Differential control of glycogenolysis and flow by arterial and portal acetylcholine in perfused rat liver

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The effects of acetylcholine on glucose and lactate balance and on perfusion flow were studied in isolated rat livers perfused simultaneously via the hepatic artery (100 mmHg, 25–35 % of flow) and the portal vein (10 mmHg, 75–65 % of flow) with a Krebs-Henseleit bicarbonate buffer containing 5 mM-glucose, 2 mM-lactate and 0.2 mM-pyruvate. Arterial acetylcholine (10 μ M sinusoidal concentration) caused an increase in glucose and lactate output and a slight decrease in arterial and portal flow. These effects were accompanied by an output of noradrenaline and adrenaline into the hepatic vein. Portal acetylcholine elicited only minor increases in glucose and lactate output, a slight decrease in portal flow and a small increase in arterial flow, and no noradrenaline and adrenaline release. The metabolic and haemodynamic effects of arterial acetylcholine and the output of noradrenaline and adrenaline were strongly inhibited by the muscarinic antagonist atropine (10 μ M). The acetylcholine-dependent alterations of metabolism and the output of noradrenaline were not influenced by the α_1 -blocker prazosin (5 μ M), whereas the output of adrenaline was increased. The acetylcholine-dependent metabolic alterations were not inhibited by the β_2 -antagonist butoxamine (10 μ M), although the overflow of noradrenaline was nearly completely blocked and the output of adrenaline was slightly decreased. These results allow the conclusion that arterial, but not portal, acetylcholine caused sympathomimetic metabolic effects, without noradrenaline or adrenaline being involved in signal transduction.

INTRODUCTION

The liver is innervated by sympathetic and parasympathetic nerves, which contain both afferent and efferent fibres [1–9]. In intact animals [10,11] pre-ganglionic stimulation of the splanchnic nerve or post-ganglionic stimulation of the hepatic nerves was shown to increase hepatic glucose output, to decrease oxygen consumption and to decrease hepatic flow. In isolated perfused liver [12] perivascular nerve stimulation caused, via α -receptors, an increase in glucose and lactate output [13,14], decreases in oxygen uptake [15], ketogenesis [16], ureagenesis [17] and ammonia uptake [17], an efflux of Ca^{2+} [18], an overflow of noradrenaline into the hepatic vein [14,19] and a decrease in arterial and portal flow [14] combined with an intrahepatic redistribution [15]. In rabbits and cats, stimulation *in vivo* of efferent fibres of the hepatic vagal branch increased glycogen synthesis by an activation of glycogen synthase, decreased glucose release from the liver and opened some sinusoids that were previously closed [10–12]. In the isolated rat liver perfused via the portal vein, only parasympathetic stimulation of the hepatic nerves, i.e. stimulation of all nerve bundles in the presence of α - and β -sympathetic antagonists, caused an increase in glucose uptake only in the presence of insulin, and antagonized the glucagon-dependent increase in glucose release [20]. Infusion of acetylcholine to mimic parasympathetic nerve actions was without effect on glucose balance in two studies [20,21], but decreased glucose output in another [22].

Certain regulatory effects in hepatic metabolism and haemodynamics appeared to be detectable in isolated rat livers only when they were perfused simultaneously via the portal vein with low pressure and via the hepatic artery with high pressure [14,23,24]. In this bivascular liver perfusion the β -blocker propranolol inhibited partially the metabolic nerve actions even when given via the portal route only, but it was ineffective in portally perfused livers [24]. Apparently β -receptors modulating

the α_1 -sympathetic actions of the liver nerves were accessible only when the artery was also perfused at high pressure.

Therefore it seemed possible that cholinergic receptors or ganglia could be reached only in rat livers which were simultaneously perfused via both hepatic inflows. Thus it was the aim of the present work to investigate whether acetylcholine might have a direct influence on carbohydrate metabolism and haemodynamics in the bivascularly perfused isolated rat liver. It was found that arterial acetylcholine caused an increase in glucose and lactate release and a slight decrease in arterial and portal flow, and that these effects were accompanied by a pronounced output of noradrenaline and adrenaline. Atropine, but not the α_1 -antagonist prazosin or the β_2 -blocker butoxamine, inhibited the acetylcholine-dependent alterations in carbohydrate metabolism. Portal acetylcholine caused only minor increases in glucose and lactate output and slight alterations in perfusion flow without any noradrenaline and adrenaline output.

