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Mediators of coronary reactive hyperaemia in isolated mouse heart

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1 Mechanisms regulating coronary tone under basal conditions and during reactive hyperaemia following transient ischaemia were assessed in isolated mouse hearts.

2 Blockade of NO-synthase (50 μM L-NAME), K_{ATP} channels (5 μM glibenclamide), A_{2A} adenosine receptors (A_{2A}ARs; 100 nM SCH58261), prostanoid synthesis (100 μM indomethacin), and EDHF (100 nM apamin + 100 nM charybdotoxin) all reduced basal flow ~40%. Effects of L-NAME, glibenclamide, and apamin + charybdotoxin were additive, whereas coadministration of SCH58261 and indomethacin with these inhibitors failed to further limit flow.

3 Substantial hyperaemia was observed after 5–40 s occlusions, with flow increasing to a peak of 48 ± 1 ml min^{-1} g^{-1}. Glibenclamide most effectively inhibited peak flows (up to 50%) while L-NAME was ineffective.

4 With longer occlusions (20–40 s), glibenclamide alone was increasingly ineffective, reducing peak flows by ~15% after 20 s occlusion, and not altering peak flow after 40 s occlusion. However, cotreatment with L-NAME + glibenclamide inhibited peak hyperaemia by 70 and 25% following 20 and 40 s occlusions, respectively.

5 In contrast to peak flow changes, sustained dilation and flow repayment over 60 s was almost entirely K_{ATP} channel and NO dependent (each contributing equally) with all occlusion durations.

6 Antagonism of A_{2A}ARs with SCH58261 reduced hyperaemia 20–30% whereas inhibition of prostanoid synthesis was ineffective. Effects of A_{2A}AR antagonism were absent in hearts treated with L-NAME and glibenclamide, supporting NO and K_{ATP}-channel-dependent effects of A_{2A}ARs.

7 EDHF inhibition alone exerted minor effects on hyperaemia and only with longer occlusions. However, residual hyperaemia after 40 s occlusion in hearts treated with L-NAME + glibenclami-glibenclamide + SCH58261 + indomethacin was abrogated by cotreatment with apamin + charybdo-charybdotoxin.

8 Data support a primary role for K_{ATP} channels and NO in mediating sustained dilation after coronary occlusion. While K_{ATP} channels (and not NO) are also important in mediating initial peak flow adjustments after brief 5–10 s occlusions, their contribution declines with longer 20–40 s occlusions. Intrinsic activation of A_{2A}ARs is important in triggering K_{ATP} channel/NO-dependent hyperaemia. Synergistic effects of combined inhibitors implicate interplay between mediators, with compensatory changes occurring in K_{ATP} channel, NO, and/or EDHF responses when one is individually blocked.
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Abbreviations: EDHF, endothelium-derived hyperpolarizing factor; K_{ATP} channel, ATP-dependent K^+ channel; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; SCH58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-*c*]pyrimidine

Introduction

Reactive hyperaemia is the temporary increase in tissue blood flow subsequent to brief periods of vascular occlusion. This graded response provides repayment of so-called 'flow debt' (reflecting O_2 or metabolic debt) incurred during occlusion, potentially hastening metabolic and functional recovery. The molecular basis for coronary hyperaemia remains unclear, with likely involvement of multiple mechanisms. From a fundamental viewpoint, reactive hyperaemia may involve mechano-sensitive processes (myogenic and flow/shear-mediated) together with metabolic regulatory processes. Initial dilatory stimuli may involve a combination of mechanical and metabolic triggers, while subsequent sustained dilation may be partially flow or shear dependent (Koller & Bagi, 2002). All of these processes and triggers may, in turn, act *via* nitric oxide (NO), ATP-sensitive K^+ (K_{ATP}) channel, and/or EDHF-dependent dilatory processes. However, although NO- and K_{ATP}-channel dependent processes are implicated, relative roles vary widely in different studies (Daut *et al*., 1990; Aversano *et al*., 1991; Clayton *et al*., 1992; Kanatsuka *et al*., 1992; Kostic & Schrader, 1992; Duncker *et al*., 1993; Gryglewski *et al*., 1995; Godecke *et al*., 1998; Gattullo *et al*., 1999; Kingsbury *et al*., 2000; 2001). There is also mixed support for a role for endogenously generated adenosine in

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triggering dilation (Kanatsuka *et al*., 1992; Kirkeboen *et al*., 1992; Otomo *et al*., 1997; Shinoda *et al*., 1997; Kingsbury *et al*., 2000; 2001). In contrast, studies generally find no evidence of a role for prostanoids (Kimura & Satoh, 1985; Gryglewski *et al*., 1995; 1996; Macho *et al*., 1995; Shinoda *et al*., 1997; Kingsbury *et al*., 2001), despite potential involvement in control of basal flow (Duffy *et al*., 1999). The role of EDHF in hyperaemia remains unknown, although *in vitro* and *in vivo* evidence does implicate EDHF in physiological regulation of coronary vascular resistance (Cohen & Vanhoutte, 1995; Nishikawa *et al*., 1999).

The aim of the current study was to examine the roles of K_{ATP} channels, NO, EDHF, A_{2A}ARs, and prostanoids in mediating peak and sustained flow changes during reactive hyperaemia. Hyperaemic mechanisms were interrogated in the increasingly investigated mouse heart, using a Langendorff perfusion model of global coronary hyperaemia employed widely in prior investigations in multiple species (Clayton *et al*., 1992; Kostic & Schrader, 1992; Gryglewski *et al*., 1995; 1996; Shinoda *et al*., 1997; Godecke *et al*., 1998; Kingsbury *et al*., 2001).

Methods

Murine Langendorff heart model

Investigations conformed with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institute of Health (NIH Publications No. 85–23, revised 1996). Hearts were isolated and perfused on a Langendorff perfusion system described in detail previously (Headrick *et al*., 2001a, b), and prepared for coronary vascular study (Flood & Headrick, 2001; Flood *et al*., 2002) Specifically, 77 adult male C57/B16 mice (8–12 weeks age, 20–25 g body weight, 118 ± 4 mg blotted heart weight) were anaesthetised with 50 mg kg^{-1} sodium pentobarbitone, a thoracotomy performed and hearts excised into ice-cold perfusion fluid. The aorta was cannulated and hearts perfused at a constant hydrostatic pressure of 80 mmHg with Krebs bicarbonate buffer containing (in mM): NaCl, 120; $NaHCO_3$, 22; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; MgCl, 1.2; glucose, 11; and EDTA, 0.5. Perfusate was equilibrated with 95% O_2, 5% CO_2 at 37°C, giving a pH of 7.4. Perfusate temperature was maintained at 37°C and hearts superfused in a water-jacketed chamber. The left ventricle was vented with a polyethylene drain to prevent Thebesian accumulation. Coronary flow was monitored *via* an ultrasonic flow-probe (1 N probe, accurate to 0.05 ml min^{-1}; Transonic Systems, Ithaca, NY, U.S.A.) located in the aortic perfusion line (Headrick *et al*., 2001a, b). Perfusion pressure was monitored using a P23XL pressure transducer (Viggo-Spectramed, Oxnard, CA, U.S.A.) connected to a MacLab (ADInstruments, Castle Hill, Australia). After 20-min stabilisation, hearts were switched to pacing using a Grass S9 stimulator (Grass, Quincy, MA, U.S.A.). Hearts were paced at 400 beats min^{-1} *via* silver left ventricular electrodes (0.5 ms square pulses, 20% above threshold, typically 2–5 V) and stabilised for a further 10 min.

