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VASOACTIVITY OF ADENOSINE IN THE TROUT (*ONCORHYNCHUS MYKISS*) CORONARY SYSTEM: INVOLVEMENT OF NITRIC OXIDE AND INTERACTION WITH NORADRENALINE

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Summary

A vasoconstrictory response to adenosine has been reported in coronary rings from fish. Since the reactivity of the large coronary arteries and the microcirculation may differ, the present study was undertaken to determine the role of adenosine in the intact coronary system of trout under constant pressure or flow using an isolated and non-working heart preparation. The involvement of nitric oxide (NO) and the interaction with noradrenaline were also studied. At 10^{-9} to 10^{-8} mol l^{-1}, adenosine caused a vasoconstrictory response, whereas between 10^{-7} and 10^{-5} mol l^{-1} the response was predominantly vasodilative. Theophylline abolished both these responses to adenosine. The vasodilation induced by adenosine (at 10^{-5} mol l^{-1}) was significantly reduced when the preparation was perfused under constant-flow than rather under constant-pressure conditions. The nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine (L-NA, 10^{-4} mol l^{-1}) partially reduced the vasodilation induced by adenosine (at 10^{-5} mol l^{-1}) under constant-pressure but not under constant-flow conditions. Perfusion of the intact coronary system with L-arginine or with adenosine significantly increased the rate of nitrite (NO_2^-) release, while perfusion with L-NA or theophylline reduced NO_2^- release. Chemical denudation of the coronary endothelium by CHAPS resulted in the loss of both the L-arginine- and adenosine-mediated vasodilation and the L-arginine-induced increase in the rate of NO_2^- release. Adenosine (10^{-5} mol l^{-1}) offset and overrode the vasoconstriction induced by 10^{-7} mol l^{-1} noradrenaline. L-NA inhibited only the adenosine-induced vasodilation but not the ability to offset noradrenaline vasoconstriction, excluding the involvement of NO in the interaction between adenosine and noradrenaline.

Key words: trout, *Oncorhynchus mykiss*, intact coronary system, adenosine, nitric oxide, N^{ω}-nitro-L-arginine, noradrenaline.

Introduction

Coronary blood flow and myocardial oxygen consumption *in vivo* are closely related *via* changes in coronary resistance in response to the metabolic demands of the myocardium (Berne, 1964). This capacity to adjust blood flow to myocardial metabolic requirements remains unaltered in isolated and perfused preparations devoid of external control mechanisms, i.e. sympathetic nerves and circulating agonists. The exact mechanism of this matching is unclear but it is axiomatic that it is due to several factors, including dilator agonists released from the contracting myocardium which provide an appropriate arteriolar dilator tone (Levick, 1991). The relationship described above between coronary flow and myocardial oxygen consumption has recently also been reported in the trout heart (Agnisola *et al.* 1998). In that study, the myocardial rates of oxygen consumption were approximately 21 and 5 μl O_2 min^{-1} g^{-1} under high-P_{O_2} (175 mmHg, 23.3 kPa) and low-P_{O_2} (76 mmHg, 10.1 kPa) perfusion conditions, respectively. An increase of 137 % in coronary flow and a decrease of 68 % in coronary resistance were observed under low-P_{O_2} compared with high-P_{O_2} perfusion condition (Agnisola *et al.* 1998).

This hypoxia-induced vasodilative response in an isolated perfused fish heart preparation is comparable with the response of isolated mammalian hearts, which exhibit hypoxia-induced vasodilation after a period of ischaemia, suggesting that factors intrinsic to the heart contribute to metabolic vasodilation (Berne, 1980). The currently accepted mechanisms for this vasodilatory response involve interactions between various autacoids. Compounds such as prostaglandins, endothelial-derived nitric oxide (EDNO) and adenosine may be released by the myocardium under changing physiological conditions (Levick, 1991). The role of these autacoids in the control of coronary blood flow and myocardial oxygen consumption in mammals is well

established, while comparable information from fish species is poor. The capacity of the fish heart to synthesize prostaglandins from arachidonic acid has been reported (Knight *et al.* 1995; Mustafa *et al.* 1992). Hypoxia-induced changes in the levels of thromboxane A_2 and prostacyclin in trout plasma have been described (Mustafa and Jensen, 1992). As in mammals, prostacyclin causes vasodilation of the intact coronary system in the isolated perfused trout heart (Mustafa and Agnisola, 1994). Recently, L-arginine-dependent, EDNO-mediated vasodilation has been documented in the trout coronary system (Mustafa *et al.* 1997). Both acetylcholine and serotonin appear to elicit nitric-oxide-dependent vasodilatory responses (Mustafa *et al.* 1997).

A number of studies from several laboratories have confirmed the role of adenosine in the modulation of blood flow in various mammalian tissues, e.g. heart, brain and skeletal muscle (Berne *et al.* 1983). The effect of adenosine on the fish heart seems to be atypical compared with its effect on mammalian coronary arteries, where it elicits a classic vasodilatory response (Berne, 1964). A strong contractile response is apparent in fish coronary preparations (rings from the coronary artery, running along the bulbus and entering the ventricle) from rainbow trout (Small *et al.* 1990), from skate (Farrell and Davie, 1991) and from steelhead and rainbow trout (Farrell and Johansen, 1995). However, the roles of the large coronary artery (conduit artery) and of the microcirculation supplying the ventricle compacta in the regulation of coronary tone may differ, as reported in mammals (DeFily and Chilian, 1995). Therefore, to assess the role of adenosine in the control of coronary circulation in fish, studies on the effects of adenosine in preparations that include resistance vessels are needed. Moreover, the metabolism, release and interactions of autacoids in the endothelial cells influence the control of blood flow through the coronary circulation or any vascular bed. All these mechanisms of control of the coronary circulation can be initiated *via* endothelial purinergic, muscarinic, kinin or other types of receptors, by shear forces and by hypoxia (Vane and Botting, 1994). Prostaglandins have been implicated in this control mechanism, and it has recently been claimed that nitric oxide is an important mediator of blood flow regulation in mammals (Parent *et al.* 1992; Steinhorn *et al.* 1994). In fish, the extent to which other vasoactive agents participate or interact with adenosine and the extent of endothelial involvement in this interaction are not known. The aims of the present study were (i) to re-evaluate the role of adenosine in the control of the trout coronary circulation in a preparation in which coronary resistance vessels are included, (ii) to evaluate the involvement of the endothelium and possibly nitric oxide in the action of adenosine, and (iii) to investigate any interaction between the effects of adenosine and noradrenaline. This study was performed on the intact perfused coronary system of the isolated non-working trout heart preparation. It is the first study of its kind to report a role of adenosine-mediated dilation in the modulation of blood flow through the fish coronary system.

