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INHIBITORS OF NITRIC OXIDE SYNTHESIS AND ISCHEMIA/REPERFUSION ATTENUATE CORONARY VASODILATOR RESPONSE TO PINACIDIL IN ISOLATED RAT HEART

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Evidence indicates that ATP-sensitive potassium channels (K_{ATP}) participate in the metabolic regulation of coronary flow and that this regulation is attenuated when endothelial production of nitric oxide (NO) is blocked. A hypothesis tested in this study was that, in hearts with the impaired NO-pathway, either with an inhibitor or as a result of ischemia/reperfusion, a coronary vasodilator response to K_{ATP} stimulation is impaired as well.

In Langendorff perfused rat hearts, a blocker of NO synthesis (N^{ω}-nitro-L-arginine, L-NOARG, 10 μM) and K_{ATP} inhibitor (glibenclamide, 0.6 μM) reduced the basal coronary flow by 44% and 29%, respectively. Glibenclamide caused a further 25% drop in the flow in L-NOARG perfused hearts.

To determine the respensiveness of coronary resistance vessels to K_{ATP} stimulation and NO, dose-response curves (DRC) for K_{ATP} opener, pinacidil-, and NO-donor, 3-morpholino-syndomine-hydrochloride (SIN-1)-induced increase in coronary flow were constructed, respectively. The pinacidil DRC was shifted to the right by glibenclamide and L-NOARG and to the left by SIN-1 and adenosine. The L-NOARG-induced effect was reversed by L-arginine. The SIN-1 DRC was shifted to the right by glibenclamide and not affected by L-NOARG. Another NO synthesis blocker, L-N^{G}-monomethylarginine (L-NMMA, 50 μM), caused a 43% drop in coronary flow in the untreated hearts and only 24% drop in the hearts subjected to 20 min global ischemia and 40 min reperfusion. The pinacidil DRC obtained at reperfusion showed a 2.3-fold rightward shift as compared to the DRC obtained before ischemia/reperfusion. Similar displacement of the pinacidil DRC was observed also in L-NMMA perfused hearts and in L-NMMA-perfused hearts which were subjected to ischemia/reperfusion. These results indicate that in the isolated rat heart: (1) NO and K_{ATP}, acting simultaneously, participate in the setting of the vasodilator component of the basal coronary flow; (2) The responsiveness of coronary microcirculation to K_{ATP} stimulation is attenuated when endothelial NO-pathway is impaired either pharmacologically or by ischemia/reperfusion.

K e y w o r d s: *isolated rat heart, coronary flow, nitric oxide, ATP-sensitive potassium channel, endothelial dysfunction, ischemia/reperfusion.*

INTRODUCTION

The action of nitric oxide (NO) produced by endothelium (1) and the hyperpolarization of the vascular smooth muscle cells caused by opening of

and placed in an ice-cold perfusion solution. The aorta was cannulated and the hearts were perfused aerobically in the Langendorff mode with a prefiltered (5.0 μM Millipore filter) perfusion fluid (containing, in mmol/l: 118 NaCl; 23.8 $NaHCO_3$; 4.7 KCl; 1.2 KH_2PO_4; 2.5 $CaCl_2$; 1.2 $MgSO_4$; 11 glucose and 0.01 indomethacin, gassed with 95% O_2+5% CO_2 gas mixture giving pH 7.4 and pO_2 530—580 mmHg at 37°C) at a constant pressure of 60 mmHg. In some experiments, the hearts were perfused at at constant flow by means of digital roller pump. A 20 ml compliance chamber along a perfusion line ensured a constant flow. The flow rate was adjusted during the stabilization period to obtain perfusion pressure of 60 mmHg. In these experiments, coronary perfusion pressure was continuously monitored by means of a pressure transducer connected to a side-arm of the aortic cannula. The hearts were enclosed in a small, water-jacketed chamber. The temperature of the perfusate, as well as that of the atmosphere surrounding the heart, was thermostatically controlled to ensure 37°C. The hearts were not stimulated. Coronary flow was determined by collecting and weighing one min samples of the coronary effuent. At the end of the experiment, the atria were trimmed and ventricles were dried to obtain their dry weight.

Drugs and solutions

L-arginine, L-N^G-monomethylarginine (L-NMMA), N^ω-nitro-L-arginine (L-NOARG) and indomethacin were purchased from Sigma, pinacidil and glibenclamide, from Research Biochemicals International (Natick, MA, USA), and 3-morpholino-syndomine-hydrochloride (SIN-1) was a gift form Cassela AG (Grankfurt/Main).

Desired amounts of L-arginine, L-NMMA and L-NOARG were added to the perfusate immediately before use. The other agents were made up as concentrated stock solutions in 96% ethanol (indomethacin), dimethyl sulphoxide (glibenclamide) or the mixture of 1.9% ethanol and 5 mM HCl (pinacidil). Dimethyl sulphoxide and ethanol in the highest concentrations used (0.0025 vol % and 0.019 vol %, respectively) caused no change in coronary flow. The glassware and tubing containing SIN-1 were protected from light.

