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# Role of the Fuel Utilized by Tissues on Coronary Vessel Response to Physical Stimuli in Isolated Rat Hearts

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## Summary

In isolated rat hearts which can or cannot utilize fatty acids (FA) as substrates the coronary responses to an increase in flow were studied under three different conditions: a) control, during perfusion with glucose-enriched Tyrode solution which allowed the hearts to utilize long-chain FA from the endogenous pool, b) during forced utilization of glucose obtained with oxfenicine, an inhibitor of long-chain FA oxidation, and c) during restored utilization of FA obtained with the addition of hexanoic acid which bypasses the blockade induced by oxfenicine. A step increase in coronary flow (50 %) induced an increase in coronary perfusion pressure whose initial slope (first 60-80 s) was similar in all the conditions of buffer perfusion, thereafter the pressure tended to further increase under control conditions (buffer *a*), but to decrease during oxfenicine (buffer *b*). The addition of hexanoic acid to the perfusion solution (buffer *c*) abolished the effect of oxfenicine. Steady-state conditions were reached after four minutes of increased flow, when perfusion pressure increased by about 70 and 65 % under control conditions and during hexanoate, respectively, but only by 45 % during oxfenicine. In isolated rat hearts during inhibition of FA utilization, an increase in flow elicited a reduced increase in perfusion pressure that resulted in delayed coronary dilation. It follows that the resulting shear stress is substrate-sensitive.

**Key words**
Coronary circulation • Fatty acids • D-glucose • Shear stress • Vascular resistance

## Introduction

Long-chain fatty acids (LCFA) are the major oxidation fuel of the healthy heart *in vivo* and *in vitro*, while carbohydrates, especially lactate and glucose, provide the remaining energy source (Neely *et al.* 1969, Simonsen and Kjekshus 1978, Stanley *et al.* 1997). However, heart metabolism can rapidly adapt to changes in substrate availability. For example, the heart shifts to preferential utilization of glucose when the arterial concentration of free fatty acids falls below 0.3 mmol/l (Nuutila *et al.* 1994).

A few studies analyzed the role of substrate utilized by vascular tissues. It has been reported that endothelial cells can use both glucose and fatty acids as fuel substrate, and that they utilize triglycerides as alternative fuel during glucose deprivation (Culic *et al.* 1999). On the other hand, glucose and glycogen storage predominate in vascular smooth muscles as oxidative substrates in many conditions of substrate availability



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(Allen and Hardin 2001). However, to the best of our knowledge, no studies have tried to clarify whether the oxidation of a given type of substrate compared to other is more or less advantageous for endothelial/smooth muscle function and whether the substrate utilized may affect vasomotor tone in response to physical stimuli.

In isolated hearts, enhanced coronary flow increases perfusion pressure and wall shear stress (Dijkman *et al.* 1996, 1997). Physical stimuli such as shear stress and stretch are sensed by the endothelium of coronary vessels and by means of a mechanotransducer apparatus lead to the synthesis and release of vasoactive factors, such as the vasodilators prostacyclin and nitric oxide, and the vasoconstrictors endothelins, superoxide anion and peroxynitrite (Rubanyi 1991, Lüscher *et al.* 1992, Busse and Fleming 1998). Smooth muscle function is also influenced by changes in transmural pressure (e.g. the myogenic response) (Davis and Hill 1999). We hypothesized that the coronary response to physical stimulation may be influenced by the fuel substrate utilized by the vessels.

To test this hypothesis we used the isolated crystalloid-perfused rat heart. In this model shear stress and stretch can be easily enhanced by incrementing coronary flow, while reducing the number of the involved variables to a reasonable minimum.

