagonists and certain statins have been reported to decrease the risk of developing diabetes in large clinical trials [5]. However, the mechanisms behind these antidiabetic effects remain elusive. We recently showed stimulatory effects of these drugs on pancreatic islet blood flow in male rats [6]. In this paper, we aimed at evaluating the influence of ACE inhibition, angiotensin receptor antagonism and pravastatin treatment on pancreatic and islet blood flow, as well as on glycemia and insulin concentrations in normal female rats.

Materials and methods

Animals and drugs

Female Wistar rats (ScanBur, Sollentuna, Sweden), weighing 300 to 350 g, were used in all experiments. The animals had free access to pelleted food (Type R34; ScanBur, Sollentuna, Sweden) and tap water at all times. All experiments were approved by the local animal ethics committee at Uppsala University. Captopril and pravastatin were graciously donated by Bristol-Myers Squibb Company (New York, NY). Irbesartan was generously given by Sanofi-Synthelabo (Paris, France).

Blood flow measurements

The experiments were performed according to a protocol previously described in detail [7]. The animals were anesthetized with an intraperitoneal injection of thiobutabarbital sodium (120 mg/kg body weight; Inactin™, Research Biochemicals International, Natick, MA), and placed on a heated operating table to maintain body temperature. Polyethylene catheters were inserted into the ascending aorta, via the right common carotid artery, and into the left femoral artery. The catheter in the aorta was connected to a pressure transducer (model PDCR 75/1, Druck Ltd., Groby, Leicestershire, UK) to allow constant monitoring of the mean arterial blood pressure. After the blood pressure was stable, the animals were injected intravenously with 1 ml of saline, 1 ml of pravastatin (0.5 mg/kg), 1 ml of irbesartan (3 mg/kg) or 1 ml of captopril (3 mg/kg). All these substances were dissolved in saline. Ten minutes later, $1.5\text{--}2.0 \times 10^5$ non-radioactive microspheres (IMT, Stason Labs., Irvine, CA), with a mean diameter of 10 μm , were injected during 10 s via the catheter with its tip located in the ascending aorta. An arterial blood sample was collected from the catheter in the femoral artery 5 s before the microsphere injection, and this process continued for a total of 60 s.

The exact withdrawal rate in each experiment was determined by weighing the sample. Additional arterial blood samples were obtained and later analyzed for hematocrit, blood glucose, and serum insulin concentrations (see below). After the animals were killed by cervical dislocation, the whole pancreas and both adrenal glands, as well as a 100-mg slice of the left kidney (including both cortex and medulla) were collected. The microsphere contents in these organs were determined separately. The organs were treated with a freeze-thawing technique[8], which enabled the visualization and localization of the microspheres from either the endocrine or the exocrine parenchyma of the pancreas. This was achieved by applying a microscope (Zeiss MB6; Leica AB, Stockholm, Sweden) equipped with both bright and dark field illumination. The former type of illumination allowed us to count the microspheres, whereas the latter enabled localization of the microspheres from either the endocrine or

exocrine parenchyma [8]. The number of microspheres in the islets and exocrine tissue was counted as previously described in detail [8]. The microsphere contents of the adrenal glands were used as a control to confirm an even distribution of the microspheres in the arterial circulation. The microsphere content of each of the arterial reference samples was determined by transferring the samples to glass microfiber filters and counting the microspheres in a stereomicroscope.

The blood flow rates were calculated according to the formula $Q_{\text{org}} = Q_{\text{ref}} \times N_{\text{org}}/N_{\text{ref}}$, where Q_{org} denotes organ blood flow (mL/min), Q_{ref} denotes withdrawal rate of the reference sample (mL/min), N_{org} denotes number of microspheres in the organ and N_{ref} denotes the number of microspheres in the reference sample.

Intraperitoneal glucose tolerance test

An intraperitoneal glucose tolerance test (IPGTT) was performed simultaneously as the blood flow measurements. The animals were injected intraperitoneally with a 30 % (wt/vol) D-glucose solution (2 g glucose/kg body wt). Blood samples were drawn from the tail vein immediately before and 10, 30, 60, and 120 min after glucose administration. Area under the curve for the IPGTT was determined by computerized image analysis.

Measurement of glucose and insulin concentrations

Blood glucose concentrations were measured with test reagent strips (Medisense, Solna, Sweden) based on the glucose oxidase method and serum insulin concentrations were monitored with ELISA kit (Rat Insulin ELISA, Mercodia, Uppsala, Sweden). The insulin ELISA showed 98 % recovery upon addition. Coefficients of variation were 3.4 % within assay, 2.2 % between assays, and 3.9 % in total assay.

