Depressed modulation of oxygen consumption by endogenous nitric oxide in cardiac muscle from diabetic dogs

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Zhao, Gong, Xiaoping Zhang, Xiaobin Xu, Michael S. Wolin, and Thomas H. Hintze. Depressed modulation of oxygen consumption by endogenous nitric oxide in cardiac muscle from diabetic dogs. Am J Physiol Heart Circ Physiol 279: H520–H527, 2000.—Our previous study indicated that nitric oxide (NO)-dependent coronary vasodilation was impaired in conscious dogs with diabetes. Our goal was to determine whether modulation of O2 consumption by NO is depressed in canine cardiac muscle after diabetes. Diabetes was induced by injection of alloxan (40–60 mg/kg iv), dogs were killed after diabetes was induced (4–5 wk), and the cardiac muscle from the left ventricle was cut into 15- to 30-mg slices. O2 uptake by the muscle slices was measured polarographically with a Clark-type O2 electrode. S-nitroso-N-acetylpenicillamine decreased O2 consumption in normal and diabetic tissues (10–4 M, 61 ± 7 vs. 61 ± 8%, P > 0.05). Bradykinin (10–4 M)- or carbachol (CCh, 10–4 M)-induced inhibition of O2 consumption was impaired in diabetic tissues (51 ± 6 vs. 17 ± 4% vs. 19 ± 3%, respectively, both P < 0.05 compared with normal). The inhibition of O2 consumption by kininogen or kallikrein was depressed in diabetic tissues as well. In coronary microvessels from diabetic dogs, bradykinin or ACh (10–5 M) caused smaller increases in NO production than those from normal dogs. Our results indicate that the modulation of O2 consumption by endogenous, but not exogenous, NO is depressed in cardiac muscle from diabetic dogs, most likely because of decreased release of NO from the vascular endothelium.

Diabetes mellitus is usually associated with coronary artery disease. Since the discovery of NO, a number of studies have revealed impaired endothelium-dependent vasorelaxation in diabetic animals (7, 11, 18, 21). Our recent study (54) has indicated that reflex, NO-dependent coronary vasodilation is depressed in conscious dogs after the development of alloxan-induced diabetes, suggesting a reduced ability of coronary blood vessels to produce NO. There is also evidence for mitochondrial dysfunction in the heart from the diabetic rat and mouse (10, 24, 29, 38, 46). On the other hand, a case report (19) showed that there was no evidence that diabetes was associated with a systematic abnormality of respiratory chain function.

There is a local kinin system in mammalian vascular tissue, and endothelial cells are capable of producing kinins (31). Our recent studies (52, 53) have indicated that kininogen, the protein precursor of kinins, and kallikrein, an enzyme responsible for kinin formation,
increase NO production in canine coronary microvessels in vitro. Furthermore, kininogen also decreases O₂ consumption in isolated canine cardiac muscle, which is mediated by a NO- and a kinin-dependent mechanism, because the inhibitory effect of kininogen on O₂ consumption was blocked by L-NNA, an NO synthase inhibitor, and HOE-140, an antagonist of bradykinin B₂ receptors (53).

The goals of the present study were to determine whether: 1) the modulation of O₂ consumption by NO in cardiac muscle from diabetic dogs is depressed; 2) stimulation of local kinin production decreases O₂ consumption in cardiac muscle from normal and diabetic dogs; and 3) NO production is altered in coronary microvessels isolated from diabetic dogs.

METHODS

The protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the Guiding Principles for the Use and Care of Laboratory Animals of the National Institutes of Health and the American Physiological Society.

Induction of Diabetes in Conscious Dogs

Mongrel dogs (weighing 22–29 kg, n = 13) were chronically instrumented for measurements of systemic hemodynamics. The control hemodynamics were recorded 10–14 days after the surgery. After the control recording, the dogs were divided into two groups: one normal (n = 7) and the other diabetic (n = 6). In the diabetic group, the dogs were injected with alloxan monohydrate (40–60 mg/kg iv) over 1 min. Alloxan was prepared as a 5% solution in citrate buffer buffered with 10 mol/l HEPES (pH 7.4).

