Serotonin inhibition of 1-methylxanthine metabolism parallels its vasoconstrictor activity and inhibition of oxygen uptake in perfused rat hindlimb

S. RATTIGAN, G. J. APPLEBY, K. A. MILLER, J. T. STEEN, K. A. DORA, E. Q. COLQUHOUN and M. G. CLARK

Division of Biochemistry, Medical School, University of Tasmania, Hobart, Australia

ABSTRACT

The effect of serotonin (5-HT) on the metabolism of infused 1-methylxanthine (1-MX), a putative substrate of capillary endothelial xanthine oxidase (XO), and on the distribution of infused fluorescent microspheres (15 μm) by the artificially constant-flow perfused rat hindlimb preparation was investigated. 1-MX (5–100 μM) caused a slight inhibition of oxygen uptake ($\dot{V}O_2$) but was not vasoactive, either alone or with 5-HT. 1-MX was converted to 1-methylurate (1-MU) and this conversion was inhibited by allopurinol and xanthine. 5-HT (0.35 μM), which caused vasoconstriction and decreased $\dot{V}O_2$, also inhibited the conversion of 1-MX, indicated by a lowered venous perfusate steady-state 1-MU:1-MX ratio from 1.14 ± 0.02 to 0.71 ± 0.02 ($P < 0.001$), which is equivalent to the rate of conversion decreasing from 0.83 ± 0.03 to 0.63 ± 0.05 nmol min^{-1} g^{-1}. This change closely followed the time course for changes in $\dot{V}O_2$ and perfusion pressure and all three changes reversed in parallel when 5-HT was removed. Recoveries of 1-MU plus 1-MX at all times were high (100 ± 5%). 5-HT did not act to inhibit XO. When compared with vehicle alone, 5-HT had either no effect (plantaris, gastrocnemius white, tibialis, extensor digitorum longus, vastus and thigh), or increased microsphere content (soleus and gastrocnemius red, $P < 0.05$) of muscles with only bone showing a significant decrease ($P < 0.05$). Since 5-HT did not inhibit XO or alter the net flow to individual muscles in this constant-flow model, the inhibition of conversion of 1-MX to 1-MU is concluded to be the result of a 5-HT-mediated decrease in the access of 1-MX to capillary XO within individual muscles. Possibilities include the redirection of flow to capillaries either in muscle or in connective tissue closely associated with muscle, where resistance is low and effective surface area is less. 1-MX has potential as a marker for muscle nutritive flow.

Keywords flow distribution, non-nutritive flow, nutrient access, nutritive flow, skeletal muscle, vasoconstrictors.

Received 2 January 1997, accepted 8 April 1997

In 1980, Rippe & Folkow (1980) reported that 5-HT apparently reduced capillary surface area in the artificially constant-flow perfused rat hindquarter vascular bed. Subsequent studies in our laboratory showed that 5-HT in a similar constant-flow hindlimb preparation caused marked metabolic and performance changes also consistent with a reduced capillary surface area. These included the demonstration of inhibited $\dot{V}O_2$ (Dora *et al.* 1991, 1992), lactate release (Clark *et al.* 1995), insulin-mediated glucose uptake (Rattigan *et al.* 1993; perfusions at 25 °C without red blood cells), as well as inhibition of $\dot{V}O_2$, lactate release and aerobic tension development (Dora *et al.* 1994; perfusions at 25 °C without red blood

cells and at 37 °C with red blood cells). Vasoconstriction was essential for the inhibitory effects of 5-HT, as indicated, for example, by the fact that carbamyl choline, when present with 5-HT, blocked both vasoconstriction and the inhibitory effect on insulin-mediated glucose uptake and $\dot{V}O_2$ (Rattigan *et al.* 1993). In addition, treatment of isolated incubated soleus or extensor digitorum longus muscles failed to show any inhibitory effect of 5-HT on either insulin-mediated glucose uptake (Rattigan *et al.* 1993) or skeletal muscle tension development during stimulation (Dora *et al.* 1994).

Whereas vasoconstriction may lead to reduced pressure in capillaries, decreased diameter and hence

Correspondence: M. G. Clark, Division of Biochemistry, Medical School, University of Tasmania, GPO Box 252–58 Hobart 7001, Australia.

surface area for exchange, there is evidence that the reduction in capillary surface area may involve a discrete change in flow pattern within skeletal muscle that is mediated by 5-HT. For example, Dora *et al.* (1992) showed that the outcome from vasoconstriction by 5-HT in the constant-flow perfused rat hindlimb differed markedly from that due to low-dose (<1 μM) noradrenaline (NA). Thus, 5-HT vasoconstriction was found to inhibit $\dot{V}_{O_2}$, but NA was stimulatory. Also 5-HT-mediated changes were extracellular Ca^{2+}- and O_2-independent, while those of NA were dependent on both. Given that previous workers had shown vasoconstrictor sites for 5-HT to be on larger vessels (Hollenberg 1985, Lamping *et al.* 1989) and adrenergic receptors to be more concentrated on smaller vessels (Hirst & Edwards 1989), it seems likely that site-specific vasoconstriction could be responsible for flow redistribution, reduced capillary surface area and thus the decreased metabolism and tension development. In another study using fluoroscein-labelled dextran, we have recently noted that 5-HT apparently closes off a vascular space (entraps dye) which is reaccessed (dye released) with a second exposure to the agonist (Newman *et al.* 1996). Furthermore, it was noted from vascular corrosion casting with 30 μm methyl methacrylate that 5-HT gave vascular casts of considerably reduced mass when compared with casts from control or NA perfusions. In addition, and in agreement with Hammersen (1970), we found no evidence in the perfused rat hindlimb for arteriovenous shunts of greater than 12 μm diameter (Newman *et al.* 1996).