Materials and methods

Animals

Specimens of rainbow trout *Oncorhynchus mykiss* (Walbaum) were obtained from a local fish farm and held in 400 l tanks with running dechlorinated water at 10–12 °C. The water was continuously aerated, and the animals were held under an artificial 12 h:12 h L:D photoperiod. They were fed twice a week and were allowed to acclimate for 10 days before the commencement of experiments. The mean mass of the fish was 0.38±0.01 kg (mean ± S.E.M., N=91).

Perfusion of the intact coronary tree

The heart was isolated and cannulated according to the method of Mustafa and Agnisola (1994) and mounted in a jacketed chamber maintained at 10±0.1 °C with a water bath. The coronary artery was perfused with a constant head pressure of 3 kPa. The perfusate from the coronary tree was drained into the atrium. Input and output pressures to the atrium and bulbus, respectively, were set to zero. In this preparation, any change in coronary resistance will result in a change in the perfusion flow. In some preparations, a constant-flow arrangement was used. In this case, saline was pumped into the coronary artery at a given rate using a Pharmacia P-3 peristaltic pump. The flow rate was set to obtain a basal input pressure of 3 kPa and was maintained at this level throughout the experiment. In the constant-flow preparations, changes in coronary resistance resulted in changes in head pressure. The ventricles were paced *via* platinum electrodes connected to a Grass S6 stimulator (stimuli 10 V, 20 ms) at a rate of 30 beats min^{-1}, which minimized interference from changes in extravascular resistance (Mustafa and Agnisola, 1994).

The preparation was perfused with Cortland saline containing (in $g\,l^{-1}$): NaCl, 7.25; KCl, 0.23; $MgSO_4{\cdot}7H_2O$, 0.23; $NaH_2PO_4{\cdot}H_2O$, 0.016; $Na_2HPO_4{\cdot}2H_2O$, 0.41; $CaCl_2{\cdot}2H_2O$, 0.37; glucose, 1.0; and polyvinylpyrrolidone, 10.0. The saline was gassed with 99.5 % O_2 and 0.5 % CO_2, and the pH was adjusted to 7.8 at 10 °C with $NaHCO_3$. Throughout the experimental protocols, the perfusate and test solutions were gassed with this mixture.

Coronary pressure was continuously recorded *via* a saline-filled sidearm with a Uniflow pressure transducer (Baxter, USA) connected to a computer for direct acquisition of data. Pressure measurements were expressed in kPa and were referenced to the level of saline in the perfusion chamber and corrected for cannula resistance. Coronary flow ($ml\,min^{-1}$) was determined according to the method described by Agnisola *et al.* (1994), modified to generate a computer-driven recording. The coronary resistance ($TPa\,s\,m^{-3}$) was calculated as: mean coronary pressure (kPa) × 0.06/coronary flow ($ml\,min^{-1}$), where 0.06 represents a factor necessary to convert coronary flow to $ml\,s^{-1}$ and pressure to TPa.

Experimental protocols

The preparation was allowed to stabilize until the coronary resistance in the presence of saline alone did not change between two measurements made 15 min apart. Because of the

high intra-heart variability in coronary resistance, basal coronary flow varied between 0.4 and 0.8 ml min^{-1} kg^{-1}. To generate a dose–response curve for adenosine, adenosine concentration was increased in a stepwise manner from 10^{-9} to 10^{-5} mol l^{-1}. Some experiments required pretreatment of the coronary tree with the adenosine receptor antagonist theophylline, the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine (L-NA) or noradrenaline, and the effects of these agents on coronary resistance were studied alone or in the presence of adenosine as indicated in the figure legends. A 15 min perfusion period was sufficient to achieve the maximal response to all agents tested.

Removal of the endothelium

The endothelium was chemically removed by intraluminal perfusion with 0.3 % 3-[(3-cholamidopropyl)dimethyl-ammonio]-2-hydroxy-1-propanesulphonate (CHAPS), a non-ionic, non-denaturing detergent, for 100–120 s (Tesfamariam and Halpern, 1987; Gustaffson *et al.* 1993). CHAPS was applied as a bolus with a Hamilton syringe inserted through a rubber septum, allowing it to be delivered close to the lumen of the coronary cannulae and thus avoiding any dilution. After denudation, the preparation was allowed to equilibrate with saline for 20 min. This method allowed responses to be observed both before and after the removal of the endothelium in the same preparation.