In the present study, the increase in flow was performed (a) in the presence of basal conditions with the heart utilizing the supplied glucose and endogenous substrate stores of LCFA, (b) during the blockade of carnitine palmitoyltransferase I (CPT-I), a mandatory enzyme for β-oxidation of LCFA by mitochondria (Molaparast-Saless *et al.* 1987, van de Velde *et al.* 1996, Kennedy *et al.* 2000), and (c) during the addition of hexanoic acid to the perfusate, a medium-chain fatty acid, which can be utilized in spite of CPT-I inhibition (Ishiwata *et al.* 1995, Pepine and Wolf 1999).

## Methods

The study was performed in 16 isolated hearts removed from 5- to 6-month old male Wistar rats (450-550 g of body weight). Each animal was pretreated with heparin (2 500 IU, i.m.), anesthetized by an intra-peritoneal injection of 2 ml of urethane (0.25 g/ml) 10 min later and then killed by decapitation. The heart was rapidly excised and weighed and the aorta retrogradely perfused with oxygenated buffer solution at 37 °C. Flow was kept constant (9±1 ml $min^{-1}$ $g^{-1}$ ww) using one of the three heads of a Watson-Marlow pump 313 (Falmouth, Cornwall, England). Each of the three heads was calibrated to generate identical flow rates (see "Experimental protocol"). During the stabilization period the flow was titrated to reach a coronary perfusion pressure (CPP) of 80 mm Hg. Perfusion and flow oscillations were limited using the 314 four roller pump-heads of the Watson-Marlow pump and by inserting a 50 ml Windkessel device into the perfusion line. A small aperture in the left ventricular wall allowed the drainage of the thebesian flow. Left ventricular pressure (LVP) was recorded by a polyvinyl-chloride balloon placed in the left ventricle *via* the mitral valve and connected to an electromanometer (Monitoring Kit mk 5-02 DTBNVF, Abbott, Milan, Italy). The balloon was filled with saline and connected to a graduated syringe filled with the same fluid. The piston of the syringe was connected to a micro-manipulator in order to adjust ventricular volume and to achieve a diastolic LVP of 5 mm Hg. A small cannula inserted into the right ventricle *via* pulmonary artery was used to remove samples of the coronary effluent for gas analysis. The heart rate was measured from individual cardiac contractions and was then increased by 15-20 % (i.e. 280-300 bpm) above the spontaneous beating frequency using a stimulator (Sane'ei Instrument Ltd, Tokyo, Japan) to avoid any secondary effects caused by changes in heart rate.

Coronary perfusion pressure and CF were monitored with an additional electromanometer and an electromagnetic flow-probe (Pencar 107/1000, Austec, Milan, Italy) respectively, both placed in the perfusion line.

LVP, CF and mean CPP were recorded using a TEAC R-71 recorder (Tokyo, Japan), digitized at 1000 Hz and analyzed off-line with a CODAS software (DATAQ Instruments, Inc. Akron, OH, USA). The oxygen content of in-flowing and out-flowing buffer solutions was measured using a Ciba-Corning 280 gas-analyzer (Halstead, Essex, England). The myocardial oxygen consumption ($MVO_2$) was calculated by multiplying coronary flow by the difference in oxygen content between in- and out-flowing buffer solutions. Animal use was in accordance with the University of Torino ethical committee guidelines and conformed to the Italian law (DL – 116, Jan. 27, 1992).

*Experimental protocol*

The heart was allowed to stabilize for 20-30 min before baseline values were recorded. During this period, the heart was perfused with glucose-enriched Tyrode buffer (total solute concentration mmol/l made up from

154 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 D-glucose, 5 HEPES, pH adjusted to 7.35 with NaOH.) along with 10 µg/ml lidocaine (Hare *et al.* 1998, Paolocci *et al.* 2000, Rastaldo *et al.* 2001). Lidocaine was used to limit the occurrence of arrhythmias. The buffer was oxygenated with 100 % $O_2$ at a pressure of 640-650 mm Hg.