5-HT-mediated decrease in metabolism and tension development (Rattigan *et al.* 1993, Dora *et al.* 1994), as well as apparent capillary surface area (Rippe & Folkow 1980), could be the result of redirection of flow from muscle to less vascularized tissue of lower metabolic activity. In the present study, our aim was to apply a novel approach to directly assess capillary exposure and confirm that 5-HT-mediated inhibition of muscle metabolism and tension development was associated with reduced capillary surface area. It was hypothesized that if 5-HT mediated a decrease in capillary exposure, there would be a marked inhibition of the metabolism of infused 1-MX, a substrate for capillary endothelial xanthine oxidase (Jarasch *et al.* 1986, Parks & Granger 1986). Fluorescent microspheres were used to determine whether 5-HT-mediated vasoconstriction caused redistribution of flow between the different muscles or between muscle and non-muscle (bone, skin, adipose) tissue.

METHODS

Animals

All rats were cared for in accordance with the principles of the Australian code of practice for the care and use of animals for scientific purposes (1990, Australian Government Publishing Service, Canberra). Experimental procedures were approved by the committee on the ethical aspects of research involving animals of the University of Tasmania. Males of a local strain of hooded Wistar rats (180–200 g) were housed at 22 °C with free access to water and a commercial rat chow (Gibsons, Hobart) containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre with added vitamins and minerals. Rats were anaesthetized with pentobarbitone sodium (5–6 mg 100 g^{-1} body wt) i.p. before all surgical procedures.

Hindlimb perfusions

Hindlimb surgery was essentially as described by others (Ruderman *et al.* 1971), with additional details as given previously (Colquhoun *et al.* 1988). The foot and contralateral leg were tied off to prevent extraneous flow. A tight tie was also positioned around the entire animal at the approximate level of L3. The hindlimb was perfused at a constant flow rate (5.0 ± 0.1 mL min^{-1}) with buffer consisting of 118 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 8.3 mM glucose, 1.27 mM $CaCl_2$ and 2% (w/v) dialysed bovine serum albumin (fraction V). The perfusate was gassed with 95% O_2:5% CO_2 via a silastic tube oxygenator and the temperature brought to 25 °C by passage through a heat exchanger coil. Venous effluent flowed through a 0.5 mL thermostatically controlled (25 °C) chamber containing a Clark-type oxygen electrode and was discarded. $\dot{V}_{O_2}$ was calculated as described previously using the appropriate Bunsen coefficient (Dora *et al.* 1992). The effluent was periodically sampled (at times indicated) for measurement of flow and purines by high-performance liquid chromatography (HPLC). Perfusion pressure was constantly monitored via a side arm proximal to the aorta. The hindlimb was allowed to equilibrate for 40 min before infusions were commenced. At the flow rate of 5.0 ± 0.1 mL min^{-1}, approximately 15 g wet wt of muscle was perfused. This was assessed by weighing stained tissue following a perfusion in which 1% Evans blue was injected into the arterial cannula.

5-HT (35 μM) in 0.9% NaCl was freshly prepared before use and infused at a rate of 1 in 100 of the flow rate. Solutions of 1-MX or XAN were made by dissolving in 0.1 N NaOH and dilution in 0.9% NaCl. Infusions were made into the perfusion line prior to a small bubble trap that was continuously mixed by a magnetic stirrer. Infusion of vehicle alone had no effect.

Microsphere infusions

Constant-flow perfusions were conducted as described above. Equilibration time was 40 min, fol-

lowed by a 10 min infusion of either 5-HT or vehicle. At steady state for either 5-HT or vehicle, a bolus of 250 000 yellow-green 15 μm FluoSpheres[®] (Fluorescent Microsphere Resource Center, University of Washington, Seattle) was injected over 10 s, inducing a pressure spike of less than 5 mmHg and no change to the trace for venous P_{O^2}. Perfusion continued for a 10 min washout period with agonist infusion unchanged.

Extraction of microspheres

Muscles, including soleus, plantaris, gastrocnemius red, gastrocnemius white, tibialis, extensor digitorum longus, remaining calf muscles, vastus, remaining thigh and trunk muscle, as well as bone, skin and subcutaneous white adipose tissue of the perfused leg were dissected free. In addition, tissues were collected from unperfused regions to check for leakage. These included the tied-off foot of the perfused leg, the contralateral (unperfused) leg, associated skin, spinal and abdominal regions and the tail. All tissue samples were briefly blotted and weighed, then digested using the method of Van Oosterhout *et al.* (1995). Tissues were placed in 50 mL Greiner centrifuge tubes and digested in 5–10 volumes of 2 M ethanolic KOH (2 M KOH in 95% ethanol) containing 0.5% Tween-80 (Sigma), with heating to 58–60 °C for 2–6 h (depending on tissue size and effectiveness of digestion).

Tubes were centrifuged for 25 min at 2000 *g*. Supernatants were aspirated, retaining 1–2 mL at the base of the tube. Pellets were washed using 0.25% Tween-80 in distilled water, followed by distilled water alone. Following the final wash, supernatants were carefully aspirated to avoid disturbing the pellet but to retain minimal aqueous phase. A volume (5 mL) of 2-ethoxyethyl acetate was used to dissolve the microspheres, releasing the lipophilic fluorescent dye. Samples were allowed to extract overnight (4 °C) before being vortexed and centrifuged (2000 *g*, 20 min).

Fluorescence intensity of the organic phase was measured against a solvent blank using an Aminco-Bowman spectrophotofluorometer with excitation and emission settings at 495 nm and 510 nm, respectively. Calculation of microsphere numbers in each sample was achieved by reference to a standard curve produced from dilution of a known number of microspheres dissolved in the solvent. The data were expressed in terms of percentage of total infused.

Purine analyses

Perfusate samples (0.5 mL) were taken from the venous outflow, centrifuged briefly to remove any remaining blood cells, added to 0.1 mL of 2 M $HClO_4$ on ice and centrifuged for 5 min at 8000 *g*. The supernatant (0.4 mL) was neutralized with 2.5 M K_2CO_3. $KClO_4$ was allowed to precipitate at 0 °C and removed by centrifugation at 8000 *g* for 5 min. This supernatant was used directly for HPLC analysis or stored at −20 °C until use.