Reactive hyperaemia protocol

Each heart studied was subjected to four occlusion periods (5, 10, 20 and 40 s) applied in random order. Each occlusion protocol was separated by a 5-min period of reperfusion during which flow recovered to preocclusion levels. Peak hyperaemic flow and the integral of coronary flow throughout the initial 1 min reperfusion (i.e. total coronary flow in ml g^{-1}, referred to as total flow repayment) were determined for each occlusion period. Peak flows and the flow integral were determined from continuous flow-meter recordings in the Chart data acquisition program (ADInstruments, Castle Hill, Australia). Since absolute coronary flow rates change proportionally with heart mass and metabolic rate, all flows were normalised to wet heart weight (ml min^{-1} g^{-1}). Reactive hyperaemic responses were assessed in the absence (control; $n = 14$) or presence of 50 μM L-NAME (NO-synthase inhibitor, $n = 10$), 5 μM glibenclamide (a nonselective K_{ATP} channel antagonist; $n = 10$), 100 nM SCH58261 (selective A_{2A}AR antagonist; $n = 8$), 100 μM indomethacin (cyclooxygenase inhibitor; $n = 6$), 100 nM apamin + 100 nM charybdotoxin (EDHF blockade; $n = 7$), 5 μM glibenclamide + 50 μM L-NAME ($n = 6$), 5 μM glibenclamide + 50 μM L-NAME + 100 nM SCH58261 + 100 μM indomethacin ($n = 8$), or 5 μM glibenclamide + 50 μM L-NAME + 100 nM SCH58261 + 100 μM indomethacin + 100 nM apamin + 100 nM charybdotoxin ($n = 8$). Untreated (control) and treated hearts were assessed in random order to minimise experimental bias. Treatment with antagonists was commenced 15 min prior to assessing reactive hyperaemic responses. Infusion rates were manually adjusted prior to occlusion, and at 5 s and then every 10 s thereafter during reperfusion (to 60 s) to reduce transient changes in infused drug concentrations during initial hyperaemia. Given the transient nature of the hyperaemic responses, intravascular and interstitial drug concentrations are not predicted to change substantially during the protocol. Since resting coronary flow was reduced by different inhibitory agents studied, calculation of % flow debt repayment (commonly assessed in studies of reactive hyperaemia) was not meaningful. Rather, we assessed and present overall hyperaemic responses (Figures 1, and 4–7), and changes in peak hyperaemic flow and total flow repayment during 60 s reperfusion (Figure 3).

Inhibitor concentrations were based in part on levels used in prior studies (including our own work in murine hearts), and were selected to inhibit targeted mediators effectively. We employ a 5 μM concentration of glibenclamide, two- to five-fold higher than in prior studies in other species (Shinoda *et al*., 1997; Kingsbury *et al*., 2001), and which we have shown abrogates coronary relaxation to K_{ATP} openers in mouse (Flood & Headrick, 2001). The 50 μM L-NAME concentration is ~2- to 5-fold higher than used in guinea-pig (Kostic & Schrader, 1992; Kingsbury *et al*., 2000; 2001), and we have shown this level eliminates maximal responses to 0.1 μM ADP in mouse without altering direct smooth muscle relaxation with nitroprusside (Flood *et al*., 2002). The 100 μM level of indomethacin used is 10-fold higher than levels applied in hyperaemia studies in other species (Gryglewski *et al*., 1995; 1996; Shinoda *et al*., 1997; Kingsbury *et al*., 2001), and up to 100-fold higher than the K_i for inhibition of murine and human cyclooxygenase/prostaglandin synthesis (Gierse *et al*., 1999; Kalgutkar *et al*., 2000), ensuring effective inhibition. Levels of apamin and charybdotoxin are equivalent to those used in other species (Shinoda *et al*., 1997). The concentration of charybdotoxin is 10-fold higher than its K_i for blockade of intermediate-conductance Ca^{2+}-activated K^+ channels (IK_{Ca})

(Nelson & Quayle, 1995). Apamin was applied at a concentration ~100-fold higher than its K_i for small-conductance K_{Ca} (SK_{Ca}) channels, and is highly selective for this target (Ciechanowicz-Rutkowska *et al*., 2003). Finally, in preliminary studies we found 100 nM SCH58261 abolishes coronary dilation with a maximally effective 2 nM concentration of the A_{2A}AR agonist CGS21680 (data not shown). Thus, levels of the varied inhibitors chosen should be sufficient to inhibit targeted mediators substantially.

Chemicals

The A_{2A}AR antagonist SCH58261 was kindly donated by the Schering-Plough Research Institute (Milan, Italy). All other drugs were purchased from Sigma/RBI (Sigma, Castle Hill, Australia). L-NAME, SCH58261, apamin, and charybdotoxin were dissolved directly in perfusion fluid while indomethacin and glibenclamide were dissolved as stock solutions in sodium hydroxide (NaOH) and dimethylsulfoxide (DMSO), respectively. All compounds were infused into the coronary circulation through a filter with a pore size of 0.22 μm at no more than 1% of coronary flow to achieve final concentrations indicated. The vehicle (NaOH or DMSO) concentrations did not exceed 0.4 mM or 0.01%, respectively, in any group. Preliminary studies verified that these low solvent levels fail to modify ventricular contractile function, coronary tone, and responses to vasodilators. As a further safeguard, we additionally assessed infusion of nongassed Krebs buffer solution, which was shown to not modify contractile function in hearts instrumented with ventricular balloons, or to modify hyperaemic responses to 5–40 s occlusions (data not shown).

Data analyses

Peak hyperaemic flows and repayment flows over 60 s reperfusion in the different treatment groups were compared by analysis of variance with Tukeys H.S.D. *post hoc* test when significant effects were detected. In all tests, $P<0.05$ was considered indicative of statistical significance. All values are reported as mean ± s.e.mean (s.e.m.).

Results

Effects of inhibitors on basal coronary tone

Baseline coronary flow was 12.9 ml min^{-1} g^{-1} in untreated hearts. Treatment with L-NAME, glibenclamide, SCH58261, indomethacin, and apamin in conjunction with charybdotoxin led to 40–50% reductions in resting coronary flow (to 7–8 ml min^{-1} g^{-1}). Combined treatment with L-NAME and glibenclamide exerted an even greater 70–75% reduction in basal flow (Table 1). Addition of SCH58261 and indomethacin with these inhibitors failed to further limit basal flow. However, administration of apamin + charybdotoxin in conjunction with these other inhibitors did slightly but significantly further attenuate flow.