Experimental design

In general, the hearts in this study were perfused at a constant pressure and changes in coronary flow were monitored. Only some experiments in a protocol 2 (see below) were done in the hearts perfused at a constant flow and therefore changes in coronary perfusion pressure were followed in those hearts. After 30 min of the equilibration perfusion the hearts were assigned to one of the following experimental protocols designed to study:

(1) Contribution of NO and K_{ATP} in the setting of coronary tone in isolated rat heart. The hearts were subjected to 50 min perfusion with NO-synthesis inhibitor, L-NOARG (10 μM) or K_{ATP} inhibitor, glibenclamide (0.6 μM) and changes in coronary flow were followed. In some L-NOARG perfused hearts, glibenclamide was applied to study an effect of simultaneous blockade of NO and K_{ATP}.

(2) Coronary responsiveness to K_{ATP} stimulation and to NO in the hearts in which either NO-pathway or K_{ATP} was inhibited. A control concentration-response curve (DRC) for pinacidil- or SIN-1-induced increase in coronary flow was first constructed. Then, the heart was exposed either to 10 μM L-NOARG or to 0.6 μM glibenclamide for 40 min and the DRC was repeated. In similar way, DRC for pinacidil-induced reduction in coronary perfusion pressure was studied in the untreated and L-NOARG-treated hearts perfused at a constant flow mode. Control experiments demonstrated that DRCs for pinacidil and SIN-1 were fairly superimposable when they were obtained twice in the same control heart (n = 3, for each drug).

(3) The reversal of the L-NOARG induced effects by L-arginine. The control pinacidil DRC was constructed, then one set of the hearts was perfused with L-arginine, 1 mM, for 15 min and the DRC was repeated. The other set was first perfused with L-NOARG for 40 min and the pinacidil DRC was constructed. Then L-arginine was applied and, after a steady coronary flow response had been obtained, the DRC was repeated in the continuing presence of L-NOARG and L-arginine.

(4) The effect of SIN-1 on the coronary responsiveness to pinacidil. After control pinacidil DRC was obtained, the hearts were perfused for 5 min with 0.8 μM SIN-1 (a concentration producing ca 30% increase in coronary flow) and the DRC was repeated in the presence of SIN-1.

(5) Coronary responsiveness to pinacidil in ischemic/reperfused hearts. After the control pinacidil DRC was constructed, the hearts were subjected to 20 min global ischemia followed by 80 min reperfusion and the DRC was repeated. To study a potential additivity of the effects of ischemia/reperfusion and NO-synthesis inhibitor, 50 μM L-NMMA was administered either 30 min before the ischemia or at 40 min of the reperfusion. The pinacidil DRC was obtained just before the ischemia and it was repeated at 80 min of the reperfusion (c.f., *Fig. 5*).

Construction and analysis of the dose-response curves

A concentrated solution of a tested substance in the perfusion buffer was delivered to the aortic cannula, by means of a microprocessor controlled syringe pump. The output of the pump was increased stepwise. In the constant pressure perfused hearts, only after a stable response had been obtained, a final concentration of the drug in the perfusate was calculated. Approximately 6—8 concentrations of the drug were tested to complete each DRC. Data for each DRC were expressed both as the per cent of the basal coronary flow or basal coronary perfusion pressure and as the per cent of the maximum amount of coronary flow increase or perfusion pressure reduction produced by a drug. Then each DRC data were fitted to a logistic equation of the form (28):

$$E = M \times A^p/(K^p + A^p)$$

where E is the observed change in coronary flow produced by the drug, M is the maximum increase in coronary flow or reduction in perfusion pressure, A is the concentration of the drug, K is the EC_{50} value and p is the slope power. From this relationship, M, K and p parameters were computed for each DRC.

Statistics

All data, including those for M, K and p parameters, are expressed as means ± s.e.m. However, for the statistical analysis of K, the pD_2 ($-\log K$) values were used. Significant differences ($P < 0.05$) among groups were calculated by one-way analysis of variance followed by Dunnet's procedure. Paired or non-paired Student's t-test was also used when appropriate.

RESULTS

Role of NO and K_{ATP} in maintaining the basal coronary flow

In the untreated hearts, L-NOARG reduced coronary flow by 43.6 ± 3%, while glibenclamide by 29.4 ± 3% (*Fig. 1*). The administration of glibenclamide to L-NOARD perfused hearts resulted in an additional 24.9% drop in coronary flow. L-NOARG and glibenclamide, caused a small significant reduction in heart rate (*Table 1*).