Methods of O₂ Consumption

Only dogs with blood glucose levels below 200 mg/dl at injection of alloxan, a second injection of alloxan was given. In some dogs whose blood glucose after alloxan injection, in other dogs whose blood glucose were measured in coronary microvessels (20 mg wet wt) from normal and diabetic dogs. S-nitroso-N-acetylpenicillamine (SNAP) was used as a NO donor in the present study, because it decomposes spontaneously in solution to release NO. SNAP at concentrations of 10⁻⁷–10⁻⁴ M (final concentration) was added to the chambers in a cumulative manner.

Inhibition of O₂ consumption by endogenous NO in cardiac muscle from normal and diabetic dogs. Inhibition of O₂ consumption by carbachol. Carbachol (CCh) activates muscarinic receptors on the endothelium to stimulate NO production. After recording baselines, cumulative concentrations of CCh at 10⁻⁷ to 10⁻⁴ M were added in the presence or absence of L-NNA (10⁻⁴ M). In another experiment, atropine at a concentration of 10⁻⁵ M was added before CCh was added at a concentration of 10⁻⁴ M.

Inhibition of O₂ consumption by bradykinin. Bradykinin stimulates kinin B₂-receptors on the endothelium to stimulate NO production. After baselines were recorded, cumulative concentrations of bradykinin at 10⁻⁷ to 10⁻⁴ M were added in the presence or absence of L-NNA (10⁻⁴ M). In another experiment, HOE-140 at a concentration of 10⁻⁵ M was added after adding SNAP at 10⁻⁴ M.

Preparation of Cardiac Muscle Slices and Measurement of O₂ Consumption

The preparation of cardiac muscle slices and the measurement of O₂ consumption were as described previously (48 and 49). In brief, normal and diabetic dogs were euthanized with an overdose of pentobarbital sodium (50 mg/kg iv), and the hearts were obtained immediately. Cardiac muscle was isolated from the left ventricle (free wall only). The muscle was freed of epicardium, endocardium, connective tissue, fat, and large arteries and was cut into 15–30 mg segments at room temperature. The muscle segments were incubated in Krebs solution (mol/l: 118 NaCl, 4.7 KCl, 1.5 CaCl₂, 25 NaHCO₃, 1.1 MgSO₄, 1.2 KH₂PO₄, and 5.6 glucose) at 37°C for 2 h and bubbled continuously with 20% O₂–5% CO₂–75% N₂.

O₂ uptake by muscle slices was measured polarographically with a Yellow Spring Instrument (YSI) apparatus consisting of a YSI model 5300 biological O₂ monitor and a Clark-type O₂ electrode (YSI model 5331). O₂ consumption studies were performed at 37°C in a stirred 10-ml bath (YSI model 5301) containing 3 ml of air-saturated Krebs solution buffered with 10 mol/l HEPES (pH 7.4).

The effect of the agents on O₂ consumption was measured. Tissue respiration (expressed as nmol O₂·min⁻¹·g of wet tissue⁻¹) was calculated as the rate of decrease in O₂ concentration after the addition of muscle segments, assuming an initial [O₂] of 224 nmol/ml. The typical observation time for each drug concentration was 5 min, and new muscle segments were used for each agent examined. Sodium cyanide (1 mol/l) was added at the end of each respiration measurement to confirm that the change in O₂ consumption was from mitochondrial sources.

Experimental Protocols

Inhibition of O₂ consumption by exogenous NO in cardiac muscle from normal and diabetic dogs. S-nitroso-N-acetylpenicillamine (SNAP) was used as a NO donor in the present study, because it decomposes spontaneously in solution to release NO. SNAP at concentrations of 10⁻⁷–10⁻⁴ M (final concentration) was added to the chambers in a cumulative manner.

Inhibition of O₂ consumption by endogenous NO in cardiac muscle from normal and diabetic dogs. Inhibition of O₂ consumption by carbachol. Carbachol (CCh) activates muscarinic receptors on the endothelium to stimulate NO production. After recording baselines, cumulative concentrations of CCh at 10⁻⁷ to 10⁻⁴ M were added in the presence or absence of L-NNA (10⁻⁴ M). In another experiment, atropine at a concentration of 10⁻⁵ M was added before CCh was added at a concentration of 10⁻⁴ M.