Statistical analysis

All values are given as means \pm SEM for the number of experiments given. Each experiment represents one animal and experiments were performed on different days. Statistical comparisons were made with one-way analysis of variance (ANOVA) (SigmaStat; SSPD,

Erfart, Germany) in conjunction with Bonferroni's post-hoc test. $P < 0.05$ was deemed statistically significant.

Results

Effects of captopril, irbesartan and pravastatin on blood flow in female rats. Intravenous injection of pravastatin (0.5 mg/kg BW), captopril (3 mg/kg BW) and irbesartan (3 mg/kg BW) significantly enhanced PBF (Figure 1a), the action of the statin being the most pronounced. IBF was also significantly augmented by all three substances (Figure 1b). Only irbesartan and captopril preferentially increased IBF, *i.e.*, relatively more than PBF (IBF as % of PBF; Figure 1c). Renal blood flow was also substantially increased after administration of all three agents (Figure 2a). Irbesartan and pravastatin augmented adrenal blood flow significantly, whereas captopril did not (Figure 2b). Interestingly and quite unexpectedly, the vascular response to pravastatin was more pronounced than that occurring after giving agents interfering in RAS in all organs tested. Additionally, this effect occurred exclusively in female rats and not in our previously studied male rats. This indicates significant vasoactive actions of pravastatin that have hitherto not received much attention but that may be involved in the anti-atherogenic effects of the statin. This finding may also have implications for gender-specific differences in the vasoactive responses to these drugs described previously [9, 10].

Blood glucose concentrations, serum insulin levels, and mean arterial blood pressure.

None of the treatments influenced non-stimulated serum insulin levels significantly (Figure 3a). Blood glucose concentrations also did not differ between any of the treatment groups in the non-stimulated state (Figure 3b). In contrast, during an IPGTT late phase insulin secretion was substantially augmented (Figure 3a) and glucose tolerance was significantly improved (Figure 3b) in animals treated with captopril or pravastatin. Separate calculations of the total amount of insulin secreted during the 120 min IPGTT (area under the curve) revealed that insulin secretion in rats treated with pravastatin ($p < 0.01$) or captopril ($p < 0.001$) were significantly higher than in control rats receiving solvent only (Figure 3c). As shown in Figure 3d, there was also a trend towards a decrease in post-load glycemia, expressed as AUC, although this difference did not attain statistical significance. No effects on mean arterial

blood pressure (averaging 110 mmHg) were detected by any of the treatments given (data not shown).

Discussion

Type 2 diabetes is increasing in the Western world and is seen in ever-younger age groups [11]. We can expect this to lead to momentous public health problems, especially in the form of premature cardiovascular morbidity. In terms of quantity, the most important complications of type 2 diabetes are macroangiopathies, *i.e.* myocardial infarction and stroke, which cause some 70 % of the deaths related to type 2 diabetes [12, 11]. In contrast to microangiopathies (*e.g.* nephropathy and retinopathy), where the causal relation to hyperglycemia is well supported, the link between hyperglycemia and macroangiopathy is uncertain, at least in terms of the possibility of reducing macrovascular morbidity solely by reducing hyperglycemia [12]. To improve prognosis of diabetic patients, several other risk factors (*e.g.* hypertension, albuminuria [a biomarker of generalized endothelial dysfunction], dyslipidemia etc) need to be treated as well [12]. The disease is characterized not only by hyperglycemia, but also insulin resistance with attendant dyslipidemia and increased levels of circulating free fatty acids. Diabetic patients whose risk factor profile is well controlled are thus being treated with one or more antidiabetic drugs, a lipid lowering statin and an ACE inhibitor or angiotensin receptor antagonist against hypertension and albuminuria. These drugs are also exerting beneficial metabolic effects, causing an improved glucose tolerance in patients, but the precise nature of the mechanisms by which this is achieved remains elusive [5]. We have now studied whether these drugs influence islet blood perfusion, glycemia and insulin levels *in vivo* in female rats.

Our present study shows a substantial increase in pancreatic blood flow, pancreatic islet blood flow, renal and adrenal blood flow by pravastatin treatment. Along with these beneficial effects, preferentially increasing islet blood flow, insulin secretion was augmented (preferentially late phase secretion) and glycemia was improved following an IPGTT. Future work, involving diabetic animals, will reveal whether stimulation of islet blood flow by similar means is sufficient to reverse experimental hyperglycemia. The antithrombotic and anti-inflammatory effects by pravastatin may play a role in enhancing endothelium-dependent vasodilation [13], lending support to our present findings of increased blood flow after pravastatin administration. Conversely, dyslipidemia – a salient feature in type 2 diabetes - is