Reverse-phase HPLC was conducted essentially as described by Wynants *et al.* (1987) with an LKB instrument fitted with a Varian Polychrom Model 9065 diode array detector equipped with LC Star Workstation. Nucleosides and catabolites were separated on a Hibar Li Chrosorb Select B column (25 cm; 5 μm particles Merck) under isocratic conditions at 0.7 mL min^{-1} and identified by retention time and absorbance spectra. For a more rapid analysis of 1-MX and 1-MU, a buffer containing 10 mM sodium acetate, pH 4.0, with 6% acetonitrile was used.

Rat hindlimbs were intentionally perfused with cell-free perfusate to avoid complications due to the release or uptake of purine and purine catabolites by red blood cells. Under the conditions chosen of constant flow at 5.0 ± 0.1 mL min^{-1} hindlimb^{-1} (0.33 mL min^{-1} g^{-1} wet wt of muscle at 25 °C) with albumin-containing buffer, responses to vasoconstrictors and indices of high-energy phosphate status were similar to blood-perfused hindlimbs at 37 °C (e.g. see review by Clark *et al.* 1995 and references therein) and oedema was not excessive (Colquhoun *et al.* 1990). However, there was a small constant release of purines and purine catabolites as previously reported (Clark *et al.* 1990). Effluent concentrations after equilibration (40 min) were (in μM): uric acid, 5.34 ± 0.28; hypoxanthine, 0.09 ± 0.05; xanthine, 0.22 ± 0.05 (*n* = 5). These concentrations remained constant for up to 2 h, providing the conditions of perfusion were unaltered. However, peak resolution on HPLC was good and determination of 1-MX and 1-MU was unhindered by the presence of endogenous purines, pyrimidines and their catabolites.

Cytochrome oxidase

Rats were anaesthetized, and tissues were removed and homogenized in 2–5 mL 50 mM potassium phosphate buffer, pH 7.35. Cytochrome C (Boehringer-Manheim) was dissolved in water to make a 2% (w/v) solution, then reduced with excess ascorbate and passed through a small column of G.25 Sephadex. The cytochrome oxidase reaction was assayed in a recording spectrophotometer set at 550 nm and 37 °C with a continuously stirred cuvette (Wharton & Tzagoloff 1967). Reactants were 50 mM potassium phosphate buffer, pH 7.35 (2.0 mL), water (250 μL), reduced cytochrome C (100 μL) and homogenate (10 μL).

Xanthine oxidase and dehydrogenase

The total rate of xanthine oxidation was monitored at 293 nm and 37 °C in 50 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA essentially as described by Wajner & Harkness (1989). Xanthine oxidase and the combined activities of oxidase plus dehydrogenase were determined using oxygen and NAD^+ as electron acceptors, respectively. Activities were determined at 37 °C to maximize potential changes.

Statistical analysis

Unless indicated otherwise, the statistical significance of differences between groups of data were assessed by unpaired, two-tailed Student's *t*-test. Significant differences were recognized at $P < 0.05$.

RESULTS

Effects and metabolism of 1-MX

Figure 1, a representative set of traces, shows the effect on venous P_{O_2} and perfusion pressure of constant infusion of 0.35 μM 5-HT or vehicle alone and the effect of stepwise increasing doses of 1-MX (5–100 μM) in combination with each of the above. 5-HT alone increased the venous P_{O_2} from 370 to 490 mmHg, resulting in a net decrease in $\dot{V}_{O_2}$ from 7.2 to 3.8 μmol h^{-1} g^{-1}. Infused 1-MX (5–100 μM) slightly inhibited $\dot{V}_{O_2}$ but showed no vasoactivity whether added alone or in combination with 5-HT.

Since 1-MX showed neither vasoconstrictor nor vasodilator activity ($\leq$100 μM), it was infused into the perfusion medium to achieve 23 μM final concentration throughout the experiment with the purpose of assessing its conversion to 1-MU by endogenous xanthine oxidase of the hindlimb. Expectations, based on findings by others (Day *et al.* 1988), were that metabolism of 1-MX would result only in the formation of 1-MU and that neither 1-MX nor 1-MU would be taken up and assimilated by tissue. The data of Figure 2 show that these expectations were confirmed and that once steady state was reached ($\approx$20 min after commencement of infusion) the sum of 1-MX + 1-MU was constant ($\approx$23 μM) and thus recovery was quantitative. Furthermore, Table 1 shows that infusion of 20 μM allopurinol resulted in a marked decrease in the ratio of 1-MU:1-MX from 1.14 $\pm$ 0.02 to 0.16 $\pm$ 0.02 ($P <$ 0.001). This change did not reverse when the allopurinol was removed (Fig. 2) and the ratio of 1-MU:1-MX continued unchanged at $\approx$0.10 for at least another 20 min. The recovery (1-MU + 1-MX) was close to 100% before, during and after the period of allopurinol infusion (Fig. 2, Table 1). Unlike 1-MX and 1-MU, the recovery of allopurinol + oxypurinol was not quanti-
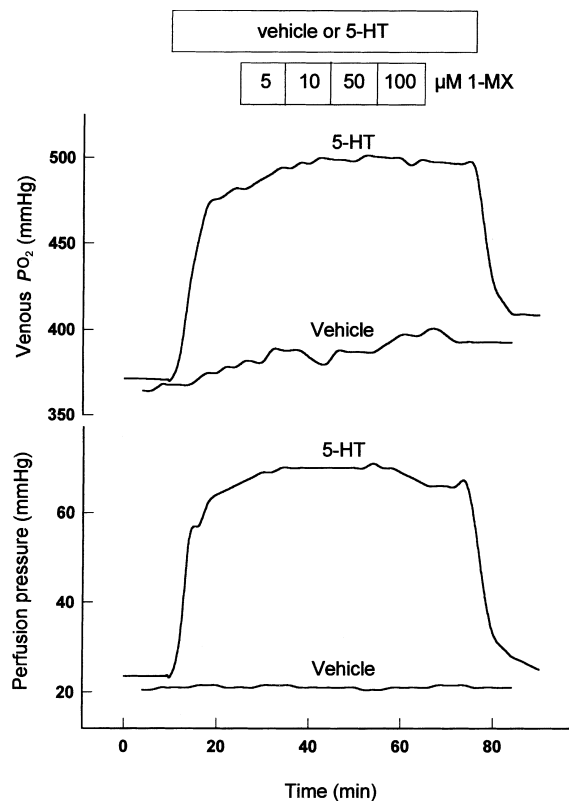

Figure 1 Time course for the effects of 1-MX on venous P_{O_2} and perfusion pressure of the constant-flow perfused rat hindlimb. Infusions of vehicle alone or 0.35 μM 5-HT were commenced at 10 min and continued for 65 min. 1-MX was infused as incremental doses of 5–100 μM starting at 25 min. Venous P_{O_2} and perfusion pressure were continuously recorded. A representative trace for each group is shown.