Inhibition of O₂ consumption by bradykinin. Bradykinin stimulates kinin B₂-receptors on the endothelium to stimulate NO production. After baselines were recorded, cumulative concentrations of bradykinin at 10⁻⁷ to 10⁻⁴ M were added in the presence or absence of L-NNA (10⁻⁴ M). In another experiment, HOE-140 at a concentration of 10⁻⁵ M was added after adding SNAP at 10⁻⁴ M.

Preparation of Cardiac Muscle Slices and Measurement of O₂ Consumption

The myocardium was cut into small pieces, chopped with a MacIlwain tissue chopper, and suspended in ice-cold phosphate-buffered saline. The resulting suspension was homogenized using a Sorvall Omnimixer for 50 s at maximum speed. The homogenate was poured over a 100-μm nylon mesh sieve, and microvessels (including arterial, venous, capillary, and lymphatic vessels; diameters <70 μm) were isolated with the method we used previously (45, 46). NO production, stimulated by ACh and bradykinin (concentration 10⁻⁵ M) and a calcium ionophore (A-23187, 10⁻⁶ M), was measured in coronary microvessels (20 mg wet wt) from normal and diabetic dogs. N⁴-nitro-L-arginine methyl ester (l-NAME) was used to block NO production in vitro, and atropine and HOE-140 were used to block muscarinic receptors and kinin B₂-receptors, respectively. The formation of NO was measured as nitrite using the Griess reaction and a spectrophotometer at 540 nm absorbance. Nitrite production is expressed as picomoles per milligram of wet tissue. Car-
Left ventricular end-diastolic pressure, from chronically instrumented dogs were used as the normal group.

Chemicals

Alloxan, A-23187, ACh, atropine, bradykinin, carbachol, and kallikrein were purchased from Sigma (St. Louis, MO). L-NNA was purchased from Aldrich (Milwaukee, WI). Kininogen was purchased from Seikagaku Kogyo. HOE-140 was generously provided by Hoechst-Marion-Roussel.

Data Analysis

All data are presented as changes from baseline (means ± SE). The statistical significance of differences was determined with a paired t-test for the response to each agent, and differences between normal and diabetic groups were evaluated with repeated measures analysis of a variance in $O_2$ consumption studies. When the ratio of F values indicated a significant difference, significance was determined using a Tukey’s test. The changes in NO production from canine coronary microvessels were determined with Student’s t-test. Changes were considered significant at $P < 0.05$. A computer-based software package (GBStat) was used for statistical analysis.

RESULTS

Changes in Hemodynamics, Arterial Blood Gases, and Arterial Glucose Levels in Conscious Dogs After Development of Alloxan-Induced Diabetes

Arterial glucose levels were significantly increased, and mean arterial blood pressure was significantly decreased in conscious dogs after the development of alloxan-induced diabetes. Other hemodynamic indexes were not altered after the development of diabetes. There were no significant changes in arterial blood gases. Table 1 shows systemic hemodynamics, arterial blood gases, and arterial glucose levels in conscious dogs before and after the development of diabetes (4–5 wk after injection of alloxan).

Inhibition of $O_2$ Consumption by Endogenous and Exogenous NO

Inhibition of $O_2$ consumption by endogenous NO in cardiac muscle from normal and diabetic dogs. There was no significant difference in baseline $O_2$ consumption in cardiac muscle from normal and diabetic dogs (204 ± 26 vs. 238 ± 29 nmol · min$^{-1}$ · g$^{-1}$, $P > 0.05$).

In cardiac muscle from normal dogs, SNAP resulted in a concentration-dependent decrease in $O_2$ consumption, as shown in Fig. 1. For example, 10$^{-4}$ M of SNAP decreased $O_2$ consumption by 61 ± 7%. SNAP still decreased $O_2$ consumption in cardiac muscle from diabetic dogs, and this decrease was not statistically different from that in cardiac muscle from normal dogs (Fig. 1).

$O_2$ consumption by endogenous NO in cardiac muscle from normal and diabetic dogs. Inhibition of $O_2$ consumption by CCh. In cardiac muscle from normal dogs, CCh caused a concentration-dependent decrement in $O_2$ consumption (Fig. 2) that was blocked by L-NNA. The inhibition of $O_2$ consumption by the highest dose of CCh (10$^{-4}$ M) was also blocked by atropine (~48 ± 4% vs. ~2.5 ± 10%, $P < 0.05$). The inhibition of $O_2$ consumption by CCh was attenuated in cardiac muscle from diabetic dogs, as shown in Fig. 2. The highest dose of CCh only decreased $O_2$ consumption by 19 ± 3% ($P < 0.05$, compared with normal).