Reactive hyperaemic responses in control hearts

All hearts showed a substantial hyperaemic response following transient occlusion. Coronary reactive hyperaemia was graded, increasing with occlusion duration (Figure 1). Representative traces from a control heart (and hearts treated with L-NAME and glibenclamide) are presented in Figure 2. Peak hyperaemic flows increased from ~30 ml min^{-1} g^{-1} after 5 s occlusion to 48 ml min^{-1} g^{-1} after 40 s occlusion (Figure 3a). Total flow repayment over 60 s reperfusion was also graded, increasing from 13 ml g^{-1} after 5 s occlusion to 33 ml g^{-1} after 40 s occlusion (Figure 3b).

Effects of NO-synthase and K_{ATP} channel inhibition on reactive hyperaemic responses

The NO-synthase inhibitor L-NAME exerted negligible effects on peak hyperaemic flows after all occlusion periods (Figures 2–4). However, L-NAME did significantly lower flow repayment by limiting flow during the period following maximal dilation (evident with all periods of occlusion) (Figures 3 and 4). The nonselective K_{ATP} channel blocker glibenclamide exerted the greatest effect on peak hyperaemic flow, and also limited prolonged dilation following initial hyperaemia (Figures 3 and 4). These latter inhibitory effects of glibenclamide were similar to those for L-NAME, and were evident with all periods of occlusions. Effects on peak flow were gradually lessened as occlusion duration increased until glibenclamide was unable to alter peak hyperaemic flow with 40 s occlusion.

Table 1 Effects of inhibitor treatment on basal coronary tone

Treatment group	Pretreatment flow (ml min^{-1} g^{-1})	Preocclusion flows (ml min^{-1} g^{-1})
Untreated ($n=14$)	13.5 ± 1.8	12.9 ± 1.9
L-NAME ($n=10$)	13.7 ± 1.5	6.8 ± 0.8*,†
Glibenclamide ($n=10$)	13.9 ± 1.4	7.2 ± 0.7*,†
SCH58261 ($n=8$)	13.8 ± 1.7	6.9 ± 1.1*,†
Indomethacin ($n=6$)	14.0 ± 1.8	7.6 ± 1.0*,†
Apamin + charybdotoxin ($n=7$)	14.0 ± 1.9	7.9 ± 0.5*,†
L-NAME + glibenclamide ($n=6$)	13.6 ± 1.7	3.8 ± 0.4*,§
L-NAME + glibenclamide + SCH58261 + indomethacin ($n=8$)	13.9 ± 2.0	4.4 ± 0.6*,§
L-NAME + glibenclamide + SCH58261 + indomethacin + apamin + charybdotoxin ($n=8$)	14.0 ± 2.0	3.1 ± 0.4*,‡

The predrug flow was measured immediately prior to drug treatment while the preocclusion flows were assessed immediately prior to the reactive hyperaemia protocol. Values are means ± s.e.m. *$P<0.05$ vs pretreatment. †$P<0.05$ vs L-NAME + glibenclamide. ‡$P<0.05$ vs L-NAME + glibenclamide + SCH58261 + indomethacin. §$P<0.05$ vs individual inhibitor treatments.

Combined treatment with L-NAME + glibenclamide (Figures 3 and 4) was significantly more effective at limiting peak flows and flow repayment than L-NAME and glibenclamide individually (Figures 3 and 4). With the longer 20 and 40 s occlusions, the effect of simultaneous treatment with L-NAME + glibenclamide on peak flow and repayment markedly exceeded the sum of the individual effects of L-NAME and glibenclamide (Figure 3).

Effects of $A_{2A}AR$ antagonism and cyclooxygenase inhibition on reactive hyperaemic responses

The $A_{2A}AR$ selective antagonist SCH58261 did not significantly attenuate peak hyperaemic flows (Figures 3a and 5). However, repayment flow over 1 min of reperfusion was significantly reduced 20–30% by SCH58261 after all occlusions (Figures 3b and 5). The cyclooxygenase inhibitor indomethacin only significantly limited peak hyperaemic flow and flow repayment after brief 5 s occlusion, with an insignificant trend to reduced repayment after 10 s occlusion (Figures 3 and 5). Coinfusion of SCH58261 + indomethacin with L-NAME + glibenclamide (Figures 3 and 5) was no more effective in limiting hyperaemic responses than L-NAME- + glibenclamide (Figures 3 and 4).

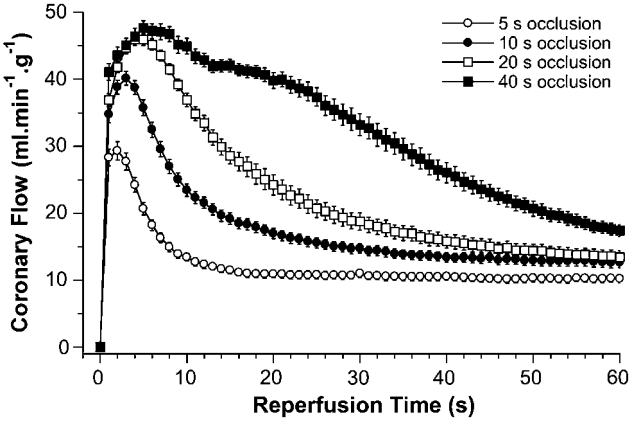

Figure 1 Reactive hyperaemic responses to 5, 10, 20, and 40 s transient coronary occlusion in control hearts ($n = 14$). All values are means ± s.e.m.

Effects of EDHF blockade on reactive hyperaemic responses

The EDHF inhibitors apamin + charybdotoxin did not significantly reduce peak hyperaemic flows (Figures 3 and 6). There was a trend to reduced initial hyperaemia with these inhibitors, which only achieved statistical significance (in terms of reduced flow repayment) after 20 s occlusions. Coadministration of apamin + charybdotoxin with L-NAME + glibenclaglibenclamide + SCH21680 + indomethacin did not further limit flow repayment beyond the inhibitory effects of the latter agents alone (Figures 3b and 5). However, coinfusion of apamin + charybdotoxin with these inhibitors did exert a significantly greater effect on peak flow after 40 s occlusion (Figure 3a and 6).