The experimental maneuvers (see below) were performed while hearts were perfused with alternating buffer solutions of different composition. Buffer "*a*": Tyrode buffer perfusion supplying only glucose as substrate. Buffer "*b*": Tyrode buffer perfusion with the addition of 4-hydroxy-L-phenylglycine (oxfenicine, 2 mmol/l), previously defined as a specific CPT-I inhibitor at this concentration (Molaparast-Saless *et al.* 1987, Van De Velde *et al.* 1996, Kennedy *et al.* 2000). Buffer "*c*": Tyrode buffer perfusion with oxfenicine and in addition hexanoic acid (1 mmol/l) in order to bypass the block of CPT-I by oxfenicine. Perfusion buffers "*b*" and "*c*" were infused in a random sequence by switching from one head to the other on the Watson-Marlow pump.

Coronary flow was increased by 50 % (from 9±1 to 14±1 ml $min^{-1}$ $g^{-1}$ ww) and for a period of 5 min the parameters were recorded. CF was then reduced to the initial control value and monitoring of variables continued for 10 min before the perfusion buffer was changed. The maximal flow applied was identical to that previously shown to be reached at the peak of coronary reactive hyperemia in crystalloid-perfused isolated hearts (Kostic and Schrader 1992), which was confirmed to hold true under the conditions of the present study (data not shown).

Hearts, which required more than 12 ml $min^{-1}$ $g^{-1}$ ww of basal CF to achieve the desired CPP during stabilization, were discarded (n=4). This was necessary in order not to exceed the upper limit during the increase in CF, previously observed by other authors during spontaneous hyperemia (e.g. Kostic and Schrader 1992).

*Data analysis*

The basal CF and the 50 % increase in flow were verified on the trace recorded by the flow-probe. The time course of CPP response following the step increase in CF obtained during control conditions (buffer a) was compared with the time course of CPP changes induced by the increase in CF during inhibition of fatty acid oxidation (buffer b) and during restored fatty acid utilization (buffer c). The ratio CPP/CF was used as index of coronary resistance (CR).

To further analyze the vascular response, according to Dijkman *et al.* (1996), we considered the coronary vascular tree as a tube system were Poiseuille's law holds, and we evaluated the variations in vessel radius and shear stress.

*Calculation of vessel radius*

We used the values corresponding to the radius calculated starting from Poiseuille's law as an index of the internal vessel radius ($r_{index}$):

$$r = \sqrt[4]{Q8\eta l/\Delta P\pi}$$

where *r* is internal vessel radius, *Q* is flow, $\eta$ is viscosity, *l* is vessel length, and $\Delta P$ is gradient of pressure in the circuit.

Since we do not know the real vessel length, we consider *l* (vessel length) as equal to one. The viscosity ($\eta$) of the buffer can also be considered equal to one and constant in all the buffer perfusion conditions. The venous pressure is considered equal to 0 mm Hg. Therefore:

$$r_{index} = \sqrt[4]{Q_c 8/P_c\pi}$$

where $Q_c$ is coronary flow, and $P_c$ is mean coronary perfusion pressure.

*Calculation of shear stress*

We used the values corresponding to the shear stress calculated starting from Poiseuille's law as an index of shear stress ($\tau_{index}$):

$$\tau = 4\eta Q/r^3\pi$$

where $\tau$ is shear stress, the other symbols are as above. Considering r (internal vessel radius) = $r_{index}$

$$\tau_{index} = 4\eta Q_c/r_{index}^3\pi.$$

*Statistics*

Data are presented as means ± S.E.M. For comparison of paired samples in each conditions of the buffer perfusion Student's t-test for paired data was used. Between-intervention effects were first tested by ANOVA, and then individual comparisons were made by Student's non-paired t-test.

*Drugs and chemicals*

The drugs used in these experiments were all freshly dissolved at the required concentration in the perfusion buffers. Most of the compounds were obtained from Sigma Chemical (St. Louis, MO, USA), heparin from Roche (Milan, Italy) and lidocaine from Astra Farmaceutici (Milan, Italy).