Inhibition of $O_2$ consumption by bradykinin. In cardiac muscle from normal dogs, bradykinin decreased $O_2$ consumption in a concentration-dependent manner, which was blocked by L-NNA, as shown in Fig. 2. The inhibition of $O_2$ consumption by bradykinin at a concentration of 10$^{-5}$ M was blocked by HOE-140 (10$^{-5}$ M) as well (~51 ± 6% vs. ~2 ± 10%, $P < 0.05$). The inhibition of $O_2$ consumption by bradykinin was attenuated in cardiac muscle from diabetic dogs. The highest concentration of bradykinin (10$^{-4}$ M) decreased $O_2$ consumption only by 17 ± 4% from the baseline (Fig. 2, $P < 0.05$, compared with normal).

Inhibition of $O_2$ consumption by kininogen and kallikrein. Kininogen decreased $O_2$ consumption in cardiac muscle in a concentration-dependent manner, and this was blocked by L-NNA and HOE-140, as shown in Fig. 3. Kallikrein also resulted in a decrease in $O_2$ consumption in cardiac muscle, which was blocked by...
L-NNA and HOE-140 (Fig. 4). The inhibition of O\textsubscript{2} consumption induced by kininogen or kallikrein was depressed in cardiac muscle from diabetic dogs (Figs. 3 and 4).

**NO Production From Coronary Microvessels**

There was a lower basal nitrite production in coronary microvessels from diabetic dogs (n = 5) compared with that from normal dogs (n = 7, 54 ± 5 vs. 75 ± 4 pmol/mg tissue, P < 0.05). In coronary microvessels from normal dogs, ACh, bradykinin, and A-23187 caused an increase in nitrite production. The actual changes in nitrite production in coronary microvessels from both normal and diabetic dogs are shown in Fig. 5. Nitrite production in coronary microvessels from diabetic dogs in response to ACh, bradykinin, and A-23187 was significantly smaller when compared with that from normal dogs. The increase in nitrite production in response to ACh, bradykinin, and A-213187 was blocked by L-NAME. The increase in nitrite production by bradykinin and A-23187 was blocked by HOE-140 in coronary microvessels from normal dogs as well as diabetic dogs.

**DISCUSSION**

The most important result of the present study is that there is depressed modulation of O\textsubscript{2} consumption by endogenous NO in cardiac muscle from diabetic dogs. This is not due to the dysfunction of mitochondria after diabetes, as evidenced by the preserved modulation of O\textsubscript{2} consumption by exogenous NO. Another important finding of the present study is that kallikrein, an enzyme responsible for kinin production, results in a significant decrease in O\textsubscript{2} consumption in cardiac muscle from normal dogs through a kinin- and NO-mediated mechanism, as shown by the blockade of the modulatory effect of kallikrein on O\textsubscript{2} consumption by HOE-140 and l-NNA.

Before the discovery of NO, Granger et al. (14) first observed that the injury of neoplastic cells by cytokinotoxin-activated macrophages was due to inhibition of mitochondrial respiration. Granger and Lehninger (13)
demonstrated that the major sites of inhibition of mitochondrial respiration were complex I and complex II in the electron transport chain. Drapier and Hibbs (8) showed that activated macrophages inhibited aconitase in tumor cells by interaction with the iron-sulfur group. It is now known that arginine is the precursor for inhibition of mitochondrial respiration, that the activity is blocked by substituted arginine molecules, and that the mechanism of the action appears to be the formation of a nitrosyl complex by NO with the iron-sulfur center of the enzymes, including aconitase, complex I, and complex II (9, 12, 27, 43–45). NO also reversibly inhibits cytochrome-c oxidase (3). It has been reported that peroxynitrite inhibits aconitase rather than NO (17).