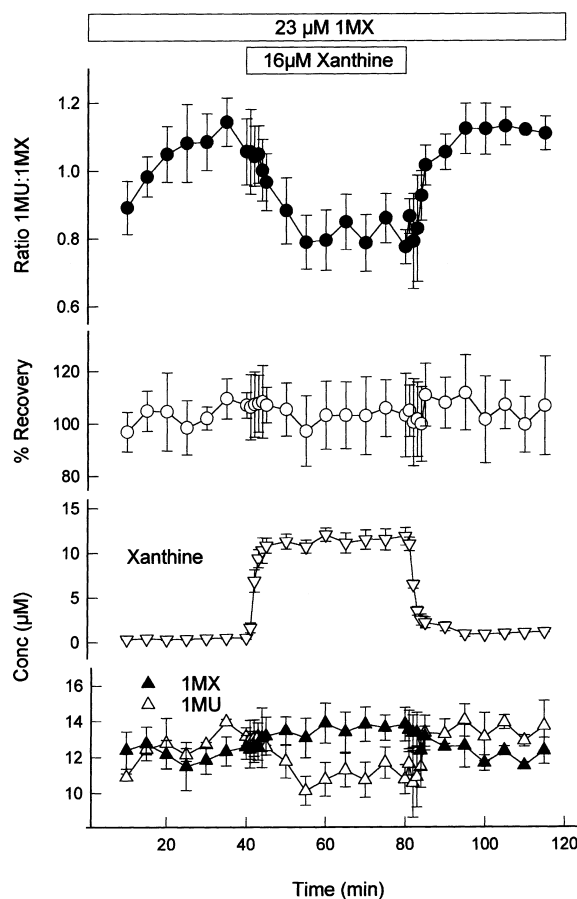tative, possibly being due to uptake and further metabolism of oxypurinol.

Infusion of xanthine (16 μM) also inhibited the conversion of 1-MX to 1-MU (Table 1, Fig. 3). However, since basal values for XAN were only 0.22 $\pm$ 0.05 μM, it appears that interference with 1-MX conversion to 1-MU would be minimal and may even be less following 5-HT when levels of XAN decrease below basal (see below).

The results in Figure 4 show the effect of 0.35 μM 5-HT on the conversion of 1-MX to 1-MU when 23 μM 1-MX was continuously infused into the constant-flow perfused hindlimb. 5-HT (0.35 μM) resulted in a marked decrease in the ratio of 1-MU:1-MX from 1.14 $\pm$ 0.02 to 0.71 $\pm$ 0.05 ($P < 0.05$) (Table 1), reflecting a decrease in conversion of 1-MX from 0.83 $\pm$ 0.03 to 0.63 $\pm$ 0.05 nmol min^{-1} g^{-1}. This was reversed upon 5-HT removal (Fig. 4). Examination of individual results for perfusate concentration of 1-MU and 1-MX indicated that the decrease in the ratio of 1-MU:1-MX due to 5-HT infusion resulted from an
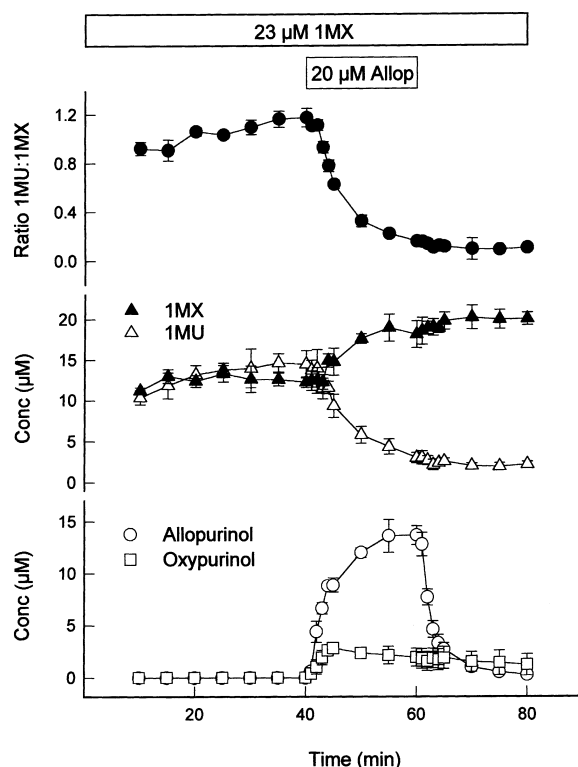
Figure 2 Effect of infusion of 20 μM allopurinol on the conversion of 1-MX to 1-MU by the constant-flow perfused rat hindlimb. 1-MX (23 μM) was infused for the duration of the perfusion ($\approx$80 min), and 20 μM allopurinol was introduced at 40 min and maintained until removal at 60 min. Samples of perfusate were collected for analysis of 1-MX, 1-MU, allopurinol and oxypurinol. Values shown are means $\pm$ SEM (n = 3). When not shown, error bars are within the symbol.