Measurement of nitrite levels

NO in oxygenated aqueous solution in the absence of oxyhaemoglobin is oxidized primarily to nitrite (NO_2^-) with little or no formation of NO_3^- (Ignarro *et al.* 1993). Measurements of NO_2^- concentration have been utilized to assess the release of NO in vascular systems (Abbe *et al.* 1995; Sessa *et al.* 1994). To measure NO production and release in the trout coronary system, we measured NO_2^- levels in the perfusate drained from the coronary preparation perfused with saline alone and then with saline containing L-arginine (L-Arg) or adenosine, and in presence of their antagonists alone or together with adenosine. At the end of each perfusion period, coronary pressure and flow were recorded and 1 ml of perfusate was collected. Nitrite was determined using the Griess reaction as described by Sessa *et al.* (1994). The perfusate was reacted with acidified sulphanilamide and *N*-(1-naphthyl)-ethylenediamine solution for 10 min at room temperature (22±1 °C). The absorption of the samples was then determined at 540 nm and compared with sodium nitrite standards. The NO_2^- standard curve demonstrated a linear relationship over the range 0.04–6.25 μmol l^{-1}.

Chemicals

The following analytical grade agents were used: adenosine (9-β-D-ribofuranosyladenine), theophylline, (–)-arterenol hydrochloride (noradrenaline), L-arginine free base, N^{ω}-nitro-L-arginine and CHAPS. All these chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA). All other chemicals were of the highest grade available. The stock solutions of adenosine, theophylline and noradrenaline were prepared in distilled water. L-Arginine and nitro-L-arginine were prepared in slightly acidic (0.03 mol l^{-1} HCl) water. CHAPS was dissolved in saline because it was applied directly as a bolus. All stock solutions were freshly prepared daily. The addition of stock solutions to the trout saline for final dilutions did not affect the final pH. Because of the sensitivity of noradrenaline to light, all containers holding this agent were wrapped in aluminium foil.

Statistics

In all the experiments, *N* represents the number of animals used for the perfused coronary preparation. Data are expressed as means ± standard error of the mean (S.E.M.). The effects on coronary resistance are presented as the percentage change from the basal coronary resistance. However, all statistical comparisons were performed on the resistance values using one-way analysis of variance (ANOVA) with replications. Tukey's or Dunnett's *post-hoc* tests for multiple comparisons were used, as appropriate. Individual values were compared using Student's paired or unpaired *t*-tests, as appropriate. Significance was accepted at $P<0.05$.

Results

Effect of adenosine on the coronary resistance

In the intact coronary system of the isolated non-working trout heart, increasing concentrations of adenosine elicited a biphasic vasoactive response (Fig. 1). At 10^{-9} mol l^{-1} adenosine, a statistically significant vasoconstriction was apparent; the effect was maximal at 10^{-8} mol l^{-1} adenosine. At 10^{-7} mol l^{-1} adenosine, a vasodilative response was elicited, which at 10^{-6} to 10^{-5} mol l^{-1} adenosine reached a value of 45 % compared with the control resistance. The percentage change in coronary resistance between 10^{-8} and 10^{-6} mol l^{-1} adenosine was more than 60 %.

The effects of theophylline, a non-specific antagonist of adenosine, were studied at both low and high concentrations. As shown in Fig. 2, 10^{-8} adenosine applied alone caused an increase

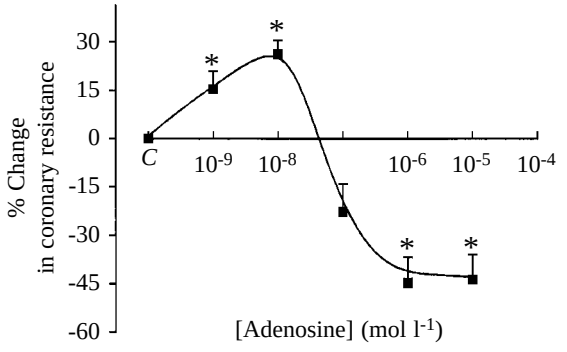

Fig. 1. Dose–response curve for adenosine of the perfused trout coronary system in the isolated non-working heart. Values are means + S.E.M. (N=9; mean animal mass 0.40±0.02 kg; basal resistance 0.53±0.12 TPa s m^{-3}). The effects were significantly dependent on the drug concentration ($P<0.001$). Asterisks indicate a significant percentage change from the control (C) value ($P<0.05$).

and 10^{-5} mol l^{-1} adenosine alone caused a decrease in resistance; these results correspond with the effects of adenosine at low and high concentrations reported in Fig. 1. The low and high concentrations of theophylline (10^{-7} or 10^{-4} mol l^{-1}) applied alone caused no significant change in the coronary resistance compared with the control value in the absence of adenosine. Pretreatment of the coronary system with 10^{-7} mol l^{-1} theophylline abolished the vasoconstrictory effect of 10^{-8} mol l^{-1} adenosine, and pretreatment with 10^{-4} mol l^{-1} theophylline abolished the vasodilatory effect of 10^{-5} mol l^{-1} adenosine.

The involvement of NO in adenosine-mediated vasodilation

Fig. 3 presents evidence for the involvement of NO in adenosine-mediated vasodilation. The results presented in Fig. 3A show that adenosine applied alone caused a significant vasodilation (more than 40 %) compared with the basal resistance. Pretreatment of the coronary system with L-NA caused a small but significant vasoconstriction. When the L-NA-pretreated coronary system was perfused with L-NA (10^{-4} mol l^{-1}) and adenosine (10^{-5} mol l^{-1}) together, the vasodilative response to adenosine was significantly diminished (60 %) compared with the effect of adenosine alone. These experiments (Fig. 3A) were performed at a constant intracoronary luminal head pressure so that any change in coronary resistance would change the perfusion flow.

To test whether adenosine perfusion directly accounted for the overall vasodilative response or whether the adenosine-mediated increase in flow in turn stimulated the release of NO, a constant intracoronary flow was maintained so that changes in coronary resistance resulted in changes in coronary head pressure. Under these constant-flow conditions (Fig. 3B), the vasodilatory response to adenosine alone was significantly smaller than the response to adenosine at constant pressure (Fig. 3A) and did not differ from the response in the presence of L-NA (Fig. 3B). The vasoactive responses of the coronary tree to perfusion with L-NA alone and to L-NA in the presence of adenosine at constant flow were similar to those under constant-pressure conditions.