In the present study, SNAP decomposing to release NO in the solution caused a decrease in O2 consumption in canine cardiac muscle, indicating a significant inhibitory effect of exogenous NO on tissue respiration. Furthermore, bradykinin or CCh decreased O2 consumption in cardiac muscle from normal dogs, which was blocked by L-NNA, suggesting an endogenous NO-dependent mechanism. The inhibitory effects of bradykinin and CCh on O2 consumption are mediated by kinin and muscarinic receptors, respectively, because HOE-140 and atropine blocked the inhibitory effect of bradykinin and CCh on O2 consumption, respectively. These results are consistent with our previous results (49).

The most striking finding of the present study is that the modulation of O2 consumption by endogenous NO induced by bradykinin and CCh is depressed in cardiac muscle from diabetic dogs, whereas the modulation of O2 consumption by exogenous NO is still preserved. Diabetes mellitus is usually associated with coronary artery disease. Since the discovery of NO, a number of studies (7, 11, 18, 21, 24) have revealed impaired endothelium-dependent vasorelaxation in diabetic animals. Our recent study (54) has indicated that reflex, NO-dependent coronary vasodilation is depressed in conscious dogs after the development of alloxan-induced diabetes. The present results further indicate that there is depressed modulation of O2 consumption by bradykinin and CCh in cardiac muscle from diabetic dogs. Because the modulation of O2 consumption by bradykinin and CCh was blocked by L-NNA, an endogenous NO mechanism appears to be involved.

![Fig. 4. Change in O2 consumption in isolated cardiac muscle by kallikrein (20 U/ml). In normal tissue (n = 7), kallikrein caused a significant decrease in O2 consumption, and this was blocked by HOE-140 and L-NNA, suggesting the mechanisms of kinins and NO. In diabetic tissue (n = 6), 20 U/ml of kallikrein caused a smaller decrease in O2 consumption. Values are means ± SE; *P < 0.05 compared with baseline; #P < 0.05 compared with normal.](image)

![Fig. 5. Bar graphs showing nitrite production in coronary microvessels by ACh (A), bradykinin (B), and A-23187 (C) from normal and diabetic dogs. L-NAME, Nω-nitro-L-arginine methyl ester. ACh, bradykinin, or A-23187 resulted in a significant increase in NO production in coronary microvessels from normal dogs (n = 7; A-23187, n = 5), whereas the same concentration of ACh, bradykinin, or A-23187 caused the smaller increase in NO production in coronary microvessels from diabetic dogs (n = 5). Bradykinin-and A-23187-induced increase in NO production was blocked by HOE-140, suggesting the involvement of bradykinin B2-receptor mechanism. The increase in NO production by ACh was blocked by atropine. Values are means ± SE; *P < 0.05 compared with control; #P < 0.05 compared with normal.](image)
There is evidence for mitochondrial dysfunction in the hearts from diabetic animals (10, 24, 29, 38). Savabi (38) showed that there were fewer mitochondria in the hearts from diabetic rats and lower O$_2$ consumption rates in isolated mitochondria than those from normal hearts. Tanaga et al. (46) suggested that there was no significant difference in basal O$_2$ consumption by myocardocytes suspended in medium, decreased calcium uptake by mitochondria, and reduced mitochondrial membrane potential from diabetic rats. Our results, however, showed that there was no significant difference in basal O$_2$ consumption by cardiac muscle from normal and diabetic dogs. Most importantly, exogenous NO (SNAP) still resulted in a significant decrease in O$_2$ consumption in cardiac muscle from diabetic dogs, which is not significantly different from the response observed in tissue from normal dogs. Taken together, our results suggest that mitochondrial dysfunction was not observed in cardiac muscle from diabetic dogs. We propose that the depressed modulation of O$_2$ consumption by endogenous NO in cardiac muscle from diabetic dogs is likely due to the inability to produce NO by the vascular endothelium.

A number of studies (4, 20, 28, 34, 47) suggested that superoxide is involved in the endothelial dysfunction in experimental diabetic animals and patients with diabetes because superoxide dismutase and other antioxidants could, at least in part, restore the endothelial dysfunction. However, our present results showed that exogenous NO (SNAP) still exerts inhibitory effects on O$_2$ consumption in the cardiac muscle from diabetic dogs. This suggests that superoxide might play little role in the depressed modulation of O$_2$ consumption in cardiac muscle from diabetic dogs in our experiment.