Roles of NO, K_{ATP} channels, and EDHF in responses to brief (5 s) and prolonged (40 s) occlusions

By assuming that hyperaemic responses in the presence of inhibitors of NO-synthase, K_{ATP} channels, or EDHF reflect NO, K_{ATP} channel, or EDHF 'independent' responses, respectively, one can estimate and graphically present 'dependent' and 'independent' components of hyperaemia (with the dependent component estimated by subtraction of the independent response from the control response). Data in Figure 7a demonstrate that K_{ATP} channel-dependent dilation is of chief importance in mediating initial peak hyperaemic responses to brief occlusion. The role of NO-dependent processes in initiating initial dilation appears negligible. However, the role of NO-dependent dilation increases during

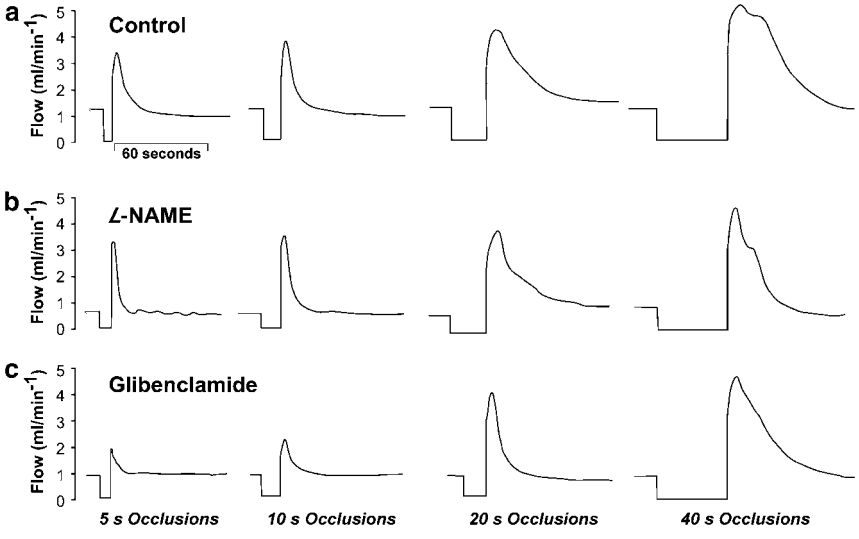

Figure 2 Representative coronary flow recordings for hyperaemic responses from (a) an untreated heart, (b) a heart pretreated with the NO-synthase inhibitor L-NAME (50 μM), and (c) a heart pretreated with the nonselective K_{ATP} channel inhibitor glibenclamide (5 μM).

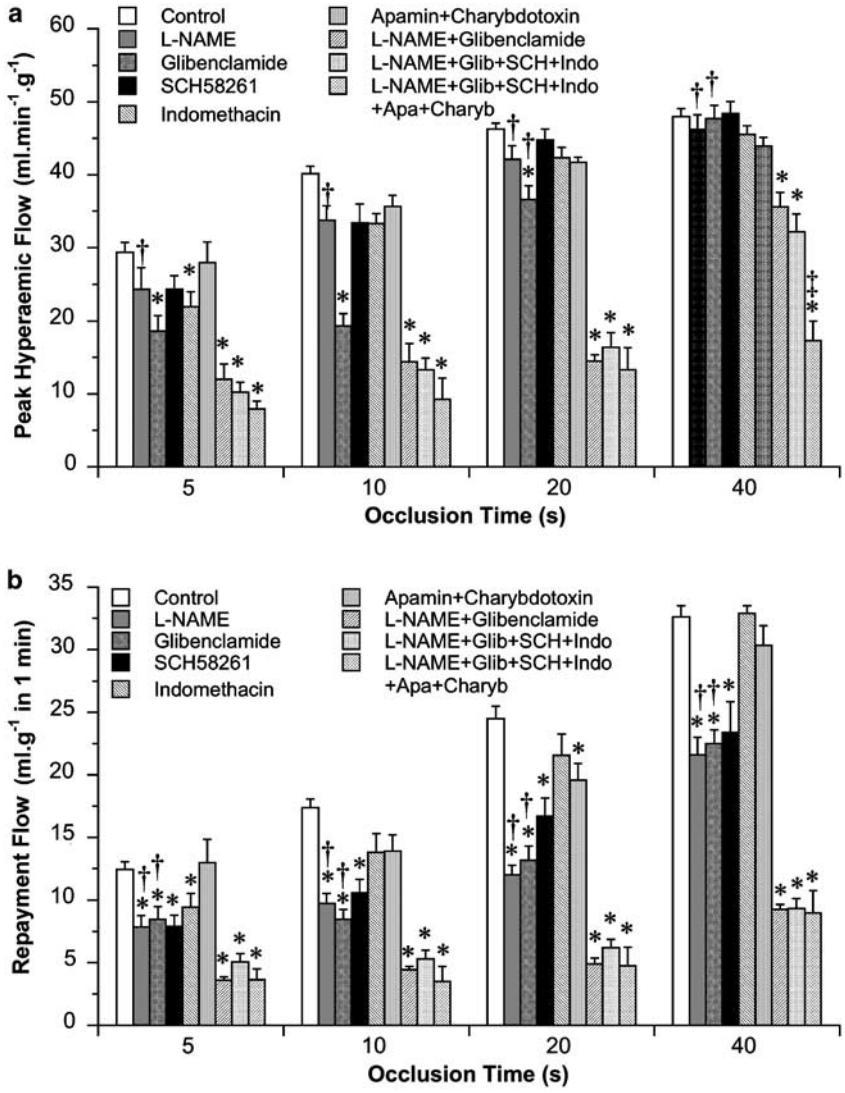

Figure 3 Effects of inhibitors on (a) peak hyperaemic flow and (b) repayment flow over the initial 1 min of reperfusion following 5, 10, 20, and 40 s occlusions. Data were acquired in the absence ($n=14$) or presence of L-NAME ($n=10$), glibenclamide ($n=10$), SCH58261 ($n=8$), indomethacin ($n=6$), apamin + charybdotoxin ($n=7$), L-NAME + glibenclamide ($n=6$), L-NAME + glibenclamide + SCH58261 + indomethacin ($n=8$) or L-NAME + glibenclamide + SCH58261 + indomethacin + apamin + charybdotoxin ($n=8$). All values are means ± s.e.m. $^{*}P<0.05$ vs control. $^{\dagger}P<0.05$ vs cotreatment with L-NAME + glibenclamide. $^{\ddagger}P<0.05$ vs L-NAME + glibenclamide + SCH58261 + indomethacin.

the postocclusion period such that NO- and K_{ATP}-channel dependent responses are of primary (and equal) importance in mediating sustained dilation over the remaining 60 s reperfusion period. With brief occlusion, there is no evidence of EDHF involvement beyond the initial 2–3 s of reperfusion. This situation changes subtly, however, with longer 40 s occlusion (Figure 7b), when initial hyperaemia (during the first 10–20 s) is partially NO, K_{ATP} channel, and also EDHF dependent (although EDHF involvement is minor in the absence of inhibitors of the other mediators, and failed to achieve significance in terms of total flow repayment over 60 s).