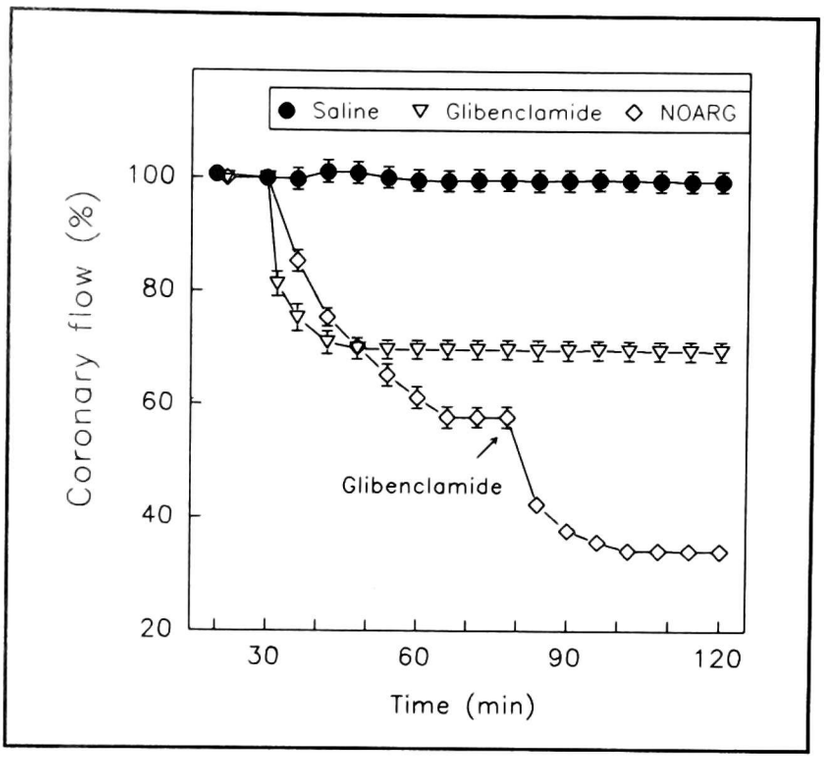

Fig. 1. Effect of L-NOARG and glibenclamide on coronary flow in Langendorff perfused rat hearts. L-NOARG (◇, 10 μM) and glibenclsmide (▽, 600 nM) were applied for 50 min, and then, in L-NOARG perfused hearts glibenclamide (600 nM) was added to the perfusate. Each data point is a mean ± s.e.m. of 6 experiments.

Table 1. Characteristics of the concentration-response curve to pinacidil as affected by glibenclamide, L-NOARG, SIN-1, L-arginine, adenosine, L-NMMA and ischemia/reperfusion in isolated rat heart

Intervention	Number of Hearts	Basal Coronary Flow ml/min/g dry wt)	Heart Rate (beats/min)	Characteristics of the pinacidil concentration-response curve		
				EC_{50}	Slope Power	Maximum Flow (ml/min/g dry wt)
Untreated	6	52.1 ± 3.5	268 + 16	176 ± 11	3.3 ± 0.4	96.6 ± 4.0
Glibenclamide (0.6 μM)	6	36.6 ± 3.2 *	238 ± 15 *	1511 ± 101*	2.6 ± 0.4	95.3 ± 4.2
Untreated	5	58.3 ± 3.1	261 ± 15	166 ± 9	3.5 ± 0.4	107.9 ± 4.1
L-NOARG (10 μM)	5	33.8 ± 2.9 *	234 ± 16 *	484 ± 23 *	3.3 ± 0.3	82.8 ± 4.8 *
Untreated	5	59.0 ± 3.7	277 ± 11	169 ± 10	3.3 ± 0.4	107.2 ± 4.3
SIN-1 (0.8 μM)	5	77.0 ± 4.5 *	275 ± 10	99 ± 5 *	3.4 ± 0.4	105.9 ± 4.2
Untreated	5	53.3 ± 3.7	281 ± 10	176 ± 10	3.4 ± 0.4	103.0 ± 3.5
L-Arginine (1 mM)	5	58.6 ± 4.2	282 ± 11	185 ± 13	3.5 ± 0.3	104.8 ± 4
Untreated	6	54.7 ± 3.2	276 ± 11			
L-NOARG	6	29.6 ± 2.8 *	238 ± 10 *	482 ± 19	3.3 ± 0.3	77.8 ± 4.0
L-NOARG + + L-Arginine	6	52.2 ± 3.8 #	261 ± 12 #	185 ± 16 #	3.4 ± 0.3	95.2 ± 4.6 #
Untreated	6	55.3 ± 4.5	280 ± 11	180 ± 11	3.3 ± 0.3	98.0 ± 5.2
Ischemia/reperfusion	6	47.0 ± 3.0	278 ± 13	406 ± 52 *	3.4 ± 0.6	78.0 ± 4.1 *
Untreated	6	51.8 ± 3.9	277 ± 12			
L-NMMA(50 μM)	6	28.3 ± 2.8 *	241 ± 13 *	525 ± 43	3.2 ± 0.4	75.9 ± 4.8
L-NMMA + Isch/ /Reperf	6	28.8 ± 3.0 *	238 ± 17 *	552 ± 61	3.1 ± 0.4	72.3 ± 3.9

All values are means ± s.e.m. * $P < 0.05$, *vs* the respective untreated hearts; # $P < 0.05$, L-NOARG *vs* L-NOARG + L-arginine

NO and coronary responsiveness to pinacidil

In the untreated hearts, pinacidil, produced a concentration-dependent increase in coronary flow in the concentration range of 0.05–0.5 μM and a reduction in the flow at the higher concentrations (*Fig. 2, upper panel*). Only the rising part of the pinacidil concentration-response curve (DRC) was analyzed. Consistent with its blocking action on K_{ATP}, glibenclamide caused a 8.6-fold rightward parallel displacement of the pinacidil DRC while the maximum pinacidil response was nor affected (*Fig. 2*).