known to impair endothelium-mediated vasodilatation [14] and impaired endothelial function has been shown to result in diminished capillary recruitment [15]. Pravastatin has known beneficial effects on endothelial function [16-18]; therefore, pravastatin may significantly enhance selective tissue perfusion by restoring endothelial function. Beyond simply lowering cholesterol, it also exerts beneficial antithrombotic actions by inhibiting platelet aggregation and promoting local nitric oxide (NO) synthesis [19-21]. Thus, it is possible – albeit not proven – that increased local NO formation might be involved in the salutary effects of pravastatin noted in the current study. There appear to be important differences between different statins in this regard, as pravastatin is in a class of its own in terms of preventing diabetes [22, 23, 5]. Pravastatin is not metabolized by hepatic CYP-450 enzymes, shows very little binding to proteins, and is markedly hydrophilic. Whether these characteristics or other attributes, such as anti-inflammatory actions, underlie pravastatin's anti-diabetogenic effect remains to be shown. Other mechanisms are conceivable, for instance direct effects on the endocrine pancreas. *In vitro* studies have shown that lipophilic statins (simvastatin) inhibit glucose-stimulated insulin secretion by blocking voltage-gated L-type Ca^{2+} channels in insulin-secreting β cells, whereas pravastatin has no such adverse effect [24]. Furthermore, pravastatin can prevent inflammation and rejection of transplanted islets of Langerhans [25], all actions that would contribute to improved glucose tolerance.

We recently reported that administration of pravastatin in male Wistar rats increased only pancreatic islet blood flow [6]. Our present results indicate qualitative and quantitative gender-specific differences between male and female rats in terms of vascular responses to pravastatin and RAS interfering agents, which may be due to the vasoactive features of estrogen or other influences conferred by female gender. For instance, while in male rats captopril was the most potent stimulus of islet blood flow [6], pravastatin evoked the strongest stimulation of islet blood flow in the presently studied female rats (Fig 1B). Likewise, captopril was a stronger stimulator of islet blood flow in male than in female rats, compared to irbesartan. Additionally, non-stimulated serum insulin levels were heightened by irbesartan and captopril in male rats [6] but not influenced by these drugs in the present work on female rats. It is becoming increasingly appreciated that gender differences exist in terms of susceptibility to and mortality from a variety of vascular diseases [26, 10, 27]. Estrogen is known to increase NO production primarily through upregulation of endothelial NO synthase (eNOS) gene expression [28]; also a gender difference exists in basal NO release by rabbit aorta [29, 30]. Alternatively, it is possible that estrogen may function by upregulating eNOS

and/or enhancing production of eNOS-derived NO [31-33], thereby producing a vasodilatory effect [27].

The systemic RAS plays a crucial role in the regulation of arterial blood pressure. In the past few years, it has become increasingly clear that local RAS also exist in various tissues, implying that high local levels of angiotensin II (Ang II) might exert paracrine influences on neighboring cells [34-38]. In the pancreas of several species, mRNA encoding angiotensinogen and renin, as well as substantial levels of angiotensin II, have been detected [34-38]. Ang II has been shown to adversely influence pancreatic and islet blood flow through vasoconstrictive effects [39, 40]. Also, high affinity binding sites for Ang II were recently localized specifically to islet β -cells by double immunostaining and Ang II was found to block glucose-stimulated insulin secretion, an event fully reversible by losartan [39, 35, 40]. It is thus conceivable that pancreatic Ang II, locally produced by intrinsic RAS, may adversely influence insulin secretion *in vivo*, either directly by suppressing β -cell insulin exocytosis or indirectly through inhibitory effects on islet blood perfusion [39, 35, 40]. This may be of particular importance in diabetic patients since hypertension is markedly overrepresented in these individuals [23, 5], and angiotensinogen expression seems to be upregulated by hypertension [23, 5]. Hence, many diabetic patients are treated with ACE inhibitors or angiotensin receptor antagonists against their hypertension or as part of a renal protection strategy. About 15 years ago, it became clear that estrogen can influence the RAS [41-47], by down-regulating renin, ACE, and Ang II receptor expression [48]. An interesting review has reported that gender and sex hormones affect the components of the RAS by multifarious mechanisms [9]. In general, estrogen inhibits Ang II action and has been shown to attenuate vascular contractility [49]. Irbesartan is an Ang II receptor antagonist, characterized by high selectivity and insurmountable blockade of the type 1 Ang II receptor. In our study, intravenous injection of irbesartan or the ACE inhibitor captopril induced robust increases in blood flow not only in the pancreas and pancreatic islets, but also (as expected) in the kidney. Pancreatic blood flow was preferentially diverted to the endocrine part, *i.e.* the islets. This can be explained by a local RAS operative in the pancreas [4]. Such a system enables local production of Ang II, yielding Ang II concentrations much higher than those encountered in peripheral blood. Some publications show that islet blood flow can be suppressed by locally produced Ang II [12, 35], lending support to our present findings. Recent evidence also suggests that certain Ang II receptor antagonists have the capacity to enhance vascular vasodilatation [12, 35], which might be another mechanism contributing to our current findings. Additionally, it was recently demonstrated that the Ang II receptor antagonist