EXPERIMENTAL

Materials

All chemicals were of reagent grade, from commercial sources. Enzymes and BSA were purchased from Boehringer (Mannheim, Germany), acetylcholine, neostigmine, atropine and propranolol were from Sigma (München, Germany), and prazosin was from Pfizer (Karlsruhe, Germany). Butoxamine was generously given by Wellcome (Grossburgwedel, Germany).

Animals

Male Wistar rats (200–250 g) were obtained from Winkelmann (Borchen, Germany). At least 1 week before the experiment they were subjected to a 12 h-day/-night rhythm with free access to food (standard diet of Ssniff, Soest, Germany). All experiments were started at 09:00 h. The animals were anaesthetized by

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intraperitoneal injection of pentobarbital (60 mg/kg body wt.).

Liver perfusion and acetylcholine infusion

The liver was perfused *in situ* without recirculation in a 37 °C cabinet either via the portal vein only or via both the portal vein and the hepatic artery. In the portal perfusion system livers were perfused under constant pressure of about 10 mmHg. For the arterial plus portal perfusion the portal vein, the aorta and the inferior caval vein were cannulated and all branching vessels were ligated. Rats were killed after cannulation of the portal vein and before cannulation of the aorta and inferior caval vein, by an intracaval injection of Nembutal (60 mg/kg body wt.). The preparation technique has been described in more detail previously [23]. Thus a bivascular liver perfusion was established with a low-pressure component via the portal vein (10 mmHg, 80–60 % of total flow) and a high-pressure component via the hepatic artery (100 mmHg, 20–40 % of total flow) [14,23,24]. In both systems a total flow rate of about 4 ml/min per g of liver was maintained. The experiments were started after a pre-perfusion period of 30 min. Acetylcholine was infused between 40 and 45 min of perfusion. Antagonists of sympathetic and cholinergic receptors were added to the perfusion medium throughout the whole experiment. Total perfusion flow was quantified by fractionating the effluent into calibrated tubes. In liver perfusions via both the portal vein and the hepatic artery, the portal flow was measured with a Hellige SMS-302 flowmeter placed into the portal inflow. The arterial flow was obtained as the difference between total and portal flow.

Perfusion medium

The basic perfusion medium was a Krebs–Henseleit buffer (24 mM- NaHCO_3 , 122 mM- NaCl , 4.7 mM- KCl , 2.5 mM- CaCl_2 , 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4) containing 5 mM-glucose, 2 mM-lactate, 0.2 mM-pyruvate, 0.5 % BSA and 1 μM -neostigmine. The medium was equilibrated with O_2/CO_2 (19:1). The effluent medium was collected after an initial period of 30 min and cooled on ice.

Determination of metabolites

Metabolites were measured by standard enzymic techniques [25]: glucose with glucose dehydrogenase and lactate with lactate dehydrogenase and glutamate-pyruvate transaminase. Adrenaline and noradrenaline were measured by h.p.l.c. with electrochemical detection using the Waters system (Eschborn, Germany). This method allowed a sensitive and selective detection of catecholaminergic metabolites [26]. The specificity of the separation of adrenaline and noradrenaline was ascertained by addition of authentic catecholamines to some samples of the effluent, which resulted in symmetrical peaks and additivity of the quantification.

Evaluation

The effects of acetylcholine on metabolism and haemodynamics were evaluated by calculating the area under the curve of the acetylcholine-dependent alterations of each metabolic and haemodynamic parameter. When baselines of metabolic and/or haemodynamic parameters after infusion differed from those of the pre-stimulation period, both baselines were connected for evaluation of the area under the curve as indicated in the Figures. Significance was tested with Student's *t* test for paired or unpaired values.