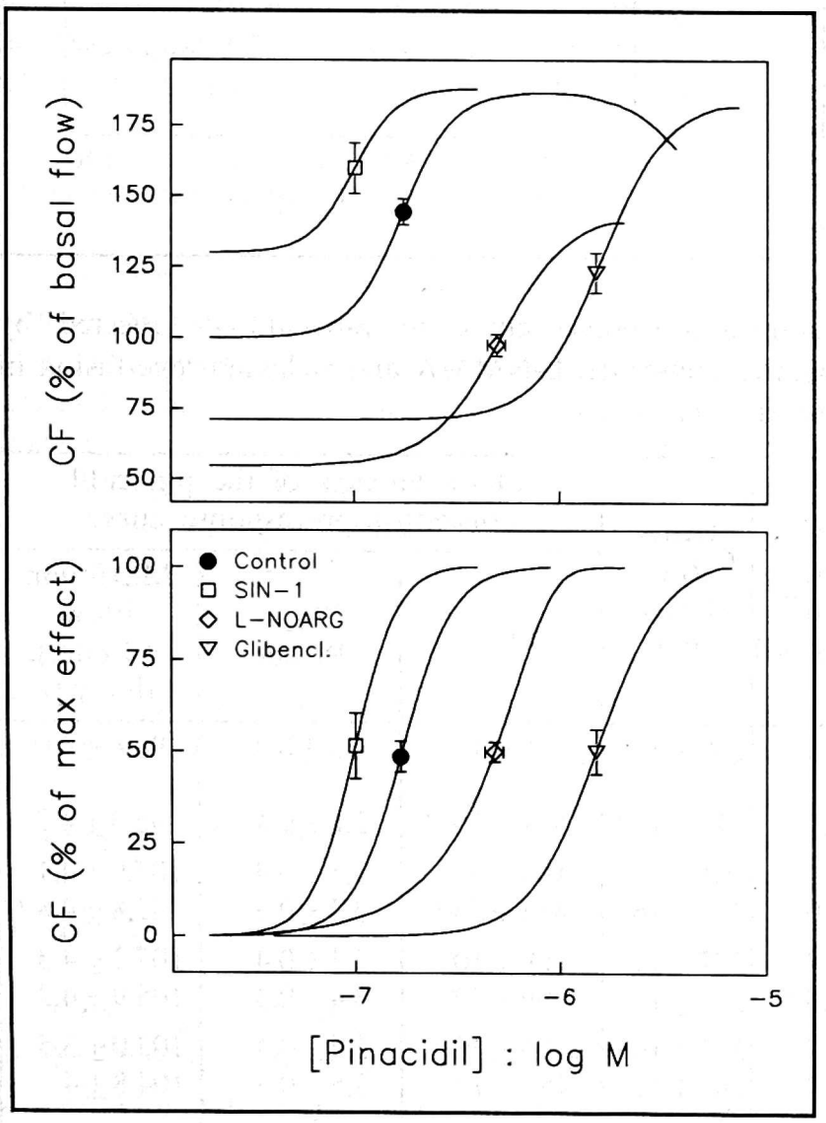

Fig. 2. Effect of L-NOARG, glibenclamide and SIN-1 on concentration-response relationship (DRC) of pinacidil for increase in coronary flow in isolated rat heart perfused at constant pressure mode. Control pinacidil DRC was first obtained and then the DRC repeated in the presence of L-NOARG, 10 μM (◇, n = 5), glibenclamide, 0.6 μM (▽, n = 6) or SIN-1, 0.8 μM (□, n = 5). Variance analysis revealed no inter-group difference between the control pinacidil DRCs obtained during different studies (*Table 1*). Therefore, the data from all control DRCs were pooled and fitted to a single curve (●, mean EC_{50} 171 ± 5 nM, n = 33). Data are expressed as the per cent of the basal coronary frlow (*upper panel*) and as the per cent of the maximum amount of coronary flow increase produced by pinacidil (*bottom panel*).

A significant ca 3-fold rightward shift of the pinacidil DRC occurred also in L-NOARG (10 μM, *Fig. 2*) or L-NMMA (50 μM, *Fig. 5*) perfused hearts. This was, however, associated with the reduction in the maximum vasodilatory response to pinacidil (*Fig. 2*). The L-NOARG-induced changes in coronary flow and in pinacidil DRC were completely reversed by L-arginine, 1 mM, a concentration that, by itself, affected neither the basal coronary flow nor the pinacidil DRC (*Table 1*). The pretreatment with 0.8 μM SIN-1, resulted in a small but significant leftward shift of the pinacidil DRC (*Fig. 2, Table 1*). Interestingly enough, a significant 2.1-fold rightward shift of the DRC for

pinacidil-induced reduction in coronary perfusion pressure (change in ED_{50} from 129 ± 13 nM to 273 ± 25 nM) was characteristic also for L-NOARG-treated hearts perfused at the constant flow (*Fig. 3*), suggesting that L-NOARG-induced reduction in coronary responsiveness to pinacidil was independent from coronary flow. Altogether, these results suggest that coronary K_{ATP} activity may be NO-dependent. If so, the K_{ATP} blockade is expected to result in the attenuated coronary responsiveness to NO. This was examined in following experiments.