Figure 3 Effect of infusion of 16 μM XAN on the conversion of 1-MX to 1-MU by the constant-flow perfused rat hindlimb. Values shown are means $\pm$ SEM (n = 3).

equal change in the concentrations of each of $\approx$2.8 μM (Fig. 4). In addition, 5-HT-induced changes in the ratio of 1-MU:1-MX coincided closely with the 5-HT-induced changes in oxygen uptake (down from 7.3 $\pm$ 0.1 to 4.1 $\pm$ 0.1 μmol h^{-1} g^{-1}) and perfusion pressure (up from 26 $\pm$ 3 to 51 $\pm$ 5 mmHg) (Fig. 4b). Infusion of 5-HT also reversibly decreased the release of total endogenous purines (XAN + HX + UA) from 6.78 $\pm$ 0.14 to 4.14 $\pm$ 0.13 μM (P < 0.05). Guanosine and adenosine were undetectable (i.e. less than 0.05 μM) and thus changes due to 5-HT were insignificant. Uric acid

accounted for $\approx$93% of the total endogenous purines released by the hindlimb, whether or not 5-HT was present.
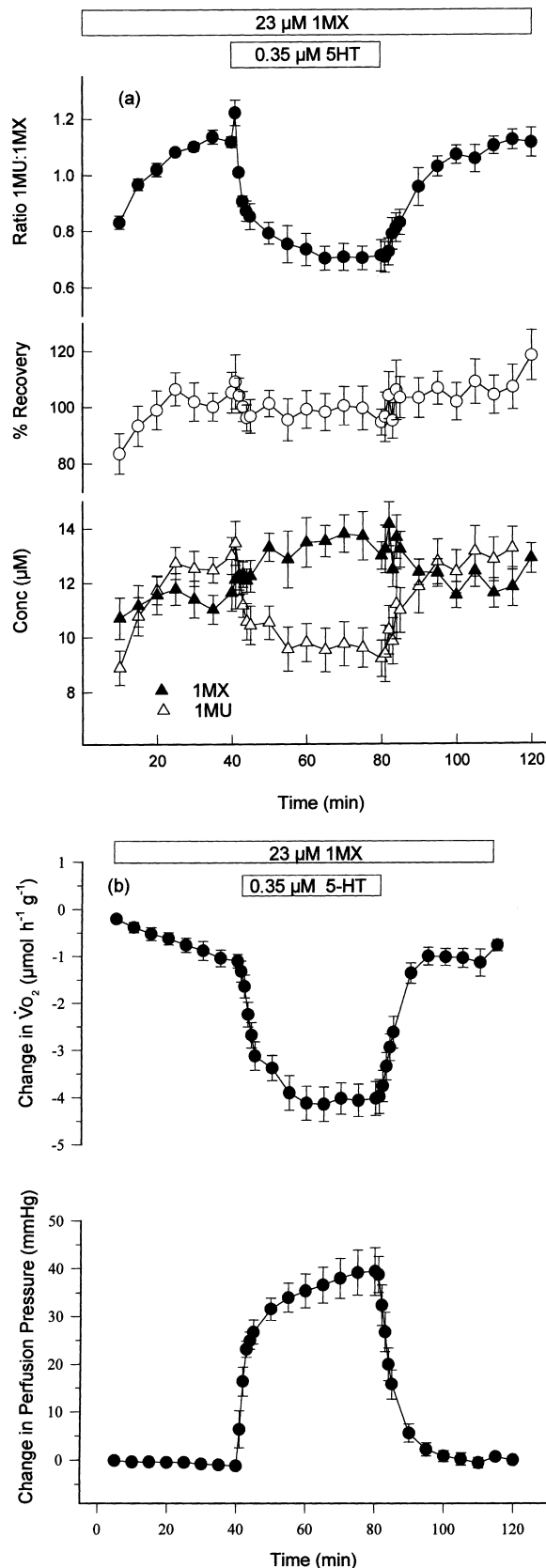
Microsphere recovery

The data of Figure 5 show that when compared with control (vehicle), 5-HT infusion did not alter microsphere recovery in muscle, spine, skin, white adipose tissue or tissues of the lower abdomen. Only bone showed a significant decrease. Of the muscles, only

Table 1 Effects of allopurinol xanthine and 5-HT infusions on the metabolism of 1-MX by the constant-flow perfused rat hindlimb

	[1-MX]	[1-MU]	[1-MU]/[1-MX]	Recovery (%)
Control	11.0 $\pm$ 0.5	12.5 $\pm$ 0.5	1.14 $\pm$ 0.02	102 $\pm$ 4
Allopurinol (20 μM)	18.1 $\pm$ 1.6*	3.0 $\pm$ 0.6*	0.16 $\pm$ 0.02*	92 $\pm$ 10
Xanthine (16 μM)	13.8 $\pm$ 1.0	10.8 $\pm$ 2.0	0.79 $\pm$ 0.17*	107 $\pm$ 9
5-HT (0.35 μM)	13.8 $\pm$ 0.7	9.5 $\pm$ 0.1*	0.71 $\pm$ 0.05*	102 $\pm$ 6

Concentrations are given as μmol L^{-1}.
*, significantly different (P < 0.05) from control values based on one-way analysis of variance and pairwise comparison procedures by Dunnett's method using SigmaStat (Jandel Scientific Software).

two, soleus and gastrocnemius red, showed a significant increase.

Most of the microspheres that were infused were recovered in the tissues shown (Fig. 5a). Thus recoveries in muscle, spine, bone, skin, white adipose tissue and the abdominal region of the perfused leg constituted 72.9 ± 4.1 and $75.7 \pm 2.6\%$ of the total infused for vehicle and 5-HT, respectively. Perfusate contained an additional 1.1 ± 0.4 (vehicle) and $0.8 \pm 0.4\%$ (5-HT), and the non-perfused regions, including the foot of the perfused leg, accounted for 2.3 ± 0.4 (vehicle) and $1.6 \pm 0.2\%$ (5-HT). Thus total recovery of microspheres was 76.4 ± 4.8 ($n = 5$, vehicle) and $77.8 \pm 2.9\%$ ($n = 5$, 5-HT). The difference was not significant. The remaining 22–23% of microspheres could not be found in other parts of the carcass and may represent the accumulated loss from processing of the 18 individual tissue samples listed in 'materials and methods'.

Of the microspheres recovered and shown in Figure 5(a), 40 ± 4.6 and $46.7 \pm 2.2\%$ were found in muscles of the perfused leg from vehicle and 5-HT infusions, respectively. The non-muscle tissues of bone, skin and white fat of the perfused leg accounted for $14.0 \pm 1.8\%$ (vehicle) and $8.6 \pm 1.4\%$ (5-HT). Again, there was no significant effect of 5-HT when compared with vehicle ($P > 0.05$). Finally, and as expected, some microspheres were recovered from perfused muscles surrounding the lower spine (below the L3 tie) and perfused tissues of the lower abdomen (below the L3 tie). For vehicle and 5-HT infusions, these represented 13.8 ± 1.4 and $11.8 \pm 2.0\%$ for spinal region, and 5.2 ± 0.9 and $8.5 \pm 1.3\%$ for the lower abdomen, respectively.