RESULTS

In the present study, the effects of the parasympathetic

transmitter acetylcholine on glucose and lactate metabolism and on perfusion flow were investigated in rat livers perfused *in situ* either exclusively via the portal vein or simultaneously via the hepatic artery and portal vein. After 40 min of perfusion, acetylcholine was infused for 5 min via the hepatic artery, the portal vein or simultaneously via both vessels. The acetylcholinesterase inhibitor neostigmine was added to the perfusion medium, to decrease acetylcholine degradation.

Lack of effects of acetylcholine in rat livers perfused via the portal vein only

Acetylcholine infused via the portal vein with a vascular and sinusoidal concentration of 10 μM did not influence glucose and lactate metabolism and perfusion flow (Fig. 1).

Effects of arterial, but not portal, acetylcholine in rat livers perfused via the hepatic artery and portal vein

Metabolism. In order to compare the results obtained in the bivascular liver perfusions with those in the monovascular system, it is possible to add effectors with equal concentrations either to the inflow vessels or to the sinusoids. In the first bivascular perfusion series with an infusion of acetylcholine via the hepatic artery, the concentration was adjusted to a sinusoidal value of 10 μM as in the monovascular portal perfusions. This concentration corresponded to 30 μM in the artery, since the arterial flow amounted to 30–35 % of total flow. This arterial addition caused a pronounced increase in glucose output and a slight enhancement of lactate release (Fig. 1). The maximal alterations in glucose and lactate balance were observed 6 min and 4 min respectively after the start of the 5 min infusion period.

In the second bivascular perfusion series acetylcholine was added via the portal vein. The concentration was adjusted to a vascular value of 30 μM , which corresponded to a sinusoidal concentration of 20 μM . Even at this higher sinusoidal concentration, portal acetylcholine had only minor effects on glucose and lactate metabolism (Fig. 1). In a third bivascular perfusion series acetylcholine was infused simultaneously via the hepatic artery and portal vein, with a vascular concentration of 30 μM in each vessel, corresponding to a sinusoidal concentration of 30 μM . As in the first arterial infusion series, acetylcholine caused an increase in glucose release and an enhancement of lactate output, which were similar to the effects caused by arterial acetylcholine alone (Fig. 1).

Haemodynamics. Arterial acetylcholine at a vascular concentration of 30 μM caused a slight decrease in arterial and portal flow, and consequently in total flow (Fig. 1). At a vascular concentration of 30 μM portal acetylcholine produced slight haemodynamic alterations, with a decrease in portal flow and an increase in arterial flow (Fig. 1). Acetylcholine added simultaneously via both vessels with a vascular concentration of 30 μM in each vessel also caused a slight decrease in portal flow, an increase in arterial flow and no alteration in total flow (Fig. 1).

Output of catecholamines. The observed metabolic and haemodynamic effects caused by acetylcholine were surprising. The transmitter of the parasympathetic nervous system would have been expected to cause a decrease in glucose release or a switch of glucose output to uptake [10–12,20–22,27,28]. The observed metabolic and haemodynamic alterations were at variance with these assumptions, and exhibited similarities to the effects caused by infusion of catecholamines or by electrical stimulation of sympathetic liver nerves [10–14]. Therefore the possibility was considered that arterial acetylcholine caused the release of catecholamines via the arterial vasculature and the sinusoids into

Fig. 1. Effects of acetylcholine on glucose and lactate balance, on arterial, portal and total flow, and on noradrenaline and adrenaline output in perfused rat liver

Livers were perfused with a Krebs–Henseleit bicarbonate buffer containing 5 mM-glucose, 2 mM-lactate, 0.2 mM-pyruvate and 1 μ M-neostigmine. Acetylcholine (AC) was added via the portal vein in the portally perfused livers and via the hepatic artery (a), portal vein (p) or jointly (j) via both hepatic inflows in the bivascularly perfused liver. Substrate balance is given by [concn. in hepatic vein (μ mol/ml) $\times$ flow (ml/min per g)] – [concn. in portal vein (μ mol/ml) $\times$ portal flow (ml/min per g)] + [concn. in hepatic artery (μ mol/ml) $\times$ arterial flow (ml/min per g)]. A positive value indicates release, a negative value uptake. Values are means $\pm$ S.E.M. of 3–5 experiments each, as indicated in parentheses.

the hepatic vein. The outputs of noradrenaline and adrenaline were estimated in order to verify whether sympathetic agonists might be involved in the actions of acetylcholine on glucose and lactate metabolism and on perfusion flow.