losartan selectively improved glucose-induced insulin release and insulin biosynthesis in islets from spontaneously diabetic mice [50]. Oral losartan treatment delayed the onset of diabetes, and improved glucose tolerance in the diabetic mice, but did not affect the insulin sensitivity of peripheral tissues, suggesting that Ang II receptor antagonism improves β -cell function and glycemia in young type 2 diabetic mice. It was also recently shown that another potent Ang II receptor antagonist, candesartan, when given orally over several weeks increased β -cell mass, increased staining intensity of insulin, and decreased oxidative stress markers in β -cells [51]. Candesartan treatment also reduced fibrosis in and around the islets and prevented the loss of endothelial cells in islets, indicating that candesartan partially prevents deterioration of glucose tolerance by providing protection against progressive β -cell damage in diabetic mice.

In conclusion, we have shown that vasoactive drugs that are frequently given to diabetic patients and improve their glucose tolerance have beneficial effects by enhancing pancreatic islet blood flow, augmenting insulin secretion and improving glycemia. Our findings moreover indicate qualitative gender differences in the vascular response to these drugs, which might be related to the ability of estrogen to produce vasodilatation. The results may prove useful in tailoring gender-specific treatment strategies that improve islet function, contributing to an improved glycemic control in diabetic patients or in subjects at risk of developing glucose intolerance.

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Legends to figures

Figure 1: Enhancement of pancreatic blood flow by captopril, irbesartan, and pravastatin.

Following i.v. injection of captopril (3 mg/kg BW), irbesartan (3 mg/kg BW), or pravastatin (0.5 mg/kg BW) into normal female rats, rates of blood perfusion in the whole pancreas (**a**), pancreatic islets (**b**), and the fraction of total pancreatic blood flow contributed by islets (**c**) were measured using a microsphere technique. Bars represent means \pm SEM for 8 independent experiments. *, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, for chance differences *vs* controls using ANOVA.

Figure 2: Enhancement of renal blood flow and adrenal blood flow by captopril, irbesartan, and pravastatin.

Following i.v. injection of captopril (3 mg/kg BW), irbesartan (3 mg/kg BW), or pravastatin (0.5 mg/kg BW) into normal female rats, rates of blood perfusion in the kidneys (**a**) and adrenals (**b**) were measured using a microsphere technique. Bars represent means \pm SEM for 8 independent experiments. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, for chance differences *vs* controls using ANOVA.

Figure 3: Captopril and pravastatin augment insulin secretion and improve glycemia during an IPGTT.

Following i.v. injection of captopril (3 mg/kg BW) or pravastatin (0.5 mg/kg BW) into normal female rats, serum insulin concentrations (**a**) were measured with ELISA and blood glucose levels (**b**) with test reagent strips during a simultaneous IPGTT over 120 min. Changes in insulin (**c**) and glucose (**d**) responses are expressed as area under the curve integrated over 120 min. Values represent means \pm SEM for 6 independent experiments. *, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, for chance differences *vs* controls using ANOVA.