A breakdown of figures for microsphere recovery in muscle (Fig. 5(a)) is shown in Figure 5(b). When compared with vehicle alone, 5-HT significantly increased microsphere recovery in soleus and gastrocnemius red muscles; all other muscles were unaffected. Although not shown, values for microspheres recovered per gram fresh weight of muscle were not uniform and showed a ranked order: sol = g. red > tib = calf = plant > g. white = vast > thigh. This order was not affected by 5-HT.

Figure 4 Effect of 5-HT on (a) the conversion of 1-MX to 1-MU, and on (b) $\dot{V}O_2$ and perfusion pressure by the constant-flow perfused rat hindlimb. Following equilibration, 1-MX (23 μM) was infused for the duration of the perfusion ($\approx$120 min) and 0.35 μM 5-HT was introduced at 40 min and maintained until removal at 80 min. Samples of perfusate were collected for analysis of 1-MX and 1-MU using HPLC. Venous P_{O_2} and perfusion pressure were continuously recorded. $\dot{V}O_2$ was calculated from arteriovenous difference and flow. Values shown are means $\pm$ SEM ($n = 5$). When not visible, error bars are within the symbol.
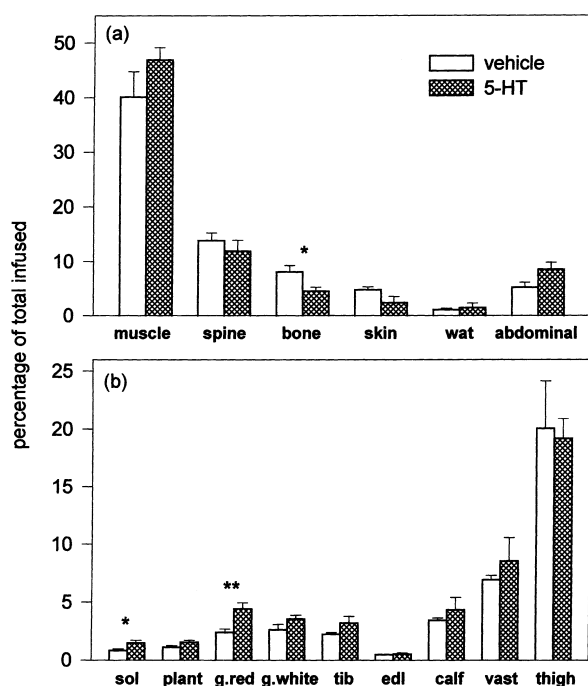
Figure 5 Recovery of fluorescent microspheres from (a) tissues, and (b) individual muscles of the constant-flow perfused rat hindlimb following 5-HT or vehicle treatment. Microspheres were recovered from individual muscles including soleus (sol), plantaris (plant), gastrocnemius red (g.red), gastrocnemius white (g.white), tibialis (tib), extensor digitorum longus (edl), remaining muscles on the lower leg (calf), vastus group (vast), remaining muscles on the upper leg and trunk after removal of the vastus group (thigh). 'Spine' included muscles of the lower back between the L3 tie and tail. 'Wat' represented subcutaneous white adipose tissue. 'Abdominal' included tissue of the urogenital region between the L3 tie and tail. Values are means ± SEM for $n = 5$. *$P < 0.05$; **$P < 0.01$ for 5-HT vs. vehicle alone.

Cytochrome oxidase

Total activity of cytochrome oxidase was determined as a potential indicator of relative oxidative capacity of the tissues of the hindlimb. Values were (μmol min^{-1} g tissue^{-1}): soleus, 25.2 ± 1.6 ($n = 9$); thigh, 7.2 ± 0.7 ($n = 3$); white adipose tissue, 3.2 ± 0.3 ($n = 9$); bone, 4.2 ± 0.6 ($n = 9$); and skin, 3.9 ± 0.4 ($n = 9$).

Xanthine oxidase

Values for the gastrocnemius-plantaris-soleus muscle group ($n = 3$), with and without 5-HT, showed xanthine oxidase to be 0.31 ± 0.05 and 0.27 ± 0.07 units g^{-1} protein and xanthine dehydrogenase 0.63 ± 0.07 and 0.65 ± 0.16 units g^{-1} protein, respectively. There was no significant effect of 5-HT on either activity.

DISCUSSION

The principal finding from this study was that 5-HT treatment of the artificially constant-flow perfused rat

hindlimb led to a marked rapid inhibition of the metabolism of infused 1-MX. The inhibition closely paralleled the 5-HT-mediated vasoconstriction and inhibition of $\dot{V}_{O_2}$, but did not appear to result from a direct inhibitory effect of 5-HT on the activity of XO when assayed in muscle extracts. In addition, there was no apparent 5-HT-mediated redistribution of flow between muscles or between muscle and non-muscle tissue.

The use of the XO substrate, 1-MX, as a marker of endothelial cell metabolism provided a novel method to assess capillary flow. XO is widely distributed amongst mammalian tissues, with liver and intestine having the highest levels (Parks & Granger 1986) but with detectable activities in heart, spleen, kidney and skeletal muscle (Jarasch *et al.* 1986). Immunohistochemical techniques suggest that the enzyme is found in the cytoplasm of capillary endothelial cells of a variety of tissues including skeletal muscle (Jarasch *et al.* 1986). The endothelium of larger vessels, however, does not contain significant amounts of XO (Jarasch *et al.* 1986). Metabolism of 23 μM 1-MX could be studied in the constant-flow perfused hindlimb under basal and 5-HT constricted states without 1-MX itself altering the haemodynamics (Fig. 1), and regardless of the perfusion conditions the sum of perfusate levels of 1-MX and 1-MU was always quantitative ($100 \pm 5\%$) during the experimental period. This metabolism of 1-MX was solely due to XO as it was blocked by the XO-specific inhibitor allopurinol (Emmerson *et al.* 1987) and reversibly inhibited by a competing substrate for XO (Figs 2 and 3). By conducting the non-recirculating, non-red blood cell perfusions at 25 °C, steady-state conditions for 1-MX metabolism could be obtained before, during and after 5-HT without deleterious effects to the hindlimb or interference from red blood cell nucleotides.