Effects of adenosine in the presence of noradrenaline and L-NA

Noradrenaline (10^{-7} mol l^{-1}) induced a significant increase in coronary resistance compared with the control value (Fig. 4A). This increase in coronary resistance was completely abolished when the noradrenaline-pretreated coronary system was perfused with noradrenaline and adenosine (10^{-5} mol l^{-1}) together, and a significant vasodilation was then observed that was similar to that in presence of adenosine alone (Fig. 3A). The difference in coronary resistance after treatment with noradrenaline alone and after treatment with noradrenaline in the presence of adenosine is highly significant (Fig. 4A). The resistance of the coronary system precontracted with noradrenaline and then perfused with L-NA remained unchanged, suggesting that NO is not involved in this response. The addition of adenosine in the presence of both noradrenaline and L-NA abolished the vasoconstriction elicited by noradrenaline alone or noradrenaline in the presence of L-NA (Fig. 4B). A significant difference between the coronary resistance in the presence of adenosine, noradrenaline and L-NA (Fig. 4B) and that in the presence of noradrenaline and adenosine (Fig. 4A) was also apparent, confirming the effects of adenosine and L-NA reported in Fig. 3.

Nitrite production by the trout coronary system

Perfusion of the intact coronary system with L-Arg, adenosine, L-NA and theophylline caused significant changes in the rate of nitrite release into the perfusate. The testing of two agonists and two antagonists and the need for pretreatment with the antagonist necessitated the use of four different groups of fish. As shown in Fig. 5A, L-Arg (10 μmol l^{-1}) caused a small but significant increase in nitrite production compared with that produced by the untreated coronary tree (untreated 0.062±0.015 nmol min^{-1}, L-Arg-treated 0.086±0.016 nmol min^{-1}). Perfusion with adenosine (10 μmol l^{-1}) caused a significant increase in the rate of nitrite production, which was twice as great as that from the untreated

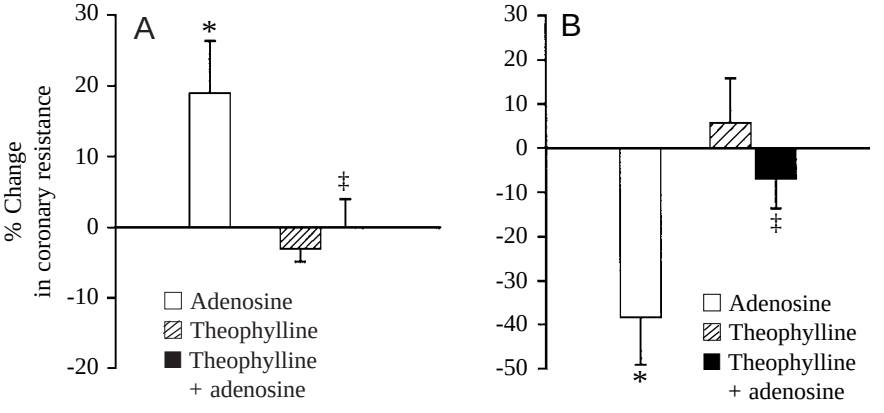

Fig. 2. Effect of theophylline on the responses to adenosine in the perfused coronary system of the isolated non-working trout heart. (A) Effect of 10^{-7} mol l^{-1} theophylline on the vasoconstrictory response induced by 10^{-8} mol l^{-1} adenosine; values are means ± S.E.M. (N=4; mean animal mass 0.39±0.02 kg; basal coronary resistance 0.422±0.048 TPa s m^{-3}). (B) The effect of 10^{-4} mol l^{-1} theophylline on the vasodilatory response induced by 10^{-5} mol l^{-1} adenosine; values are means ± S.E.M. (N=8; mean animal mass 0.36±0.01 kg; basal coronary resistance 0.604±0.081 TPa s m^{-3}). The perfusion with adenosine and theophylline together was always preceded by a perfusion with theophylline alone. The responses to adenosine in the presence of theophylline are compared with the responses to adenosine obtained in a different group of animals at the same concentrations but without pretreatment with theophylline (control, N=4; mean animal mass 0.41±0.02 kg; basal coronary resistance 0.413±0.042 TPa s m^{-3}). Asterisks indicate significant percentage changes from control values (P<0.05). ‡A significant difference in the effects of adenosine in the presence and absence of theophylline (P<0.05).