Discussion

The current study demonstrates that multiple mediators contribute to coronary reactive hyperaemia in mouse, with roles varying depending upon occlusion duration and the stage of reperfusion (initial vs late). Specifically, K_{ATP} channels mediate the majority of initial hyperaemic responses to brief (5–10 s) occlusions, with K_{ATP} channels and NO contributing equally to subsequent more sustained dilation. With more prolonged occlusions (20–40 s), initial peak flows are partially K_{ATP} channel-/NO-dependent and also modestly EDHF-dependent, with later sustained dilation again solely K_{ATP} channel- and NO-dependent. Data support a significant role for A_{2A}ARs in hyperaemia (in a K_{ATP} channel-/NO-dependent manner). Finally, we present evidence for compensatory changes in K_{ATP} channel, NO, and EDHF responses when one of these is pharmacologically limited.

Regulation of basal coronary tone

There is little information regarding control of resting coronary tone in the increasingly studied mouse heart. It is

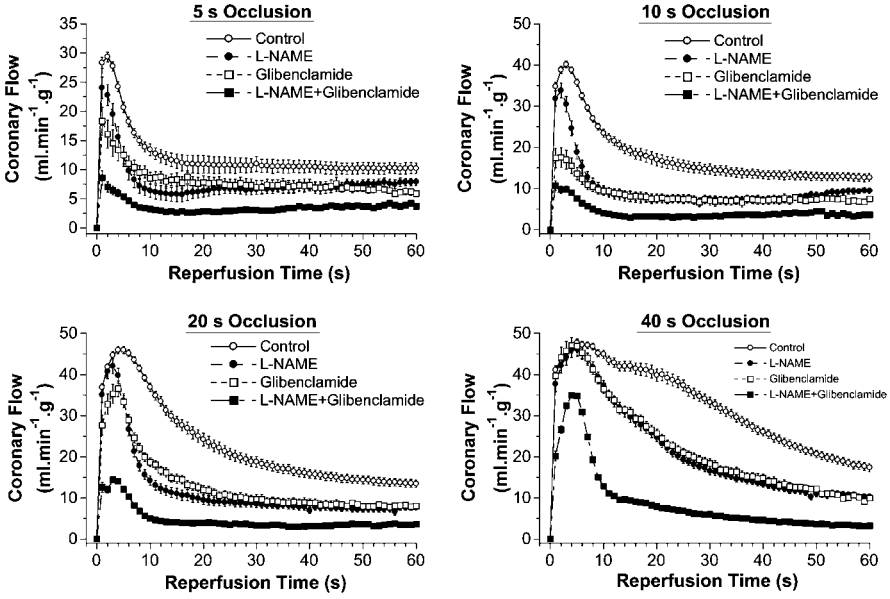

Figure 4 Contributions of NO and K_{ATP} channels to coronary reactive hyperaemia. Shown are hyperaemic responses to 5, 10, 20, and 40 s occlusions in untreated hearts ($n=14$) and hearts treated with the NO-synthase inhibitor L-NAME ($n=10$), K_{ATP} channel inhibitor glibenclamide ($n=10$), or simultaneous NO-synthase and K_{ATP} channel inhibition with L-NAME + glibenclamide (L-NAME + Glib; $n=6$). All values are means ± s.e.m.

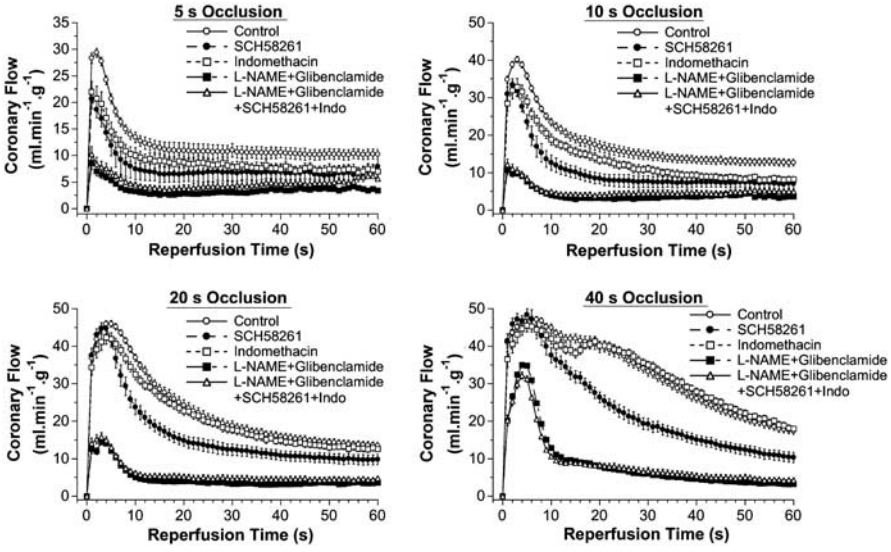

Figure 5 Contributions of $A_{2A}AR$ activation and cyclooxygenase products to coronary reactive hyperaemia. Shown are hyperaemic responses to 5, 10, 20, and 40 s occlusions in untreated hearts ($n=14$) and hearts treated with the $A_{2A}AR$ antagonist SCH58261 ($n=8$), cyclooxygenase inhibitor indomethacin ($n=6$), or simultaneous NO-synthase, K_{ATP} channel, $A_{2A}AR$, and cyclooxygenase inhibition (L-NAME + glibenclamide + SCH58261 + indomethacin; $n=8$). Effects of simultaneous NO-synthase and K_{ATP} channel inhibition with L-NAME + glibenclamide (L-NAME + Glib; $n=6$) are again shown for comparison. All values are means ± s.e.m.

possible that endogenously released adenosine (Lee *et al*., 1992; Edlund *et al*., 1995; Flood *et al*., 2002; Talukder *et al*., 2002; Rose'meyer *et al*., 2003) and prostanoids (Duffy *et al*., 1999) activate NO- and/or K_{ATP} channel-dependent dilation (Hein & Kuo, 1999; Hein *et al*., 1999; Flood & Headrick, 2001) to modulate basal tone. Our data show NO and K_{ATP} channels contribute equally (and additively) to control of basal tone (Table 1), and suggest effects of adenosine (at A_{2A}ARs) and prostanoids are NO- and/or K_{ATP} channel-dependent (since effects of $A_{2A}AR$ and cyclooxygenase inhibition are absent during NO and K_{ATP} channel inhibition). In addition, EDHF appears to modify basal tone, although not to the same extent as NO and K_{ATP} channels.