Figure 1a

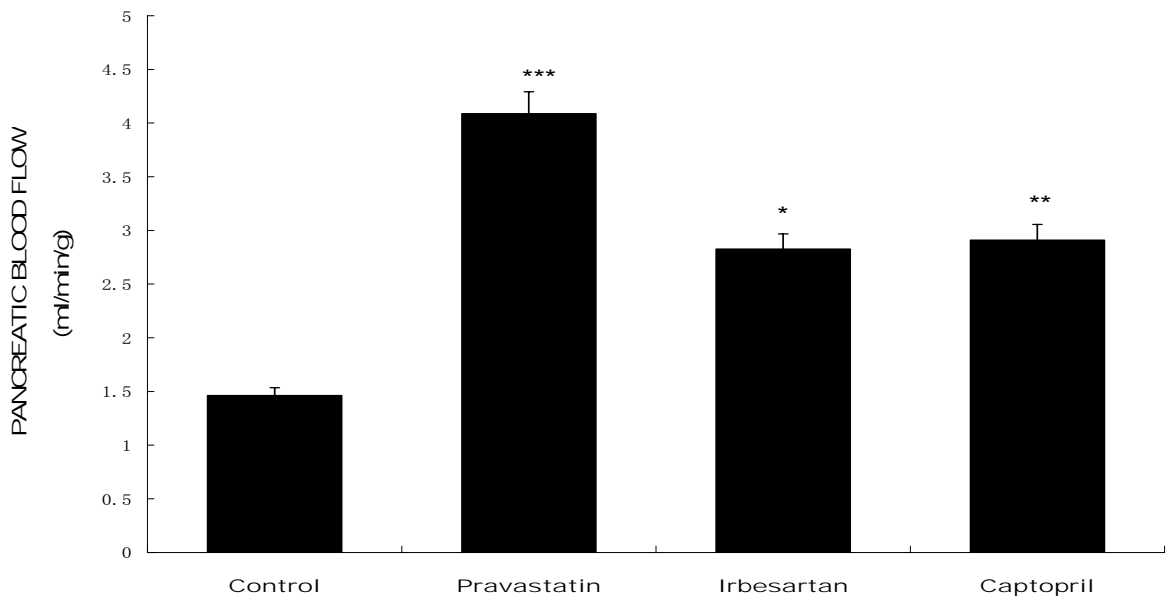


Figure 1b

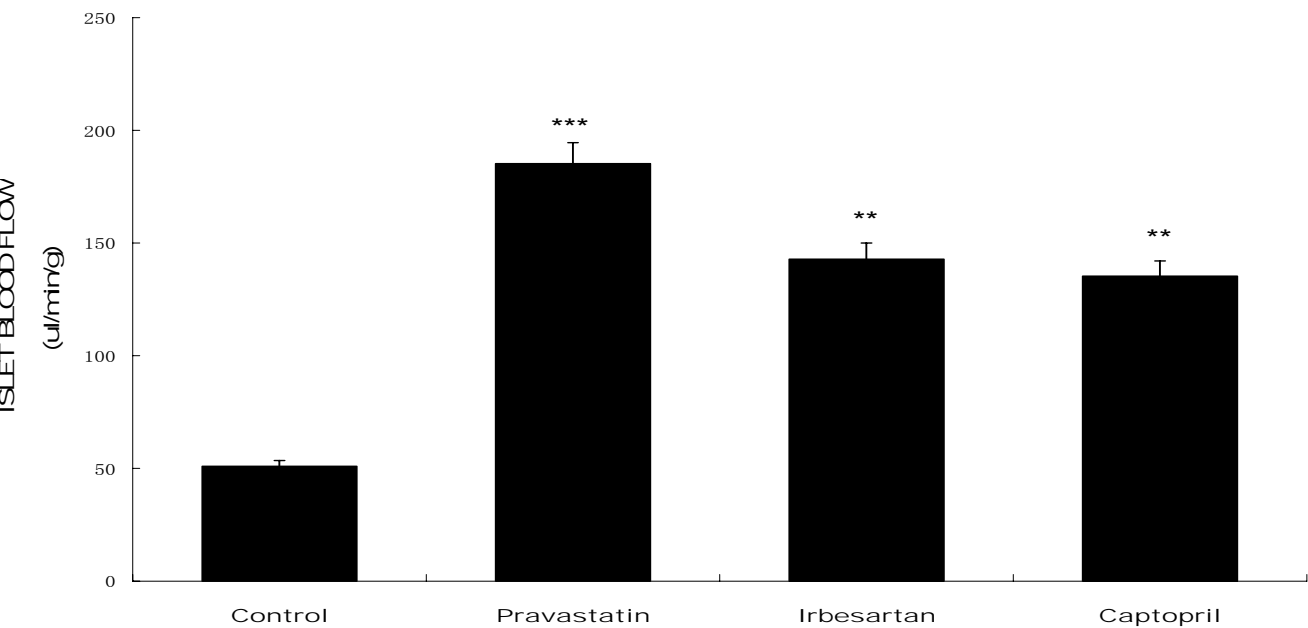