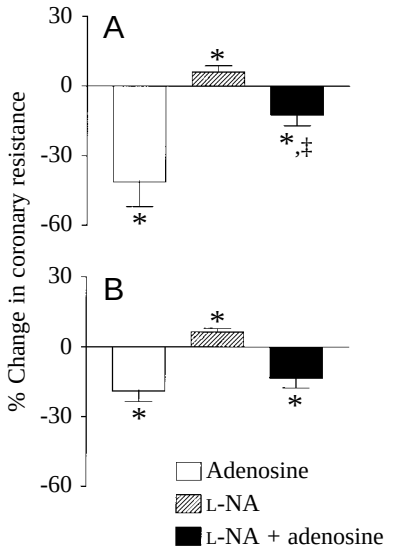

Fig. 3. Effect of L-NA (10^{-4} mol l^{-1}) on the vasodilatory response induced by adenosine (10^{-5} mol l^{-1}) in the coronary system of the isolated non-working trout heart, perfused under constant-pressure (A) and constant-flow (B) conditions. The perfusion with L-NA preceded the treatment with L-NA and adenosine together. The response to adenosine in the presence of L-NA is compared with the responses obtained in a different group of animals at the same concentration but without pretreatment with L-NA. (A) Values are means ± S.E.M. of six preparations (mean animal mass 0.29±0.02 kg; basal coronary resistance 0.432±0.072 TPa s m^{-3}; control, *N*=4, mean animal mass 0.41±0.02 kg; basal coronary resistance 0.392±0.048 Tpa s m^{-3}). (B) Values are means ± S.E.M. of six preparations (mean animal mass 0.40±0.01 kg; basal coronary resistance 0.657±0.086 TPa s m^{-3}; control, *N*=5, mean animal mass 0.40±0.02 kg; basal coronary resistance 0.95±0.09 Tpa s m^{-3}). Asterisks indicate a significant percentage change from the control value (P<0.05). ‡A significant difference between the effect of adenosine alone and in the presence of L-NA (P<0.001).

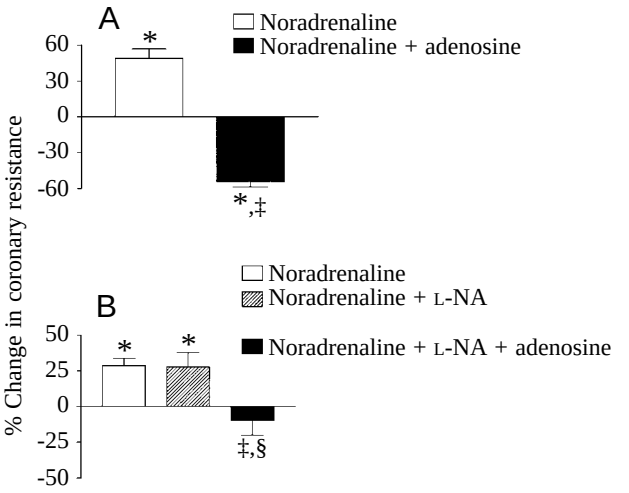

Fig. 4. (A) Effect of adenosine (10^{-5} mol l^{-1}) on the perfused coronary system of trout precontracted with noradrenaline (10^{-7} mol l^{-1}). Values are means ± S.E.M. (*N*=4; mean animal mass 0.41±0.01 kg; basal coronary resistance 1.29±0.22 TPa s m^{-3}). Asterisks indicate a significant percentage change from the control value (P<0.05). ‡A significant difference between the response to noradrenaline alone and in the presence of adenosine (P<0.05). (B) Effects of adenosine (10^{-4} mol l^{-1}) in the presence of noradrenaline (10^{-7} mol l^{-1}) and L-NA (10^{-4} mol l^{-1}) on the perfused coronary system of the isolated non-working heart. Values are means ± S.E.M. (*N*=6; mean animal mass 0.37±0.02 kg; basal coronary resistance 0.91±0.20 TPa s m^{-3}). Asterisks indicate a significant percentage change from the control value (P<0.05). ‡A significant difference between the effects of noradrenaline alone and in the presence of adenosine + L-NA (P<0.05). §A significant difference between the effects of adenosine in the presence of L-NA and noradrenaline and in the presence of noradrenaline only (see A) (P<0.05).

coronary tree (Fig. 5B, untreated 0.125±0.036 nmol min^{-1}, adenosine-treated 0.212±0.06 nmol min^{-1}). Pretreatment of the coronary tree with L-NA alone or in the presence of adenosine resulted in a significant decrease in the rate of nitrite release into the perfusate compared with the control (Fig. 5C, untreated 0.130±0.050 nmol min^{-1}, L-NA-treated 0.040±0.020 nmol min^{-1}, L-NA+adenosine-treated 0.034±0.010 nmol min^{-1}). Lastly, theophylline pretreatment alone or in the presence of adenosine caused a significant, but smaller, decrease in the rate of nitrite production compared with the control (Fig. 5D, untreated 0.106±0.028 nmol min^{-1}, theophylline-treated 0.060±0.019 nmol min^{-1}, theophylline+ adenosine-treated 0.058±0.007 nmol min^{-1}).

Removal of the endothelium by CHAPS and the effects of L-Arg and adenosine on coronary resistance

The involvement of the endothelium in L-Arg- and adenosine-mediated vasodilation was investigated in the coronary tree after the endothelium had been chemically denuded by CHAPS. The effects of L-Arg (Fig. 6A) and adenosine (Fig. 6B) were evaluated in two different groups of preparations. Both L-Arg and adenosine (10 μmol l^{-1}) perfusion elicited significant vasodilation compared with their respective basal coronary resistance (Fig. 6; controls, open columns). The preparation was washed with aerated Ringer's solution and a bolus of CHAPS was introduced. After CHAPS treatment, the preparation was allowed to equilibrate for another 20 min. After chemical denudation of the endothelium, the coronary resistance returned to the basal value (Fig. 6, left-hand hatched columns). At this stage, perfusion with 10 μmol l^{-1} L-Arg did not cause a vasodilation but resulted in a significant increase in coronary resistance compared with the basal value (Fig. 6A, filled column). The vasodilative response to adenosine also disappeared but the resistance was comparable to the basal value (Fig. 6B, filled column). The removal of the endothelium was considered successful because the vasodilative responses to L-Arg and adenosine disappeared while the response to noradrenaline was unaffected (Fig. 6, right-hand hatched columns). The effects of chemical denudation on nitrite production were evaluated in the perfusate from one of the above experiments (Fig. 6A), and the results are presented in Fig. 7. The