Mixed data continue to be acquired regarding NO and K_{ATP} channel dependence of resting tone, and roles of adenosine receptors and prostanoids. A majority of studies verify K_{ATP}

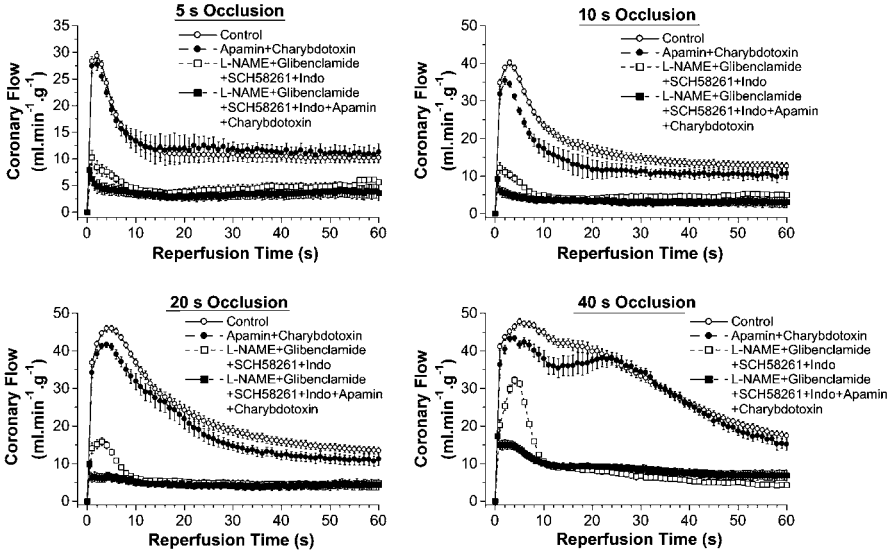

Figure 6 Contribution of EDHF to coronary reactive hyperaemia. Shown are hyperaemic responses to 5, 10, 20, and 40 s occlusions in untreated hearts ($n=14$), hearts treated with the EDHF inhibitors apamin and charybdotoxin ($n=7$), or with simultaneous NO-synthase, K_{ATP} channel, $A_{2A}AR$, cyclooxygenase, and EDHF inhibition (L-NAME + glibenclamide + SCH58261 + indomethacin + apamin + charybdotoxin; $n=8$). Simultaneous NO-synthase, K_{ATP} channel, $A_{2A}AR$, and cyclooxygenase inhibition (L-NAME + glibenclamide + SCH58261 + indomethacin; $n=8$) are again shown for comparison. All values are means ± s.e.m.

channel inhibition reduces basal coronary flow (Imamura *et al*., 1992; Samaha *et al*., 1992; Duncker *et al*., 1993; Richmond *et al*., 1999; Phillis *et al*., 2000; Yamamoto *et al*., 2000; Chen *et al*., 2001; Kingsbury *et al*., 2001; Farouque *et al*., 2002; Zhang *et al*., 2003). However, a small number find no effects of K_{ATP} inhibition (Mathew & Lerman, 2001; Tune *et al*., 2001). Similarly, inhibition of NO-synthase has been found to limit basal coronary flow in different species including mice (Chu *et al*., 1991; Kostic & Schrader, 1992; Otomo *et al*., 1997; Veronneau *et al*., 1997; Godecke *et al*., 1998; Goodhart & Anderson, 1998; Duffy *et al*., 1999; Gattullo *et al*., 1999; Jakovljevic *et al*., 1999; Andrews *et al*., 2001; Flood & Headrick, 2001; Kingsbury *et al*., 2001; Mathew & Lerman, 2001; Thornburg *et al*., 2002; Zong *et al*., 2002). Other studies document no effect of NO-synthase blockade in canine and human hearts (Egashira *et al*., 1996; Parent *et al*., 1996; Nishikawa & Ogawa, 1997; Tune *et al*., 2001). Reasons for these discrepancies are unclear. One issue we address (see below) relates to redundancy and compensation by other dilatory mechanisms when one process is blocked.

There are also mixed reports regarding the role of A_2ARs in regulating basal tone. A majority of studies fail to identify a key role for endogenous adenosine (Dole *et al*., 1985; Hanley *et al*., 1986; Bache *et al*., 1988; Tune *et al*., 2001). However, there is support for a role in humans (Edlund *et al*., 1995) and animal models (Lee *et al*., 1992; Rose'meyer *et al*., 2003), including mice (Flood *et al*., 2002; Talukder *et al*., 2002). An issue that should not be overlooked when studying rodent hearts is the difference in mass-specific metabolic rate. As body mass declines, mass-specific metabolic rate (and heart rate) rises predictably. Thus, murine myocardial VO_2 is predicted to be 1.6-fold higher than in rat, and 2.4-fold higher than in guinea-pig (Headrick *et al*., 2001a, b). A greater contribution of A_2ARs to basal tone in mice could reflect increased importance of adenosine as metabolic rate rises. This is consistent with data on coronary regulation during periods of enhanced metabolic rate in larger species (Kang *et al*., 1990; Karim & Goonewardene, 1996; Ishibashi *et al*., 1998).

Inhibition of prostanoid synthesis has been shown to increase basal tone in some models (Duffy *et al*., 1999), but particularly in hearts from subjects with coronary artery disease (Edlund *et al*., 1985; Pacold *et al*., 1986) and animal models of coronary disease (Lane & Bove, 1985; Altman *et al*., 1993). A majority of studies reveal no effects of cyclooxygenase inhibitors on resting flow in nondiseased models (Dai & Bache, 1984; Veronneau *et al*., 1997; Godecke *et al*., 1998; Jakovljevic *et al*., 1999). These data suggest a compensatory function for prostanoids in diseased hearts. It should be noted that indomethacin might exert direct coronary effects, as suggested by Edlund *et al*. (1985). This might explain the lack of effect of other cyclooxygenase inhibitors (such as diclofenac) in mouse heart (Godecke *et al*., 1998) vs reduced flow with indomethacin observed here (Table 1). We also employ a relatively high concentration of indomethacin to ensure maximal cyclooxygenase inhibition.

We also present preliminary evidence for a role for EDHF in control of basal coronary flow in mouse (Table 1). Since effects of EDHF inhibition are still evident in the presence of NO, K_{ATP} channel, $A_{2A}AR$, and cyclooxygenase inhibition, it is likely that mechanisms of EDHF-mediated dilation are at least partially distinct from these mediators. Prior studies indicate that EDHF-mediated dilation plays an important role in physiological regulation of coronary resistance vessels (Cohen & Vanhoutte, 1995; Nishikawa *et al*., 1999).