Arterial acetylcholine caused an output of noradrenaline, which was in the same range as that observed after stimulation of sympathetic liver nerves under saturating conditions [14,29], and of adrenaline, which had not been observed during nerve stimulation. Both noradrenaline and adrenaline output showed an 'escape' phenomenon, i.e. they returned to basal values in spite of continued acetylcholine infusion. Adrenaline output exceeded that of noradrenaline by a factor of 4 (area under the curve) (Fig. 1). Portal acetylcholine did not elicit an overflow of noradrenaline or adrenaline, in either the bivascular or the monovascular perfusions (Fig. 1). Acetylcholine infused simultaneously via the hepatic artery and portal vein produced an output of both catecholamines which was twice the overflow caused by arterial acetylcholine alone. Again, adrenaline release exceeded that of noradrenaline by a factor of 3 (Fig. 1).

Receptor involvement of the effects caused by arterial acetylcholine

Acetylcholine was infused in this experimental series exclusively via the hepatic artery. Antagonists of cholinergic and sympathetic receptors were added to the perfusion media throughout the whole experiment. Atropine was used as inhibitor of muscarinic receptors at a sinusoidal concentration of 10 μ M. Antagonists of sympathetic receptors were applied in another series of experiments, because the observed overflow of noradrenaline and adrenaline indicated that catecholamines might be involved in the acetylcholine-dependent alterations of hepatic glucose and lactate metabolism and haemodynamics. As rat liver was reported to contain predominantly α_1 - and also β_2 -receptors [30], the α_1 -antagonist prazosin and the β_2 -antagonist butoxamine were applied as specific inhibitors for these receptor types at sinusoidal concentrations of 5 μ M and 10 μ M respectively. These concentrations were shown to inhibit completely the actions of the α -agonist phenylephrine and the β -agonist isoprenaline

Fig. 2. Effects of atropine, prazosin and butoxamine on the acetylcholine-dependent alterations in glucose and lactate balance, in arterial, portal and total flow, and in the output of noradrenaline and adrenaline in perfused rat liver.

Rat livers were perfused via the hepatic artery and portal vein as described in Fig. 1; when indicated, $10 \mu\text{M}$ of the muscarinic antagonist atropine, or $5 \mu\text{M}$ of the α_1 -antagonist prazosin, or $10 \mu\text{M}$ of the β_2 -antagonist butoxamine was added via both inflows. Acetylcholine was infused via the hepatic artery (arterial acetylcholine = AC_7). Substrate balance is defined in Fig. 1. A positive value indicates release, a negative value uptake. Values are means $\pm$ S.E.M. of 4–5 experiments each, as indicated in parentheses.

respectively in the perfused rat liver (V. Ulken, G. Püschel & K. Jungermann, unpublished work). As the unspecific β -blocker propranolol was shown to inhibit partially the sympathetic stimulation of glucose and lactate production [24] in the bi-vascular perfusion system, this sympathetic antagonist ($10 \mu\text{M}$) was applied in an additional series of experiments ($n = 3$; results not shown).

Metabolism. Atropine completely inhibited the acetylcholine-dependent increases in glucose and lactate release, indicating that muscarinic receptors were involved in the actions of this transmitter (Fig. 2). The α_1 -sympathetic antagonist prazosin (Fig. 2) and the β -blocker propranolol (results not shown) had no inhibitory effects. The β_2 -receptor antagonist butoxamine even slightly increased the alterations in glucose and lactate metabolism (Fig. 2).

Haemodynamics. Atropine completely blocked the acetylcholine-dependent decrease in arterial and portal flow (Fig. 2). Prazosin prevented the slight decreases in portal flow and shifted

the slight decrease in arterial flow to a minor increase (Fig. 2). Butoxamine (Fig. 2) and propranolol (results not shown) inhibited the slight decrease in arterial flow, but not that in portal flow.