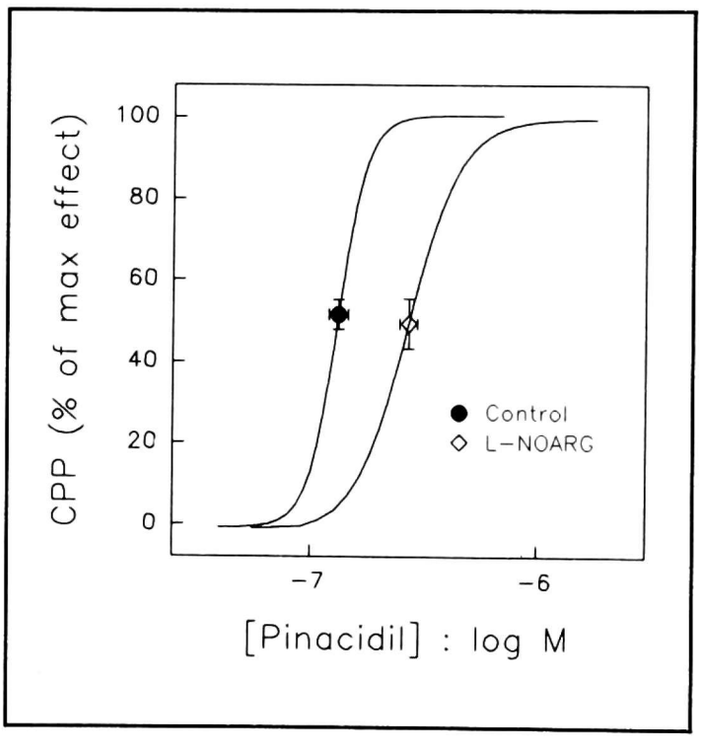

Fig. 3. Effect of L-NOARG on concentration-response relationship (DRC) of pinacidil for decrease in coronary perfusion pressure in isolated rat heart perfused at constant flow mode. Control pinacidil DRC was first obtained (●) and then the DRC was repeated in the presence of L-NOARG, 10 μM (◇, n = 5). Data are expressed as the per cent of the the maximum amount of coronary perfusion pressure reduction produced by pinacidil.

Responsiveness to SIN-1 in glibenclamide-vs L-NOARG-perfused hearts (Fig. 4)

In glibenclamide perfused hearts, the SIN-1 DRC was shifted to the right (an increase in ED_{50} from 1.35 ± 0.21 μM to 3.56 ± 0.53 μM, respectively, $p < 0.05$) and it was not affected by L-NOARG treatment (ED_{50} of 1.36 ± 0.30 μM vs 1.38 ± 0.28 μM, respectively, $p < 0.05$).

NO-pathway in ischemic/reperfused heart

In the hearts subjected to 20 min ischemia and 40 min reperfusion, the recovery of coronary flow amounted to $84.0 \pm 3.2\%$ (*Fig. 5, upper panel*). When applied at 40 min of the reperfusion, an inhibitor of NO-synthesis, L-NMMA (50 μM) caused a $23.6 \pm 3.2\%$ drop in coronary flow which resulted in a $39.1 \pm 2.8\%$ total flow reduction from the preischemic values. However, L-NMMA-induced drop in coronary flow amounted to as much as $43.2 \pm 3\%$

when the drug was applied before the ischemia ($p < 0.05$, *Fig. 5*, bottom panel). Interestingly enough, in L-NMMA perfused hearts subjected to ischemia/ /reperfusion, reperfusion coronary flow recovered to the pre-ischemic values