Roles of NO and K_{ATP} channels in reactive hyperaemia

Coronary dilation mediated *via* G-protein coupled receptors such as those for adenosine (and other endogenous dilators) act, at least in part, *via* triggering release of NO and/or

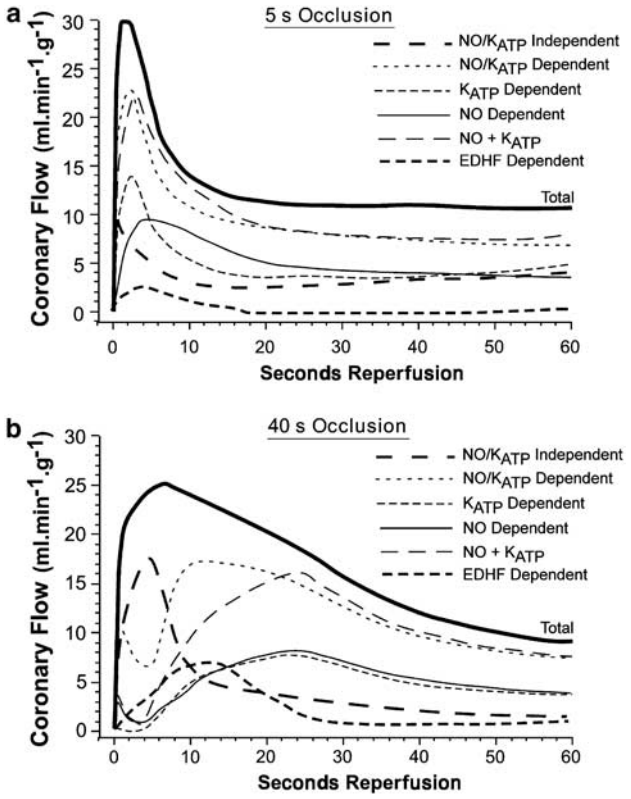

Figure 7 Relative contributions of NO-, K_{ATP} channel-, and EDHF-dependent and independent mechanisms to control of coronary flow during reactive hyperaemic responses to (a) brief 5 s occlusion and (b) prolonged 40 s occlusion. Data were calculated from hyperaemic responses in untreated (Total), L-NAME-treated (NO independent flow), glibenclamide-treated (K_{ATP} channel-independent flow), L-NAME + glibenclamide-treated hearts (NO- and K_{ATP} channel-independent flow), and apamin + charybdotoxin-treated hearts (EDHF independent flow). The NO-, K_{ATP} channel-, and EDHF-dependent responses were calculated by subtraction of independent flows from control (total) flow responses. Also shown is the summed NO- and K_{ATP} channel-dependent response (from individual effects of L-NAME and glibenclamide, respectively), demonstrating agreement between individual and combined treatments throughout hyperaemia following brief occlusion, and for the later stages of hyperaemia following prolonged occlusion. Note that EDHF inhibition altered the pattern of early hyperaemia despite no significant reduction in total repayment.

activation of K_{ATP} channels (Boulanger & Vanhoutte, 1997; Hein & Kuo, 1999; Hein *et al*., 1999; Flood & Headrick, 2001). Moreover, mechano-sensitive dilation during occlusion-reperfusion may involve NO release (Koller & Bagi, 2002). Prior studies confirm roles for NO (Kostic & Schrader, 1992; Gattullo *et al*., 1994; Gryglewski *et al*., 1995; Godecke *et al*., 1998; Gattullo *et al*., 1999) and K_{ATP} channels (Daut *et al*., 1990; Aversano *et al*., 1991; Clayton *et al*., 1992; Kanatsuka *et al*., 1992; Duncker *et al*., 1993; Kingsbury *et al*., 2001) in coronary reactive hyperaemia. Our data reveal that with brief 5–10 s occlusions, K_{ATP} channel-dependent (glibenclamide-sensitive) dilation accounts for at least 30–50% of initial hyperaemia (Figures 3, 4, and 7). Effects of glibenclamide wane with increasing occlusion duration, while NO inhibition has negligible effects on peak flows with all durations. Thus, one might conclude, as have Kingsbury *et al*. (2001), that K_{ATP} channels contribute to peak hyperaemia while NO plays no role. However, effects of combined K_{ATP} and NO synthase inhibition reveal this may be an erroneous conclusion (Figures 3 and 4). Cotreatment, particularly in the 20 and 40 s occlusion groups, is synergistic surpassing the sum of the individual effects of the two inhibitors (Figure 3). Despite no effect of either inhibitor on peak flow after 40 s occlusion, for example, cotreatment reduced dilation by 25%. Synergistic actions indicate NO does play an appreciable role, and that: (i) inhibition of the response is effectively compensated for by enhanced K_{ATP} channel activity; and/or (ii) the role of NO is compensatory in nature and only significant when K_{ATP} channel responses are blocked. It is problematic to determine which of these possibilities holds.

As opposed to initial peak hyperaemia, data clearly indicate that both NO and K_{ATP} channels mediate the major proportion of prolonged dilation during the subsequent 40–50 s of reperfusion. This is reflected in flow repayment, which is reduced by 35% by both L-NAME and glibenclamide after 5 and 40 s occlusions (and 50% after 10 and 20 s occlusions). Since inhibitory effects are additive (cotreatment limiting repayment 70–80%), we conclude both NO and K_{ATP} channels contribute equally to repayment. Relative roles of NO and K_{ATP} channel responses are diagrammatically presented in Figure 7. These data show an increased role for NO and K_{ATP} channel independent processes in peak dilation to longer occlusions, but a generally conserved contribution of NO and K_{ATP} channels to overall flow repayment.

Prior data both agree with and contrast our findings in murine hearts. In contrast to a role for NO, Shinoda *et al*. (1997) observed minor (though poorly documented) effects of L-NAME on reactive hyperaemia in rat, and Kingsbury *et al*. (2001) found negligible effects of NO inhibition in guinea-pig. Other studies document no effects of NO inhibition on hyperaemic flows (Chu *et al*., 1991; Gattullo *et al*., 1994). However, Gryglewski *et al*. (1996) found that L-NAME largely inhibited hyperaemia after 1–60 s occlusions in guinea-pig. Thus, while some differences in findings may be species-dependent, quite contradictory findings emerge within the same species (Gryglewski *et al*. 1996; Kingsbury *et al*. 2001), the origins of which are unclear though again possibly reflecting compensation and redundancy in hyperaemic mediators.

Roles for adenosine and prostanoids in mediating hyperaemia

Adenosine has received considerable attention as an endogenous mediator of hyperaemic responses (Curnish *et al*., 1972; Saito *et al*., 1981; Bache *et al*., 1988; Mainwaring *et al*., 1988; Gidday *et al*., 1990; Kanatsuka *et al*., 1992; Kirkeboen *et al*., 1992; Gryglewski *et al*., 1995; 1996; Otomo *et al*. 1997; Kingsbury *et al*., 2001), and we have shown that the A_{2A}AR is the adenosine receptor subtype almost exclusively responsible for coronary vascular control in the murine heart (Flood & Headrick, 2001; Flood *et al*., 2002). We thus chose to assess the roles of this receptor in the coronary hyperaemic response. Early studies with relatively poor antagonist treatments show coronary hyperaemic responses are mediated in part by adenosine (Curnish *et al*., 1972; Saito *et al*., 1981; Bache *et al*., 1988), with flow repayment and hyperaemia duration reduced up to 30%, and minor (if any) changes in peak flows.