Overflow of catecholamines. Atropine decreased the overflow of noradrenaline to 30 % and the output of adrenaline to 10 % (Fig. 2). In the presence of prazosin, the output of noradrenaline remained unaltered, whereas the overflow of adrenaline was increased to 220 % (Fig. 2). In the presence of butoxamine (Fig. 2) and propranolol (results not shown), the overflow of noradrenaline was decreased to 15 % and 30 % respectively, whereas the output of adrenaline was slightly decreased to 70 % and 50 % respectively. Thus propranolol inhibited the noradrenaline output to a similar extent as butoxamine and atropine.

DISCUSSION

Cholinergic effects on hepatic metabolism *in vivo* and *in vitro*

The influence of vagal-nerve stimulation and acetylcholine infusion on liver metabolism and haemodynamics has been

investigated in the intact animal and in the isolated systems of liver perfusion and hepatocyte suspension.

In intact animals, the effects on liver functions of central stimulation of the parasympathetic nerves at the lateral nucleus of the hypothalamus and of selective activation of the anatomically variable hepatic branch of the vagus have been studied [10,11]. With these experimental systems possible interactions of other organs, which may in part cause the observed effects, are difficult to exclude. This reservation holds true even for the selective stimulation of the hepatic vagal branch by placing the electrode around the bifurcation of these nerve fibres from the anterior oesophageal trunk, because it is known from human anatomical studies that the hepatic branch forms a complex in the lesser omentum and then projects to the biliary tract, pyloric antrum, pylorus, proximal duodenum, ventral pancreas, celiac plexus, the sphincter of Oddi and the liver [4]. Activation of the lateral hypothalamus or of efferent fibres of the hepatic vagal branch in rabbits or cats caused an increase in glycogen synthesis by an activation of glycogen synthase and a decrease in glucose release from the liver [10,11,27]. Vagotomy of the hepatic branch in rats diminished the rate of liver glycogen deposition after a glucose load [27]. It was concluded from these studies that the parasympathetic liver nerves and its transmitter acetylcholine are functionally related to the insulin system and act synergistically with this hormone in activating hepatic glycogen synthesis in hyperglycaemic conditions.

In the isolated rat liver perfused via the portal vein, either acetylcholine was infused [21,22] or parasympathetic nerves were stimulated after pharmacological inactivation of the sympathetic liver nerves [20]. Alternatively, acetylcholine was added to rat hepatocyte suspensions in order to mimic parasympathetic nerve actions [31]. In these studies acetylcholine was applied to final concentrations of 10 μM [22] as in the present investigation, 300 μM [21], 500 μM [20] and even 5.5 mM [31]. It is difficult to assess which of these concentrations may be physiological. Neurotransmitter levels within a synapse appear to be in the range between 100 μM and 1 mM [32]. In the portally perfused rat liver parasympathetic stimulation of the hepatic nerves, i.e. stimulation of all nerve bundles in the presence of α - and β -sympathetic antagonists, caused a small increase in glucose uptake only in the presence of insulin and antagonized the glucagon-dependent increase in glucose release [20]. Addition of acetylcholine decreased basal glucose output in one investigation [22], but was without effect on glucose balance in two other studies [20,21]. The latter data are in line with the present results that in the portally perfused rat liver acetylcholine did not influence basal glucose balance. Thus these studies with perfused livers stimulated with acetylcholine via the portal vein appear to confirm the functional relation between the parasympathetic liver nerves and the insulin system [20–22,31].

The effects of hepatic arterial acetylcholine on liver metabolism have not been investigated previously. Therefore the present study does not contrast with previous observations, but extends them by showing that arterial acetylcholine was able to act via muscarinic receptors to cause an increase in glucose and lactate output. These actions of arterial acetylcholine are at variance with a functional relationship between vagal stimulation and insulin.