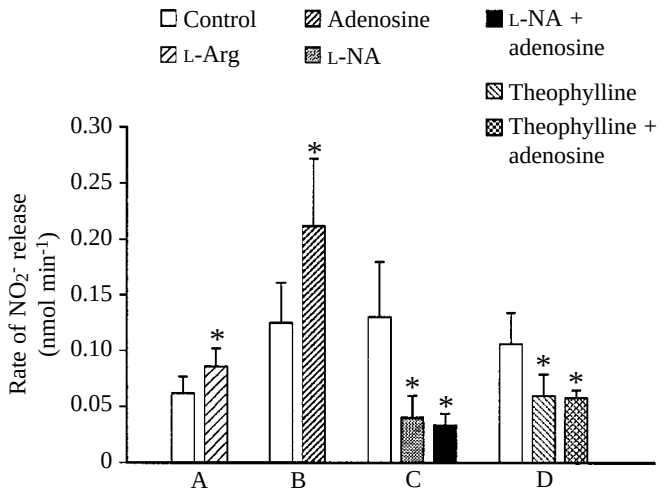

Fig. 5. Effect of L-arginine (L-Arg), adenosine, L-NA and theophylline on the rate of release of NO_2^- by the perfused coronary system in the isolated non-working trout heart. The effects of L-NA and theophylline on the adenosine-induced release are also reported. (A) Effect of 10^{-5} mol l^{-1} L-arginine. Values are means + S.E.M. (N=8; mean animal mass 0.41±0.03). (B) Effect of 10^{-5} mol l^{-1} adenosine. Values are means + S.E.M. (N=6; mean animal mass 0.34±0.02 kg). (C) Effects of 10^{-5} mol l^{-1} L-NA in the absence and in the presence of 10^{-5} mol l^{-1} adenosine. Values are means + S.E.M. (N=6; mean animal mass 0.40±0.02 kg). (D) Effects of 10^{-5} mol l^{-1} theophylline in the absence and in the presence of 10^{-5} mol l^{-1} adenosine. Values are means + S.E.M. (N=8; mean animal mass 0.33±0.01 kg). Asterisks indicate a significant difference from the control value (P<0.05).

rates of nitrite production under three different conditions, the basal level after perfusion with control saline (0.080±0.013 nmol min^{-1}), after CHAPS treatment (0.087±0.019 nmol min^{-1}) and after treatment with CHAPS and L-Arg together (0.079±0.016 nmol min^{-1}) were not significantly different. From these results, it is evident that CHAPS treatment causes some structural alterations as a result of which the endothelium loses its ability to respond to L-Arg and adenosine in a vasodilative manner.

Discussion

Vasomotive effects of adenosine in the intact coronary tree of trout

The present report demonstrates a biphasic response to adenosine in the intact coronary system of trout. The antagonistic effects of theophylline at both low (10^{-8} mol l^{-1}) and high (10^{-5} mol l^{-1}) levels of adenosine in abolishing the respective vasoconstrictory and vasodilative responses strongly suggests that the effect of adenosine may be mediated *via* A_1 and/or A_2 purine receptors. The observation of a biphasic effect of adenosine in the trout coronary system could be explained if the A_1 and A_2 receptors act in different ways according to the circulating concentration of adenosine. Studies concerning the effect of adenosine on the circulatory system of fish are scant and mostly provide support for a vasoconstrictory response to adenosine. In our hands, a significant vasodilatory response to adenosine is also apparent. This dissimilarity in vascular response to adenosine between earlier studies and the present report may be due to the two different types of preparation employed. In the studies of Small *et al.* (1990) on trout *Oncorhynchus mykiss*, Farrell and Davie (1991) on skate *Raja nasuta* and Farrell and Johansen (1995) on rainbow trout and steelhead trout (*Oncorhynchus mykiss*, sea-going trout), a 2–3 mm long section of the main coronary artery just before the bifurcation into the coronary tree was used for ring preparations. Furthermore, the response to adenosine in these preparations was studied after precontraction with KCl. Small *et al.* (1990) also raise the possibility that the structure of the fish coronary wall may be more delicate than that of mammalian

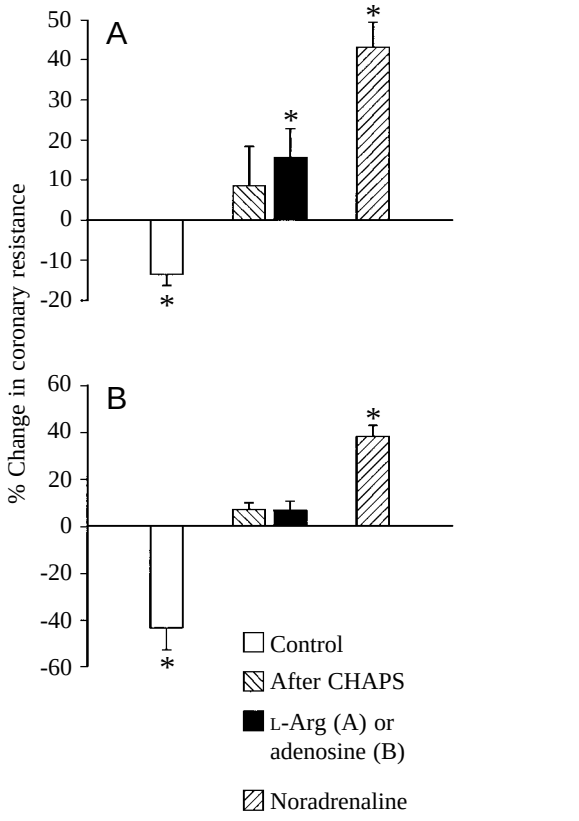

Fig. 6. Effect of chemical denudation of the endothelium by CHAPS (100 µl of 0.3 % solution, bolus injection) of the perfused coronary system of the isolated non-working trout heart on the vasodilation mediated by L-arginine (L-Arg) (10^{-5} mol l^{-1}) and adenosine (10^{-5} mol l^{-1}). The control columns represent the effects of L-arginine or adenosine before CHAPS treatment. The responses to 10^{-5} mol l^{-1} noradrenaline at the end of each experiment on CHAPS-treated preparations are also presented. Percentage values are relative to the resistance measured immediately before the treatment. Values are means ± S.E.M. (A) L-Arginine; N=10; mean animal mass 0.42±0.02; basal coronary resistance 0.414±0.052 TPa s m^{-3}; (B) adenosine; N=10; mean animal mass 0.30±0.01 kg; basal coronary resistance 0.548±0.041 TPa s m^{-3}. Asterisks indicate a significant percentage difference from the control value (P<0.05).