More recent studies also support a role for adenosine in repayment but not peak dilation (Kanatsuka *et al*., 1992; Shinoda *et al*., 1997), or verify a minor role in peak dilation (Otomo *et al*., 1997). Our data are largely consistent with these observations, supporting modest effects of A_{2A}AR antagonist with SCH58261 on peak flow with brief (but not prolonged) occlusion, and significant 25–30% reductions in flow repayment after all occlusions (Figures 3 and 5). Most studies report 30–35% reductions in flow repayment with adenosine antagonism in other species (Curnish *et al*., 1972; Saito *et al*., 1981; Bache *et al*., 1988; Gidday *et al*., 1990; Gryglewski *et al*., 1995; 1996; Macho *et al*., 1995; Otomo *et al*., 1997; Kingsbury *et al*., 2001), in good agreement with current reductions in repayment (Figure 3).

The studies of Gryglewski *et al*. (1996) and Otomo *et al*. (1997) both support the involvement of adenosine in reactive hyperaemia, with a more pronounced role with more severe ischemic episodes. Yamabe *et al*. (1992) acquired support for roles for both adenosine and NO, and found the two compounds exert additive effects. In the present study, the much greater effects of K_{ATP} and NO-synthase inhibition vs A_{2A}AR antagonism support a role for other triggers in mediating K_{ATP}- and NO-dependent hyperaemia. Prostanoids are unlikely to play a major role since indomethacin failed to substantially modify hyperaemia (Figures 3 and 5), in agreement with prior work (Kimura & Satoh, 1985; Gryglewski *et al*., 1995; 1996; Macho *et al*., 1995; Shinoda *et al*., 1997; Kingsbury *et al*., 2001). Other possible triggers for K_{ATP}- and NO-dependent dilation include haemodynamic or mechanical forces such as flow or shear-stress, which trigger 'flow-dependent' coronary dilation (Kuo *et al*., 1991; Duffy *et al*., 1999).

From analysis of hyperaemia in isolated skeletal vessels, Koller & Bagi (2002) suggest that endothelial deformation during occlusion triggers NO synthesis to initiate peak dilation, that a pressure-induced response triggers further NO release together with myogenic constriction on reperfusion, and finally that increased flow/shear during reperfusion further stimulates NO synthesis to prolong hyperaemia. They recognised that in intact tissue other factors, notably metabolic in nature, will augment dilation. As in prior studies (Clayton *et al*., 1992; Kostic & Schrader, 1992; Gryglewski *et al*., 1995; 1996; Shinoda *et al*., 1997; Godecke *et al*., 1998; Kingsbury *et al*., 2001), pressure and flow will both change in the vessels of the isolated heart, and may therefore elicit this sequence of events suggested by Koller & Bagi (2002). In agreement, we observe reduced flow repayment with NO inhibition. However, we acquire evidence for a major role for K_{ATP} channels and metabolically coupled adenosine in peak dilation as opposed to NO. This difference may reflect the more complex nature of *in vivo* responses, or differences in skeletal vs coronary regulation.

Evidence for EDHF-mediated responses in reactive hyperaemia

One potential mediator underlying reactive hyperaemia, not adequately assessed previously, is EDHF. Since we were unable to abolish completely hyperaemic responses *via* cotreatment with K_{ATP} channel, NO, A_{2A}AR, and cyclooxygenase inhibitors, other mediators must be involved. EDHF triggers vasodilation independently of NO and prostanoids, largely within the resistance vasculature. The identity of EDHF is unknown, although K_{Ca} are implicated in responses, including IK_{Ca} channels (sensitive to charybdotoxin) and SK_{Ca} channels (sensitive to apamin). Based on endothelial localisation of IK_{Ca} and SK_{Ca} channels, it is thought they modulate formation and/or release of EDHF (Edwards *et al*., 1998). We find EDHF inhibition alone is without effect on peak flows, and exerts only minor effects on flow repayment (Figures 3 and 6). Again, one might therefore conclude that EDHF plays a minor role in the murine hyperaemic response. However, it is interesting that in the 40 s occlusion group cotreatment with apamin + charybdotoxin plus all other inhibitors did largely eliminate the hyperaemia remaining in the presence of NO, K_{ATP}, A_{2A}AR and cyclooxygenase inhibitor alone (Figure 3a). This synergistic effect suggests: (i) effects of EDHF inhibition alone are masked by compensatory changes in NO/K_{ATP} channel dependent responses; and/or (ii) EDHF only plays a compensatory role when other dilatory processes are eliminated. In this respect, there is evidence NO and EDHF are intimately connected, with NO generation exerting negative-feedback inhibition of EDHF-induced vasodilation (Bauersachs *et al*., 1996; Huang *et al*., 2000; Nishikawa *et al*., 2000; Thollon *et al*., 2002). As suggested by Nishikawa *et al*. (2000), EDHF may indeed be a 'second line of defence' when NO-dependent paths are compromised (Figures 3 and 6).

Evidence of vasoregulatory 'compensation'

One important observation from this work is the ability of certain combinations of drugs to generate inhibitory actions that are synergistic in nature. As already noted, inhibitory effects of L-NAME and glibenclamide applied simultaneously are greater than the sum of individual effects of these inhibitors after 20 and 40 s (but not 5 and 10 s) occlusions. Synergistic effects of L-NAME and glibenclamide are evident for peak flow but not flow repayment. These observations, more pronounced with prolonged occlusions, are most readily explained by compensatory changes in either NO- or K_{ATP} channel-dependent dilation when one alone is limited. There is support for similar compensatory changes from other studies (Kostic & Schrader, 1992; Duncker *et al*., 1995; Ishibashi *et al*., 1998; Tayama *et al*., 1998). This interplay may well explain differences between the current findings and prior studies of coronary hyperaemia, where potential mediators have almost universally been individually targeted (Clayton *et al*., 1992; Gryglewski *et al*., 1995; 1996; Shinoda *et al*., 1997; Godecke *et al*., 1998; Kingsbury *et al*., 2001).

Conclusions

In summary, our data indicate that multiple mechanisms contribute in a complex manner to coronary reactive hyperaemia in mice. Vascular K_{ATP} channels, but not NO, mediate a major fraction of initial peak hyperaemic responses to brief (5–10 s) occlusions but become less important with longer (20–40 s) occlusions. This decline is due in part to compensatory changes in L-NAME-sensitive (NO-dependent) dilation. Prolonged dilation after initial flow adjustments is almost entirely K_{ATP} channel- and NO-dependent (each contributing equally). The NO and K_{ATP} channel responses are triggered in part by A_{2A}AR activation, since A_{2A}AR

antagonism reduces hyperaemia 20–30% only in the absence of NO and K_{ATP} inhibition. Finally, EDHF may contribute to hyperaemic responses to more prolonged occlusions (20–40 s), although this contribution is more evident when NO and K_{ATP} channels are simultaneously blocked. Whether this latter observation reflects a primarily compensatory function for EDHF, suppression of EDHF bioactivity by NO, or masking of the effects of EDHF inhibition *via* compensatory increases in NO/K_{ATP} channel responses requires further investigation.

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