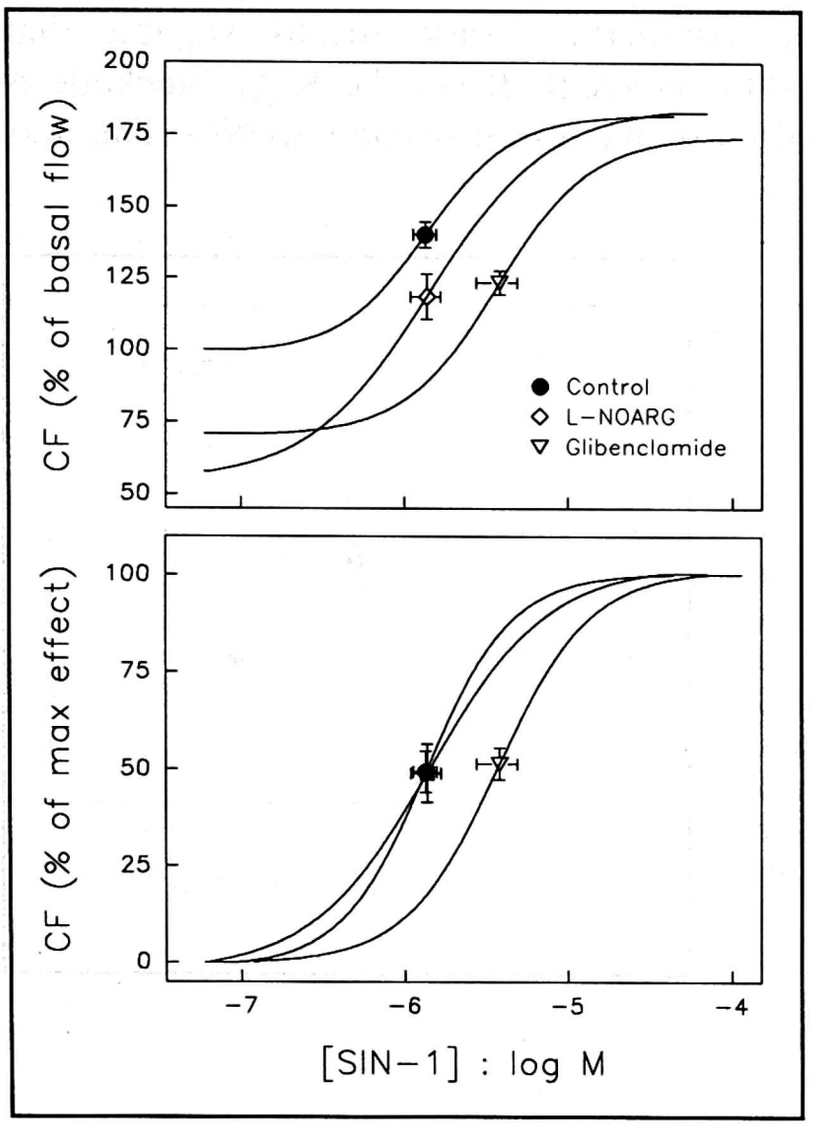

Fig. 4. Effect of L-NOARG and glibenclamide on concentration-response relationship (DRC) of SIN-1 for increase in coronary flow in isolated rat heart. Control SIN-1 DRC was first obtained and then the DRC was repeated in the presence of L-NOARG, 10 μM (◇, n = 8) or glibenclamide, 0.6 μM (▽, n = 5). The data from the control DRCs were pooled and fitted to a single curve (●).

(42.4 ± 4% reduction from the basal coronary flow) indicating that the effects of NO-blockade and of ischemia/reperfusion on coronary flow are not additive. Thus, the impairment of the post-ischemic recovery of coronary flow in our experimental model is likely to be related to the ischemia/reperfusion--induced impairment of NO-pathway.

Responsiveness to pinacidil in the ischemic/reperfused heart. Comparison to that in L-NMMA-perfused heart

The pinacidil DRC obtained at 40 min reperfusion showed a 2.3-fold rightward shift as compared to the control DRC obtained before ischemia/reperfusion (*Fig. 5*). Similar displacement of the pinacidil DRC was observed also in L-NMMA perfused hearts and in L-NMMA-perfused hearts which were subjected to 20 min ischemia and 40 min reperfusion.