Direct evidence for the involvement of NO in the modulation of O$_2$ consumption by muscarinic and kinin B$_2$-receptors comes from our observations of NO production from coronary microvessels in response to these agonists. The present results further demonstrate that ACh and bradykinin caused smaller increases in NO production in coronary microvessels from diabetic dogs compared with those from normal dogs. In the present study, the increases in NO production in coronary microvessels from both normal and diabetic dogs in response to the agonists were blocked by L-NAME, indicating that nitrite production reflects the activity of NO synthase in coronary microvessels. Bradykinin- or ACh-induced increase in NO production was blocked by HOE-140 or atropine, respectively, indicating the involvement of the bradykinin B$_2$, or muscarinic receptors. Our results indicate that there is a decreased release of NO from coronary microvessels after the development of diabetes. More recently, we (54) have demonstrated that the protein for endothelial constitutive NO synthase in the aortic endothelium from diabetic dogs is decreased by 66% compared with that from normal dogs.

Our previous results (52) showed that the calcium ionophore, A-23187, released NO from canine coronary microvessels, which is mediated by kinin formation, because HOE-140 (bradykinin B$_2$-receptor antagonist) blocked the increase in NO production by A-23187. The present results confirmed our studies. Furthermore, the same concentration of A-23187 resulted in a smaller increase in NO production in coronary microvessels from diabetic dogs. This indicates that there is a smaller release of NO in response to different agonists in coronary microvessels from diabetic dogs.

The second important finding of the present study is that kallikrein, an enzyme responsible for kinin formation, caused a significant decrease in O$_2$ consumption in canine cardiac muscle. Our recent studies (52, 53) have indicated that kallikrein, the protein precursor of kinins, and kallikrein increase NO production in canine coronary microvessels in vitro. Furthermore, kallikrein also decreases O$_2$ consumption in the isolated canine cardiac muscle, which is mediated by a NO- and a kinin-dependent mechanism, because the inhibitory effect of kallikrein on O$_2$ consumption was blocked by L-NAME and HOE-140 (53). Interestingly, the modulation of O$_2$ consumption by kallikrein and kallikrein was depressed in cardiac muscle from diabetic dogs. Because HOE-140 blocked the modulation of O$_2$ consumption by kallikrein, it is likely that activation of kinin B$_2$-receptors mediates the modulation of O$_2$ consumption by these agents, and the loss of stimulation of endogenous NO production is responsible for the changes observed in cardiac muscle from diabetic dogs.

There are some limitations in our study. First of all, although the present study observed the depressed modulation of O$_2$ consumption by endogenous NO stimulated by bradykinin and CCh in cardiac muscle from diabetic dogs, our results could not determine the exact site(s) responsible for the decreased release of NO. The defect(s) could occur in any step from stimulation of receptors to synthesis of NO from the vascular endothelium. However, the present study suggests the defect probably did not occur on the receptors themselves, because it is unlikely that both kinin B$_2$-receptors and muscarinic receptors were downregulated after diabetes. The second limitation is the diabetic model and species. In those studies showing mitochondrial dysfunction, diabetes was induced by streptozotocin in rats (24, 38, 46) or genetically diabetic mice (11), whereas in our study diabetes was induced by alloxan in conscious dogs. Finally, the present results suggest that the highest concentration of SNAP decreased O$_2$ consumption in cardiac muscle by as high as 60%, much higher than that observed in conscious dogs (35, 41). It should be pointed out that the baseline of O$_2$ consumption in cardiac muscle is very low compared with myocardial O$_2$ consumption in conscious dogs (~6%) (2). In addition to NO, a number of other factors are involved in the regulation of myocardial O$_2$ consumption in vivo, such as adenosine, the working condition of the heart, and the activity of cardiac sympathetic nerves. Therefore, our present results may not be compared with the results from the working heart.

In summary, our study demonstrates that 1) kallikrein causes a decrease in O$_2$ consumption in cardiac muscle from normal dogs, which is mediated by kinin...
and NO mechanisms; and 2) the modulation of O2 consumption by endogenous NO is depressed in cardiac muscle from diabetic dogs, whereas the modulation of O2 consumption by exogenous NO is still preserved. The mechanism responsible for the depressed modulation of O2 consumption by endogenous NO is likely because of the decreased release of NO from the vascular endothelium rather than mitochondrial dysfunction.

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