Thus overall the findings in this study confirm that the 5-HT effects on muscle previously reported, including inhibition of $\dot{V}_{O_2}$ (Dora *et al.* 1991, 1992, 1994), are unlikely to be the result of a direct inhibitory effect of this agonist on muscle. Rather, the data point to the importance of the vasoconstrictor action of 5-HT where haemodynamic changes contribute concomitantly to decreases in metabolism derived from both muscle and capillary endothelium. As such, the findings are consistent with a decrease in capillary surface area (Rippe & Folkow 1980), thus accounting for decreased nutrient availability for muscle metabolism and for decreased metabolism of infused 1-MX by capillary endothelium. Two possibilities could account for these effects. One is that, as a result of vasoconstriction by 5-HT, flow is redirected to vessels ending in low resistance/high capacity capillaries where transit time from arteriole to venule is low. Average capillary surface area would be low and thus exchange of sub-

strate (e.g. O$_2$) from lumen to surrounding parenchyma would be reduced, as would exchange of 1-MX with capillary endothelial cells. This would imply a heterogeneity of flow in the vascular circuits within each muscle, where haemodynamics and variations in local receptor activity interact to favour a given response. This notion is consistent with measurements of microsphere distribution (Fig. 5) and rests well with current views of the microvascular system of skeletal muscle (Duling & Damon 1987).

A second possibility accounting for the present data, including the apparent absence of a change in microsphere distribution at total constant flow, involves redirection of flow by 5-HT to functional vascular shunts. Some workers in the past have regarded these as separate anatomical entities where flow is not necessarily high but nutrient exchange is poor because of the relative isolation from muscle cells. Capillaries of these functional shunts may be shorter or contain lesser amounts of XO, and therefore potential exposure of a circulating substrate to endothelial enzymes would be less. Candidate vessels may be those located on septa and tendon of each muscle as described by Barlow *et al.* (1961) and Grant & Payling Wright (1970) and referred to earlier as 'metarteriolar thoroughfare channels in the cleavage planes' by Zweifach & Metz (1955). Although sufficiently insulated from muscle to prevent nutrient exchange, these vessels are likely to be removed intact with the muscle during dissection. Since only total microsphere recovery for each muscle was examined in the present study, a change in the proportion of microspheres lodging in muscle connective tissue or muscle itself would not be apparent.

A precedent for redistribution of flow between muscle capillaries and adjacent connective tissue is the intravital microscopy study of rabbit tenuissimus muscle by Borgström *et al.* (1988). These authors showed that β-adrenergic stimulation by topically administered isoproterenol caused a fractional redistribution of microvascular blood flow from the muscle tissue proper to the adjacent connective tissue. Similarly we have noted that isoproterenol opposed noradrenaline-mediated vasoconstriction and the accompanying increased $\dot{V}$O$_2$ in the constant-flow perfused rat hindlimb (Colquhoun *et al.* 1990). Since the vascular arrangement of the tenuissimus muscle may be representative of the basic unit present in hind leg musculature (Myrhage & Eriksson 1980), it is possible that vessels of the connective tissue associated with each muscle have been those to receive preferred flow as a consequence of 5-HT-mediated vasoconstriction.

Thus, true arteriovenous shunting in muscles may be a property of vessels of enveloping fascia and connective tissue septa (Hudlická 1973). The question of whether these vessels are capable of carrying sufficient flow to significantly affect nutrient access to muscle would, however, seem to be unresolved. On the one hand, Hammersen (1970) stated that such shunts were much too small to have any functional importance in muscle circulation. On the other hand, Grant & Payling Wright (1970) claimed that the arteriovenous channels of rat biceps femoris tendon were numerous, ranged in size from 12 to 25 μm (when dilated), and were 200–600 μm in length from their origin to where they widen to become venous. Recently, we have noted that apparent flow in these tendon vessels is increased when 5-HT vasoconstricts to decrease $\dot{V}$O$_2$ in the constant-flow perfused rat hindlimb (Newman *et al.* 1997).

In conclusion, the present findings suggest that 1-MX metabolism to 1-MU is a potential marker for the decrease in muscle nutritive flow or nutrient access that accompanies 5-HT-mediated vasoconstriction. The findings are also consistent with a previous report by Rippe & Folkow (1980) that 5-HT treatment of the constant-flow perfused rat hindlimb leads to a decrease in capillary surface area, but the microsphere data demonstrate that no redistribution of the bulk of flow from one muscle type to another or from muscle to non-muscle tissues such as skin, bone or adipose occurs.

REFERENCES

Barlow, T.E., Haigh, A.L. & Walder, D.N. 1961. Evidence for two vascular pathways in skeletal muscle. *Clin Sci* **20**, 367–385.

Borgström, P., Lindbom, L., Arfors, K-E. & Intaglietta, M. 1988. β-Adrenergic control of resistance individual vessels in rabbit tenuissimus muscle. *Am J Physiol* **254**, H631–H635.

Clark, M.G., Colquhoun, E.Q., Rattigan, S., Dora, K.A., Eldershaw, T.P.D., Hall, J.L. & Ye, J. 1995. Vascular and endocrine control of muscle metabolism. *Am J Physiol* **268**, E797–E812.

Clark, M.G., Richards, S.M., Hettiarachchi, M., Ye, J.-M., Appleby, G.J., Rattigan, S. & Colquhoun, E.Q. 1990. Release of purine and pyrimidine nucleosides and their catabolites from the perfused rat hindlimb in response to noradrenaline, vasopressin, angiotensin II and sciatic-nerve stimulation. *Biochem J* **266**, 765–770.