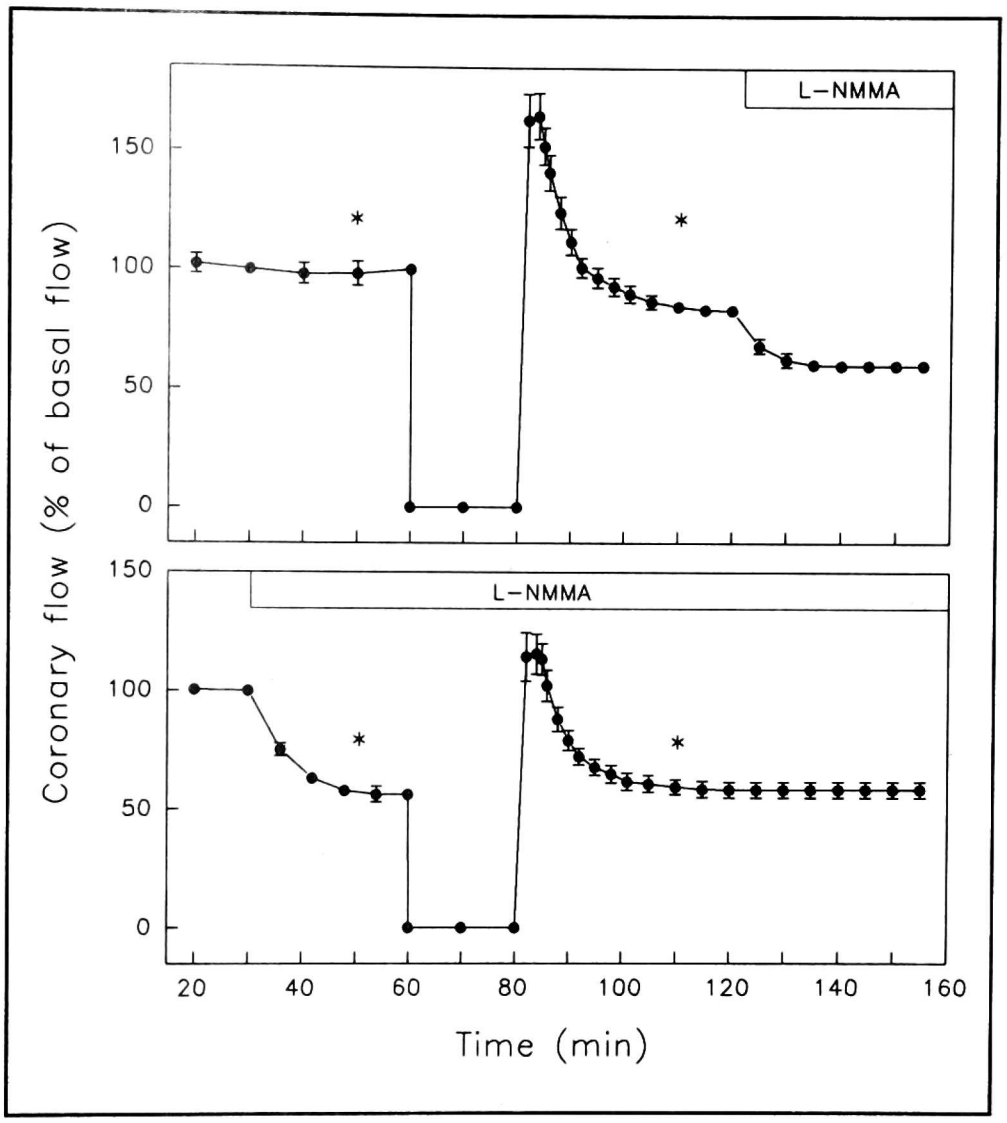

Fig. 5. Coronary flow responses to L-NMMA in isolated rat hearts subjected to 20 min ischemia followed by 80 min reperfusion. As indicated by the respective bars at a top of each panel, L-NMMA (50 μM) was administered either at 40 min of the reperfusion (*upper panel*) or 30 min before ischemia (*bottom panel*) and its administration was continued throughout the rest of the experiment. * indicates the moment when the pinacidil DRC was constructed. Each data point is a mean ± s.e.m. of 6 experiments. * $p < 0.05$ vs. respective pre-ischemic values.

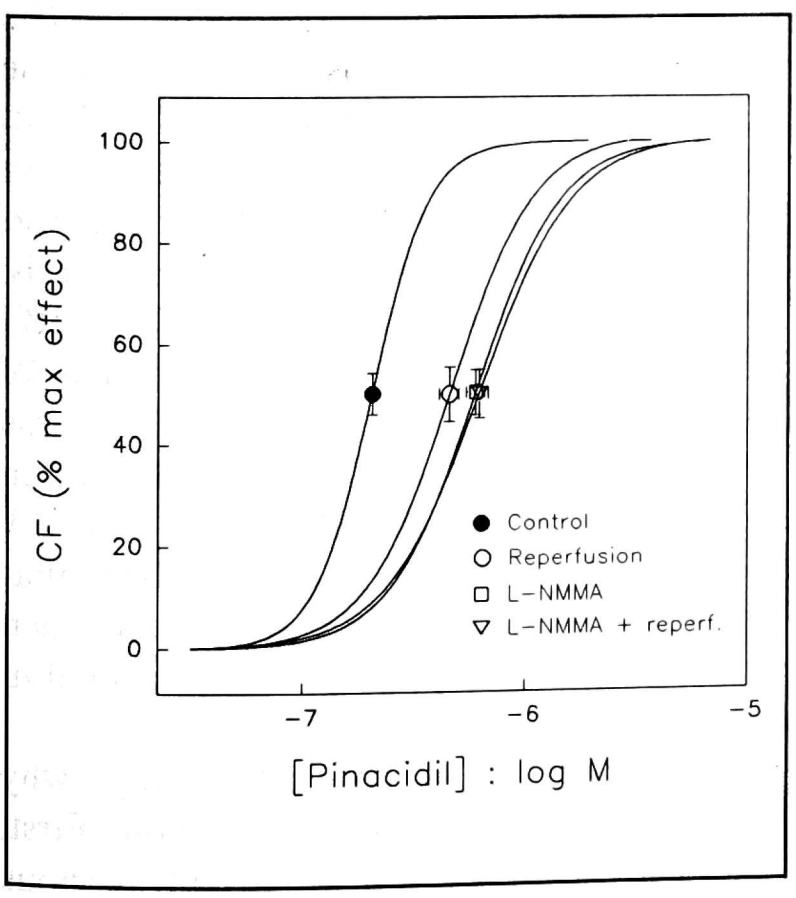

Fig. 6. Effect of ischemia/reperfusion and L-NMMA on the concentration-response relationship (DRC) of pinacidil for increase in coronary flow in isolated rat heart. In one set of hearts, the control pinacidil DRC was obtained (●, n = 6) then the hearts were subjected to 20 min ischemia + 30 min reperfusion and the DRC was repeated (○, n = 6). The other set was first perfused with 50 μM L-NMMA for 30 min and the pinacidil DRC was obtained (□, n = 6). Then the hearts were subjected to ischemia/reperfusion in the continuing presence of L-NMMA and the pinacidil DRC was repeated, (▽, n = 6) (see *Fig. 5* for the respective protocols). Data are expressed as the per cent of the maximum amount of coronary flow increase produced by pinacidil.