Figure 1c

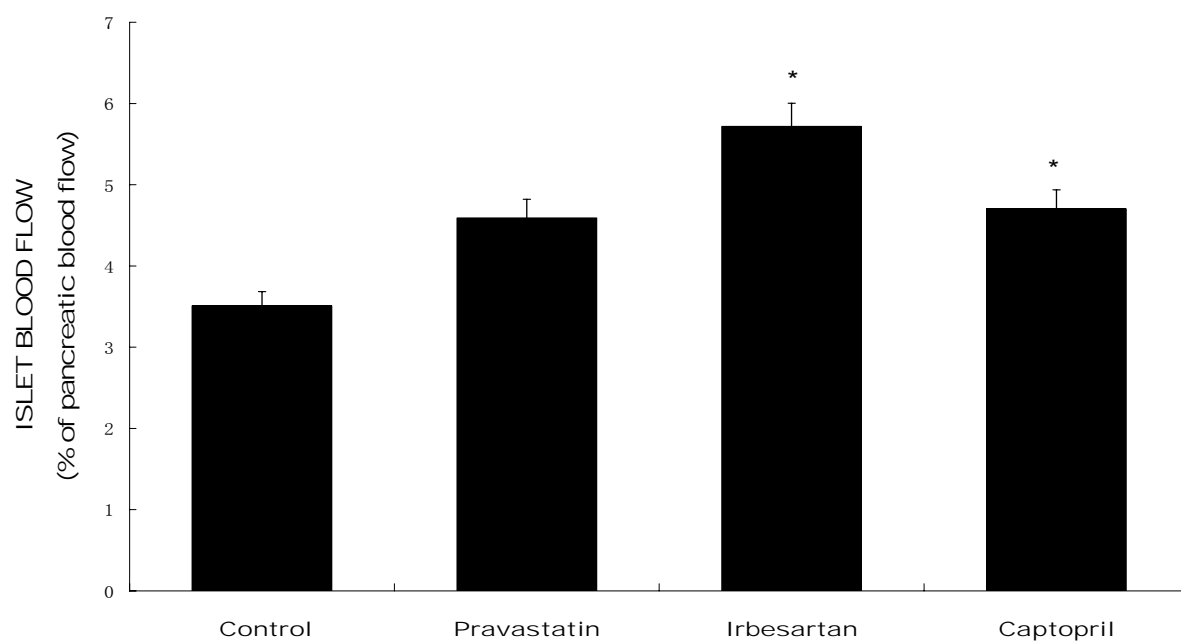


Figure 2a

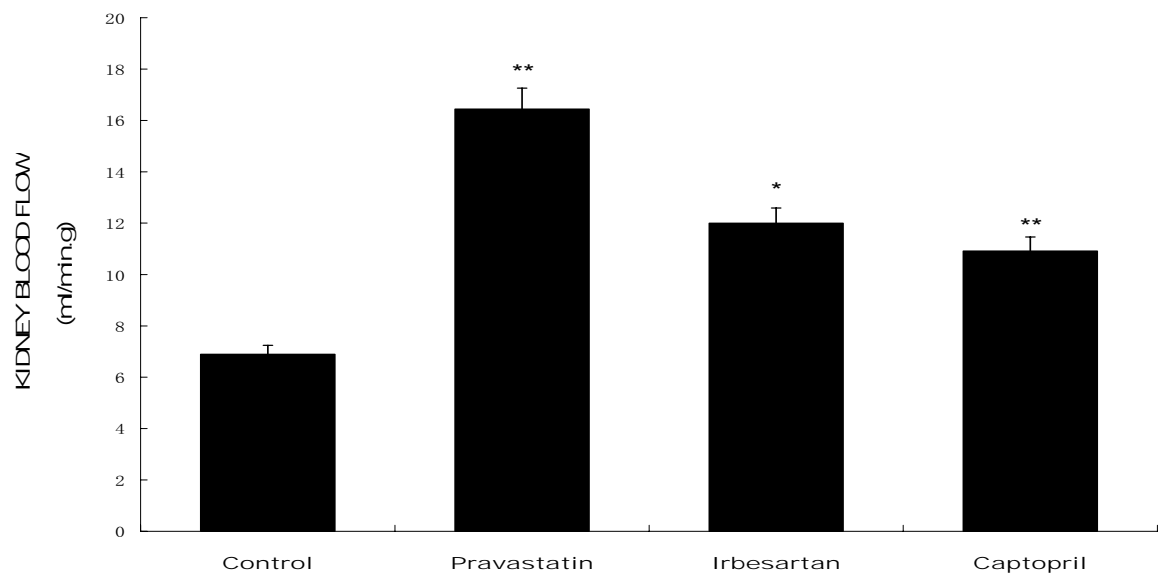