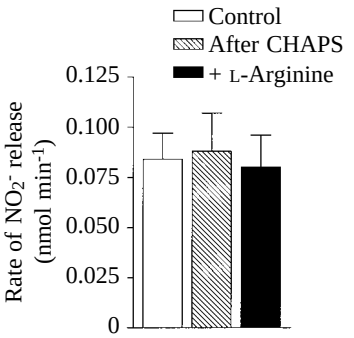

Fig. 7. The rate of NO_2^- release from the perfused coronary system of trout before and after treatment with CHAPS. The effect of L-arginine (10^{-5} mol l^{-1}) after CHAPS treatment is also presented. Values are means + S.E.M. (N=8; mean animal mass 0.42±0.02; basal coronary resistance 0.41±0.05 TPa s m^{-3}).

arteries and that the endothelium could be destroyed during the ring mounting process.

In contrast to these ring preparations from the main coronary artery, the present study describes vasoactive responses from the complete and intact coronary tree perfused at a physiological luminal pressure. Accordingly, the difference in response to adenosine between the earlier studies and the results presented here suggests that purine receptor distribution is not homogeneous in the fish coronary system. The coronary preparation used in the present study reflects the contribution to resistance changes from the arterioles and the microcirculation in addition to the main coronary artery. On the basis of the earlier results and the present studies, it may be assumed that a distinct vasoconstrictory response is dominant in the main coronary artery while a vasodilative response is predominant in the complete and intact coronary tree, which includes the microcirculation. This is in accordance with the suggestion of Chilian (1997) that various regulatory factors affect the coronary microvascular resistance with differing sensitivities related to the size of the vessels. For example, metabolic factors appear to have a dominant effect in the smallest coronary arterioles, and the response diminishes progressively in the upstream arterioles and small arteries. This is probably also true for fish species and, in our opinion, it was as a result of this heterogeneity that Farrell and Johansen (1995) were unable to show any endothelium-derived relaxing factor (EDRF)-mediated responses in their ring preparation of coronary artery from rainbow trout, steelhead trout and dogfish.

The involvement of nitric oxide in adenosine vasomotion of the trout coronary tree

Recently, the involvement of NO in adenosine-mediated vasodilation in coronary arteries has been reported for dogs (Parent *et al.* 1992; Zanzinger and Bassenge, 1993), for juvenile rabbits (Steinhorn *et al.* 1994) and for pigs (Abbe *et al.* 1995). In the trout coronary artery, the biphasic response to adenosine (Fig. 1) raises an interesting possibility. Assuming that this is not simply a function of differing adenosine doses, one explanation for this finding would be that the decrease in resistance (vasodilation at 10^{-5} mol l^{-1} adenosine) is partly flow-dependent. We have tried to investigate this problem by comparing the vasodilative responses to adenosine in preparations in which a constant pressure or a constant flow (Fig. 3A,B) is maintained throughout the experiment. Although the constant-flow preparation is non-physiological, it does resolve the role of flow as a confounding variable in measuring changes in resistance. When the flow is held constant, the adenosine response is halved (Fig. 3B) compared with the response to adenosine (Fig. 3A) at constant pressure, where any change in coronary resistance will result in a change in the perfusion flow. Under both these conditions, L-NA alone caused a slight but significant increase in the basal tone of the system. The vasodilatory response to adenosine in the presence of L-NA is significantly smaller than the response to adenosine alone under constant-pressure conditions (Fig. 3A), and it is similar to the response to adenosine alone under constant-flow conditions (Fig. 3B). These results indicate that the apparent response to adenosine in the presence of L-NA represents the vasodilation caused by the direct effect of adenosine. Under constant-pressure conditions, this vasodilation would be the stimulus for a flow-mediated vasodilatory response (Shen *et al.* 1995). The inhibition of such a response by L-NA (Fig. 3) suggests the existence of a shear-stress-dependent, NO-mediated vasodilation in the trout coronary system.

Release of nitric oxide from the intact coronary system

To confirm the involvement of nitric oxide in the vasodilation in response to adenosine, the stable product of nitric oxide, NO_2^-, was analysed in the perfusate from the intact coronary system of the isolated fish heart. Upon perfusion with L-Arg, the rate of release of NO_2^- increased significantly compared with the basal level of NO_2^- release in the presence of saline alone. Comparably, in the presence of adenosine alone, the rate of NO_2^- production was nearly doubled compared with the rate of NO_2^- release in the absence of adenosine. These results confirm the presence of L-Arg- and adenosine-mediated NO-producing systems in the trout coronary system. A further confirmation of this observation is provided by the demonstration of the attenuation of NO_2^- release in the presence of L-NA and theophylline, the respective antagonists of L-Arg and adenosine. Both antagonists inhibited the basal rate of NO_2^- release and the ability of adenosine to stimulate NO_2^- release. From these results, it can be concluded that nitric oxide is continuously released by the coronary artery under basal conditions, thus maintaining vascular tone and regulating the coronary blood flow. Since, as in mammals (Abbe *et al.* 1995), vasodilation in response to adenosine in the fish coronary preparation is accompanied by a concomitant and pronounced release of NO, it was important to evaluate the degree of involvement of the endothelium.