DISCUSSION

This study provides evidence that in isolated rat heart: (1) NO and K_{ATP} participate in maintaining the basal coronary tone (2). The responsiveness of coronary microcirculation to K_{ATP} stimulation is attenuated when endothelial NO-pathway is impaired either pharmacologically or due to prior ischemia/ /reperfusion.

NO and vascular responsiveness to K_{ATP} stimulation

In this study, the hearts were either perfused with NO-synthase inhibitors or subjected to ischemia/reperfusion to impair NO-pathway. Indeed, as in studies of other authors (7—9), also in our hands ischemia/reperfusion resulted in the attenuation of the basal NO production. The indirect evidence for this is that the vasoconstrictive response to L-NMMA has been attenuated in the reperfused hearts as compared to the untreated hearts (*Fig. 5*).

Glibenclamide as well as L-NOARG and L-NMMA have been demonstrated here to produce a rightward displacement of the pinacidil DRC. It is noteworthy, that L-NOARG produced this kind of the displacement in the hearts perfused at the constant pressure as well as at the constant flow mode suggesting that its effect was independent from coronary flow (*Fig. 2 vs Fig. 3*). However, in contrast to glibenclamide, L-NOARG and L-NMMA depressed also the maximal response to pinacidil (*Fig. 2*). These data seem to suggest that both glibenclamide and NO-synthesis inhibitors decreased activity of coronary K_{ATP}, although the mechanisms of their action seem to be different. The concept that the vasodilator function of coronary K_{ATP} is regulated by the endothelial NO finds further support in the following results of this study: (i) NO-synthesis inhibitors and the NO-donor, SIN-1, caused opposite effects on the pinacidil DRC (*Fig. 2 and 5*); (ii) The L-NOARG-induced displacement of the pinacidil DRC was completely reversed by the precursor of NO-synthesis, L-arginine (*Table 1*). These two effects suggested that the effect of L-NOARG was specific for NO; (iii) Glibenclamide produced a rightward displacement of the SIN-1 DRCi (iv) The rightward displacement of the pinacidil DRC was quantitatively similar in the hearts perfused with L-NOARG, L-NMMA and in those subjected to ischemia/reperfusion. Furthermore, the effects of L-NMMA and of ischemia/reperfusion appeared not to be additive. Thus we believe that it is the impairment of NO-pathway caused by these interventions rather then their nonspecific actions (e.g. those related to coronary flow), which resulted in the impaired responsiveness to pinacidil.

Several mechanisms may be considered when explaining why NO-deficiency compromises vasodilation induced by K_{ATP} activation. First, NO-induced stimulation of K_{ATP} has been demonstrated in various

experimental models (4, 13, 14) and the involvement of cGMP-dependent protein kinase in the mechanism was suggested (4). Thus, it is attractive to speculate that also in rat heart the coronary K_{ATP} is partially stimulated by the endothelial NO. Consequently, K_{ATP} would be less active in the absence of NO. Second, there is growing evidence that vasoconstrictors acting through stimulation of protein kinase C inhibit K_{ATP} in vascular smooth muscle cells (4). The attenuated responsiveness to K_{ATP} stimulation may, therefore, reflect a buildup of a hypothetical inhibitor of K_{ATP} in NO-deficient hearts. Third, evidence indicates that in dog heart, the inhibition of NO synthesis attenuates coronary responses to adenosine because NO blockade results in a preferential constriction of small coronary arteries but dilation of arterioles, thereby shifting the major site of coronary resistance from arterioles to small arteries (26). An important element of this reasoning is a smaller adenosine sensitivity of small arteries as compared to that of arterioles (26). Similar reasoning can be used in case of the attenuated responsiveness to pinacidil in NO-deficient heart, because, at least in dog heart, the longitudinal gradients for vasodilator responses to adenosine and K_{ATP} stimulation seem to be similar (22, 29).

In conclusion, this study provides the evidence that NO blockade results in attenuated responsiveness of coronary resistance vessels to K_{ATP} stimulation. No matter what the mechanism of this attenuated responsiveness is, its occurrence may be expected to attenuate metabolic regulation of coronary flow, a process which seems to involve the activation of vascular K_{ATP} (3, 5, 16, 21, 22). In fact, the blockade of NO synthesis has been demonstrated to attenuate metabolic regulation of myocardial perfusion (14, 24—27). It is tempting to speculate that part of this effect may be related to the attenuation of K_{ATP} mediated responses. However, a limitation of the above reasoning is: (i) the reliance on the specificity of glibenclamide for inhibition and pinacidil for activation of the K_{ATP} and (ii) the assumption that exogenous (e.g. pinacidil) and endogenous activators of K_{ATP} act similarly and at the same segment of the vascular tree. The possible practical implication of the study is that, in patients with the impaired NO-pathway, a vasodilating potency of K_{ATP} openers may be limited as compared to that of NO donors (cf. *Fig. 4*).

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