Cholinergic effects on hepatic adrenaline and noradrenaline release

Close anatomical connections between catecholaminergic and vagal nerves have been demonstrated [4,33]. It has been shown that abdominal vagal resection decreased gastric catecholamines by 50 % [33]. In dissection studies of mammals the hepatic vagal branch or its derivatives have been described as communicating

or intermingling with abdominal sympathetic nerves [4]. It has also been shown that the hepatic vagal branch carries tributaries to the perivascular plexus of the hepatic artery and that it contains, besides peptidergic afferents from the dorsal root ganglia, also catecholaminergic efferents to the celiac ganglion [4]. This close connection between the parasympathetic and sympathetic nerves in the plexus around the hepatic artery can be expected to be the anatomical substratum for the potential interactions between cholinergic receptors and catecholamine release. Clearly, the muscarinic receptors, the occupation of which triggered the output of noradrenaline and adrenaline, must be localized within the range of the hepatic arterial bed not accessible from the portal vein.

Noradrenaline release has been observed previously during sympathetic nerve stimulation [14,19]. Noradrenaline could be released from sympathetic nerve endings after muscarinic activation at axoaxonal acetylcholine receptors of nerves with perikarya situated outside the liver in hilar ganglia around the hepatic artery and in the celiac ganglion or at axosomatic acetylcholine receptors of neurons with perikarya located in hilar ganglia around the hepatic artery [34]. The cell type within the range of the hepatic artery that could synthesize and release adrenaline after this activation of cholinergic receptors is not known. One possible explanation might be the assumption that acetylcholine activated a direct release not only of noradrenaline but also of adrenaline from sympathetic nerve endings. This hypothesis seems unlikely, as the adrenaline content of liver parenchyma is only 5 % of the noradrenaline content [35,36], and as hepatic adrenaline does not appear to be stored in sympathetic nerves. Treatment of rats with 6-hydroxydopamine and reserpine, which was designed to deplete sympathetic nerve endings and to prevent catecholamine storage in any remaining nerve terminals, decreased hepatic noradrenaline, but failed to decrease hepatic adrenaline content [36]. An alternative explanation might be the assumption that acetylcholine caused locally a methylation of noradrenaline to adrenaline, which in turn was released into the hepatic vein. The liver was reported to contain, besides noradrenaline, also the methyl donor *S*-adenosylmethionine in its sympathetic nerves [37]. Moreover, a non-specific *N*-methyltransferase catalysing the methylation of noradrenaline to adrenaline was demonstrated in rat liver [36]. Thus the liver appears to contain all the reactants necessary to synthesize adrenaline from stored noradrenaline. Circulating noradrenaline was not converted into adrenaline (A. Gardemann, U. Jahns & K. Jungermann, unpublished work). Since a specific inhibitor of the hepatic unspecific *N*-methyltransferase is not known, the proposal that the acetylcholine-dependent release of adrenaline into the hepatic vein was due to a methylation of stored noradrenaline could not be tested directly.

Lack of a role of noradrenaline and adrenaline in the metabolic actions of arterial acetylcholine

The sympathomimetic metabolic effects of arterial acetylcholine were not inhibited by the α_1 -blocker prazosin and the β_2 -antagonist butoxamine, yet the weak haemodynamic actions were influenced (Fig. 2). Even high concentrations of the unspecific β -antagonist propranolol, which is known to have an affinity to β_2 -receptors about 1000-fold higher than that of butoxamine [30,38,39], were not effective in inhibiting the acetylcholine-dependent stimulation of glucose and lactate production. Therefore it can be concluded that both catecholamines were not directly involved in the metabolic action of arterial acetylcholine and that they might be involved in the haemodynamic alterations. Apparently, a non-adrenergic, perhaps peptidergic [40,41], signal chain regulating metabolism was activated via muscarinic receptors within the range of the hepatic arterial bed. As indomethacin,

the inhibitor of prostanoid synthesis, did not modify the acetylcholine-dependent metabolic and haemodynamic alterations (results not shown), it seems unlikely that the actions of acetylcholine were mediated by prostaglandins or thromboxane A_2 , which were shown to enhance glucose and lactate output in the perfused rat liver [42,43].

Function of metabolic and haemodynamic control by arterial acetylcholine

Apparently, arterial acetylcholine did not act as a terminal cholinergic parasympathetic neurotransmitter in a classical sense, but as an intermediate transmitter within a so-far unknown non-adrenergic metabolic signal chain, the actions of which were in a first approximation sympathomimetic. Administration of arterial acetylcholine thus mimics the actions of this unknown nervous signal chain located solely around the hepatic artery.

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