Endothelium-dependent mechanisms mediate the response to adenosine

To investigate whether smooth muscle relaxation in response

to adenosine is due to triggering of an extracellular purine receptor at the luminal surface and a NO-mediated vasodilation located in the endothelium, we evaluated the role of the endothelium. To this end, we treated the intracoronary lumen with CHAPS, which causes a functional removal of the endothelium in mammalian vascular preparations (Randall and Griffith, 1991; Gustaffson *et al.* 1993). We predicted that treatment of the fish coronary system with CHAPS would result in total removal of the vasodilative action of L-Arg and adenosine. After CHAPS treatment, the coronary resistance was similar in the absence or presence of adenosine, suggesting that this chemical treatment of the endothelium somehow resulted in an alteration to the structure of adenosine receptors making them unresponsive to adenosine. The coronary resistance in the presence of L-Arg after CHAPS treatment was 15–20 % higher than that in the absence of L-Arg after CHAPS treatment. This increase in resistance in response to L-Arg after CHAPS treatment is comparable with that induced by L-Arg perfusion after pretreatment with L-NA or its methyl ester (L-NAME) reported previously (Mustafa *et al.* 1997). This suggests that chemical alteration by CHAPS or the inhibition of nitric oxide synthase may result in the loss of the L-Arg-induced vasodilative response. This functional loss of the L-Arg response is also reflected when NO_2^- release after CHAPS treatment in the presence of L-Arg is evaluated. After CHAPS treatment, the ability of the coronary system to produce NO_2^- is comparable with that of the untreated preparation; however, after CHAPS treatment, L-Arg failed to cause any further increase in the rate of NO_2^- release. The constant rate of NO_2^- release we observed after CHAPS treatment in the absence and in the presence of L-Arg may be due to contributions from non-endothelial sources, including myocardial cells (Yamamoto *et al.* 1997). Perfusion of guinea pig and rat hearts with CHAPS is known to produce functional damage to the endocardial endothelium (McLeod and Piper, 1992; Schwarz *et al.* 1995). In our preparation, the perfusate draining from the coronary system was the only flow entering the atrium. Accordingly, since CHAPS empties into the atrium, it would also render the endocardial endothelium nonfunctional. Despite these limitations, the strict relationship observed between the rate of NO_2^- release and the coronary resistance suggests that NO_2^- measurements in the present study may be a fairly good index of NO production from the coronary endothelium.

Interplay between adenosine and noradrenaline in the trout coronary system

In the intact coronary system of trout, both noradrenaline and the specific α-adrenergic agonists phenylephrine and clonidine have been shown to cause coronary constriction (Agnisola *et al* .1996). The present study shows that the site of adrenergic coronary vasoconstriction probably lies at the level of the vascular smooth muscle (Fig. 6). After CHAPS treatment, a maximal response to noradrenaline was maintained, suggesting that the structure of the vascular smooth muscle of the coronary system was intact (Gustaffson *et al.* 1993). One of the aims of this study was to determine whether adenosine vasodilation can offset the adrenergic coronary constriction and whether NO is involved in the noradrenaline response in the fish coronary system. The addition of adenosine in the presence of noradrenaline completely offsets the increase in coronary resistance caused by noradrenaline (Fig. 4A), and adenosine-mediated relaxation prevails. This could imply that adenosine-mediated vasodilation competes with adrenergic-mediated vasoconstriction. The vasoconstriction caused by noradrenaline was not further potentiated when endogenous nitric oxide synthesis was inhibited with L-NA (Fig. 4B), excluding the possibility of the participation of NO in noradrenaline-mediated β-adrenergic vasoaction (Jones *et al.* 1993). Treatment with adenosine in the presence of noradrenaline and L-NA abolishes the vasoconstrictory response to noradrenaline, although the vasodilatory response is not as large as that in the presence of noradrenaline alone (Fig. 4A). This further supports the notion that it is adenosine alone and not NO that offsets the vasoconstrictory response to noradrenaline.

In conclusion, the data presented in this report demonstrate that the relaxant responses of the intact trout coronary tree to adenosine are endothelium-dependent. Theophylline and L-NA inhibited this vasodilative response. In addition, adenosine perfusion enhanced the rate of release of NO, which was diminished in the presence of theophylline and L-NA. This enhanced release of NO in response to adenosine was probably a flow-mediated effect of adenosine vasodilation, as shown by a comparison of the vasodilatory responses to adenosine under constant-pressure and constant-flow perfusion conditions. This effect would amplify the adenosine response when flow is allowed to increase. The relaxation response and NO production were endothelium-dependent, as indicated by the responses to treatment with CHAPS.

From this study, it may be inferred that adenosine may not be the sole metabolite to set the basal coronary tone. A mediator such as NO may be a more important autacoid that, at the arteriolar level, initiates vasodilatory changes, thus facilitating the blood flow in the wall of the ventricle (compacta). This is very likely to be the situation in trout because, in the conduit coronary ring preparations, adenosine causes a vasoconstritory response and nitric-oxide-mediated relaxation is not present (Farrell and Johansen, 1995). The vasodilatory response to adenosine described here suggests its involvement in matching coronary and myocardial function in fish under both normoxia and stress conditions, such as hypoxia and exercise. The observation that adenosine alone was able to offset the increases in coronary resistance elicited by noradrenaline could be of particular significance. In trout subjected to increased swimming activity or under hypoxia, levels of noradrenaline are known to increase to 10^{-7} mol l^{-1} (Butler *et al.* 1986; Perry and Reid, 1994), so an adrenergic constriction could predominate under these conditions, resulting in coronary hypoperfusion and a diminished blood supply to the compacta. The expected increased levels of adenosine would easily circumvent this situation.

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