Figure 2b

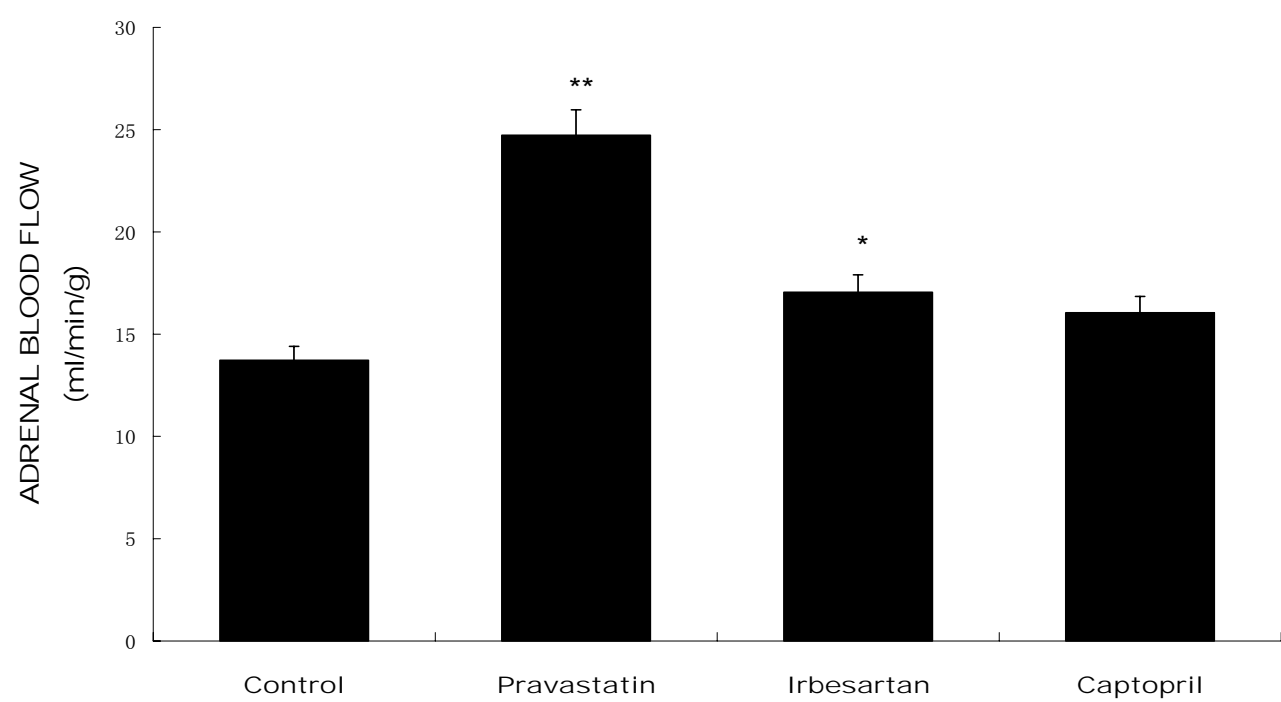


Figure 3a

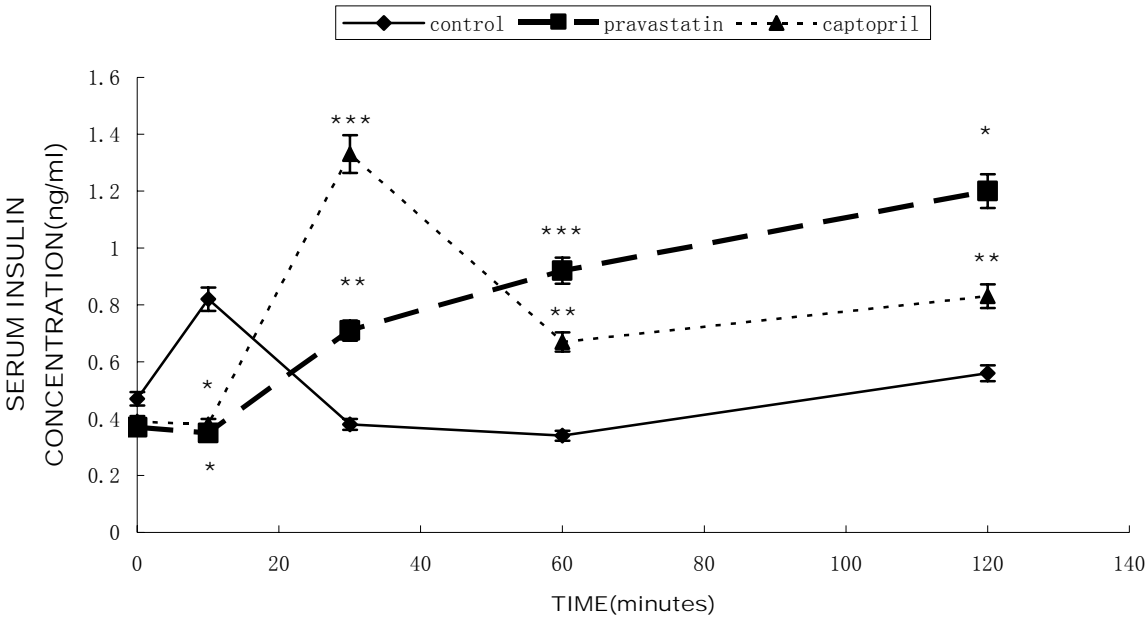


Figure 3b