Colquhoun, E.Q., Hettiarachchi, M., Ye, J.-M., Rattigan, S. & Clark, M.G. 1990. Inhibition by vasodilators of noradrenaline and vasoconstrictor-mediated but not skeletal muscle contraction-induced oxygen uptake in the perfused rat hindlimb; implications for non-shivering thermogenesis in muscle tissue. *Gen Pharmacol* **21**, 141–148.

Colquhoun, E.Q., Hettiarachchi, M., Ye, J.-M., Richter, E.A., Hniat, A.J., Rattigan, S. & Clark, M.G. 1988. Vasopressin and angiotensin II stimulate oxygen uptake in the perfused rat hindlimb. *Life Sci* **43**, 1747–1754.

Day, R.O., Miners, J., Birkett, D.J., Graham, G.G. & Whitehead, A. 1988. Relationship between plasma oxipurinol concentrations and xanthine oxidase activity in volunteers dosed with allopurinol. *Br J Clin Pharmac* **26**, 429–434.

Dora, K.A., Colquhoun, E.Q., Hettiarachchi, M., Rattigan, S. & Clark, M.G. 1991. The apparent absence of serotonin-mediated vascular thermogenesis in perfused rat hindlimb may result from vascular shunting. *Life Sci* **48**, 1555–1564.

Dora, K.A., Rattigan, S., Colquhoun, E.Q. & Clark, M.G. 1994. Aerobic muscle contraction impaired by serotonin-mediated vasoconstriction. *J Appl Physiol* **77**, 277–284.

Dora, K.A., Richards, S.M., Rattigan, S., Colquhoun, E.Q. & Clark, M.G. 1992. Serotonin and norepinephrine-mediated vasoconstriction in the perfused rat hindlimb have different oxygen requirements. *Am J Physiol* **262**, H698–H703.

Duling, B.R. & Damon, D.H. 1987. An examination of the measurement of flow heterogeneity in striated muscle. *Circ Res* **60**, 1–13.

Emmerson, B.T., Gordon, R.B., Cross, M. & Thomson, D.B. 1987. Plasma oxipurinol concentrations during allopurinol therapy. *Br J Rheumatol* **26**, 445–449.

Grant, R.T. & Payling Wright, H. 1970. Anatomical basis for non-nutritive circulation exemplified by blood vessels of rat biceps femoris tendon. *J Anat* **106**, 125–133.

Hammersen, F. 1970. The terminal vascular bed in skeletal muscle with special regard to the problem of shunts. In: C. Crone & N.A. Lassen (eds) *Capillary permeability: the transfer of molecules and ions between capillary blood and tissue*, pp. 351–365. Munksgaard, Copenhagen.

Hirst, G.D.S. & Edwards, F.R. 1989. Sympathetic neuroeffector transmission in arteries and arterioles. *Physiol Rev* **69**, 546–605.

Hollenberg, N.K. 1985. Large and small vessel responses to serotonin in the peripheral circulation. *J Cardiovasc Pharmacol* **7**, S89–S91.

Hudlická, O. 1973. *Muscle blood flow. Its relation to muscle metabolism and function*. Swetz & Zeitlinger BV, Amsterdam.

Jarasch, E.-D., Bruder, G. & Heid, H.W. 1986. Significance of xanthine oxidase in capillary endothelial cells. *Acta Physiol Scand* **548**, 39–46.

Lamping, K.G., Kanatsuka, H., Eastham, C.L., Chilian, W.M. & Marcus, M.L. 1989. Nonuniform vasomotor responses of the coronary microcirculation of serotonin and vasopressin. *Circ Res* **65**, 343–351.

Myrhage, R. & Enriksson, E. 1980. Vascular arrangements in hindlimb muscles of the cat. *J Anat* **131**, 1–17.

Newman, J.M.B., Dora, K.A., Rattigan, S., Edwards, S.J., Colquhoun, E.Q. & Clark, M.G. 1996. Norepinephrine and serotonin vasoconstriction in rat hindlimb control different vascular routes. *Am J Physiol* **270**, E689–E699.

Newman, J.M.B., Steen, J.T. & Clark, M.G. 1997. Vessels supplying septa and tendons as functional shunts in perfused rat hindlimb. *Microvasc Res* (in press).

Parks, D.A. & Granger, D.N. 1986. Xanthine oxidase: biochemistry, distribution and physiology. *Acta Physiol Scand* **548**, 87–99.

Rattigan, S., Dora, K.A., Colquhoun, E.Q. & Clark, M.G. 1993. Serotonin-mediated acute insulin resistance in the perfused rat hindlimb but not in incubated muscle: a role for the vascular system. *Life Sci* **53**, 1545–1555.

Rippe, B. & Folkow, B. 1980. Simultaneous measurements of capillary filtration and diffusion capacities during graded infusions of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) into the rat hindquarter vascular bed. *Acta Physiol Scand* **109**, 265–273.

Ruderman, N.B., Houghton, C.R.S. & Hems, R. 1971. Evaluation of the isolated perfused rat hindquarter for the study of muscle metabolism. *Biochem J* **124**, 639–651.

Van Oosterhout, M.F.M., Willigers, H.M.M., Reneman, R.S. & Prinzen, F.W. 1995. Fluorescent microspheres to measure organ perfusion: validation of a simplified sample processing technique. *Am J Physiol* **269**, H725–H733.

Wajner, M. & Harkness, R.A. 1989. Distribution of xanthine dehydrogenase and oxidase activities in human and rabbit tissues. *Biochim Biophys Acta* **991**, 79–84.

Wharton, D.C. & Tzagoloff, A. 1967. Cytochrome oxidase from beef heart mitochondria. In: R.W. Estabrook & M.E. Pullman (eds) *Methods in enzymology (oxidation and phosphorylation),* vol. **10**, pp. 245–250. Academic Press, New York.

Wynants, J., Petrov, B., Nijhof, J. & Van Belle, H. 1987. Optimization of a high-performance liquid chromatographic method for the determination of nucleosides and their catabolites. *J Chromatogr* **386**, 297–308.

Zweifach, B.W. & Metz, D.B. 1955. Selective distribution of blood through the terminal vascular bed of mesenteric structures and skeletal muscle. *Angiology* **6**, 282–289.