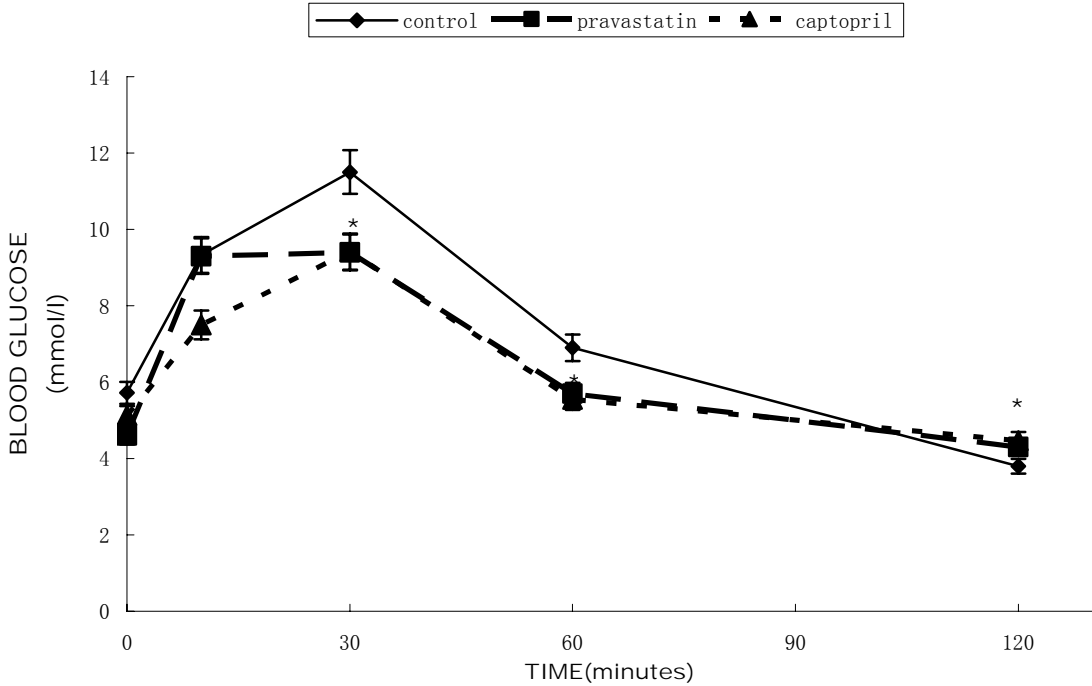


Figure 3c

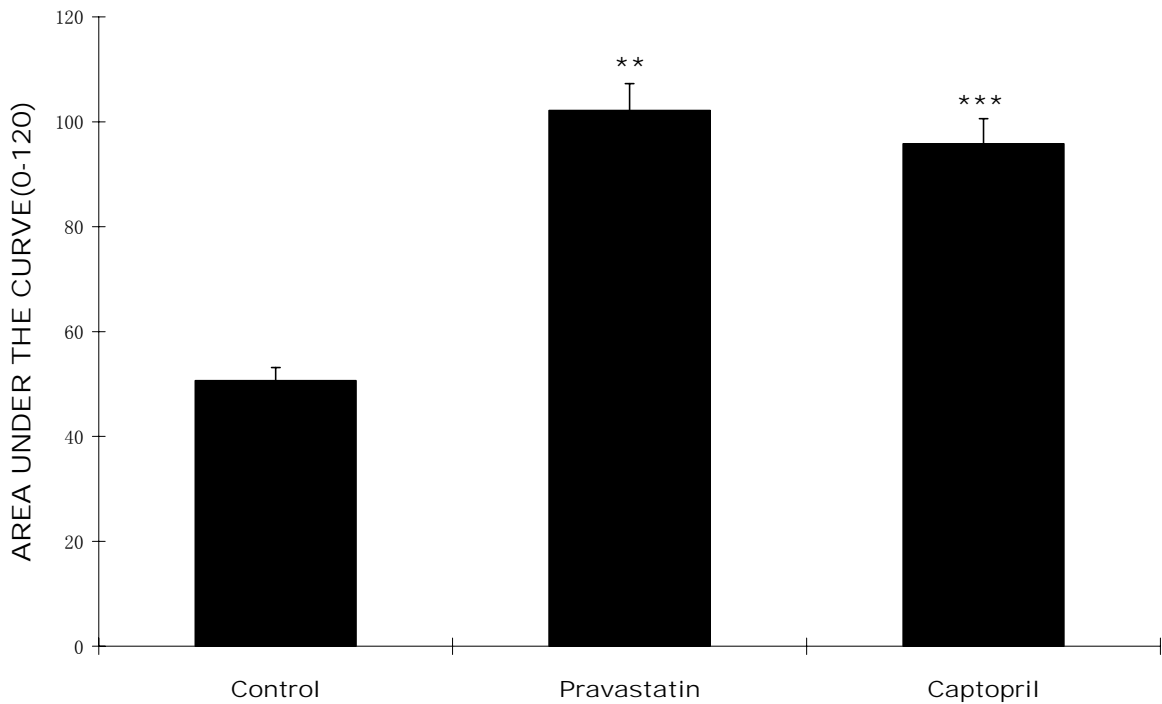


Figure 3d