## Results

Baseline values at basal flow and the values obtained 5 min after an increase in flow (+50 %) of CPP, CR, $r_{index}$, $shear_{index}$, diastolic and developed LVP, and $MVO_2$ at different perfusion conditions are shown in Table 1. Oxfenicine (buffer *a*) and hexanoate addition (buffer *c*) *per se* did not significantly alter the baseline values of any of the considered parameters.

In control conditions (buffer *a*) the 5 min of 50 % increase in flow resulted in an increase of CR by 14±2 % ($p<0.02$). On the contrary, during forced utilization of glucose by oxfenicine (buffer *b*) the increase in flow induced a decrease of CR by about 5±2 % ($p<0.05$). During the addition of hexanoate (buffer *c*) the CR-response (+10±6 %, $p< 0.05$) was similar to that observed in the control conditions. Accordingly, the $r_{index}$ was significantly reduced during the increased flow under control conditions (–5±1 %, $p<0.02$) and during hexanoate (–4±1 %, $p< 0.05$), while it was raised during the increase in flow during forced utilization of glucose by oxfenicine (+3±1 %, $p<0.05$).

The elevation of coronary flow augmented shear stress. At the steady state, $shear_{index}$ increased by about 70 % under control conditions (buffer *a*). During forced utilization of glucose by oxfenicine (buffer *b*) the $shear_{index}$ augmentation was 40% only, so that the increase in shear stress was blunted by about 45 %. During the addition of hexanoate (buffer *c*), flow-induced increase in shear (+65 %) was similar to that observed in the control conditions.

**Table 1.** Considered parameters at baseline and after 5 min of 50 % increase in coronary flow (Flow+50 %).

| | **Control (A)** | | **Oxfenicine (B)** | | **Hexanoate (C)** | |
|---|---|---|---|---|---|---|
| | **Basal Flow** | **Flow+ 50%** | **Basal Flow** | **Flow+50 %** | **Basal Flow** | **Flow+50 %** |
| *CPP (mm Hg)* | 77±3 | 133±4† | 76±3 | 118±3†* | 78±3 | 129±4† |
| *CR (mm Hg ml $min^{-1}$)* | 8.4±1 | 9.3±2† | 8.5±1 | 7.9±1†* | 8.3±1 | 8.9±1† |
| *$r_{index}$ (arbitrary unit)* | 0.74±0.03 | 0.72±0.02† | 0.74±0.02 | 0.76±0.03†* | 0.74±0.04 | 0.725±0.01† |
| *$Shear_{index}$ (arbitrary unit)* | 28±2 | 47±4† | 28±3 | 40±2†* | 28±3 | 46±3† |
| *Diastolic LVP (mm Hg)* | 5±1 | ±7±2 | 4±2 | 6±2 | 4±1 | 6±2 |
| *Developed LVP (mm Hg)* | 77±4 | 88±8† | ±76±4 | 81±5†* | 78±5 | 87±6† |
| *$MVO_2$ (ml $min^{-1}$ 100$g^{-1}$ )* | 44±0.6 | 62±0.7† | 45±0.8 | 54±0.7†* | 41±0.7 | 59±0.8† |

CPP, coronary perfusion pressure, CR, coronary resistance, $r_{index}$, index of internal vessel radius, $Shear_{index}$, index of shear-stress, LVP, left ventricular pressure, $MVO_2$, myocardial oxygen consumption, n=12. See also text for further explanations. † $p< 0.05$ vs. baseline in each conditions of buffer perfusion, * $p< 0.05$ vs. buffers a and c.

During the 5th minute of 50 % CF augmentation, the induced increase in CPP was greater during control conditions (+70 %, buffer a) than during forced utilization of glucose by oxfenicine (+45 %, buffer *b*). During the addition of hexanoate (buffer *c*), flow-induced percentage increase in CPP (+65 %) was similar to that observed in the control conditions. Changes of CPP over

the time in response to the increase in CF are shown in Figure 1.

During the first 40-60 s of the step increase in flow, the slopes of increase in CPP are similar during the three different conditions of buffer perfusion. Thereafter, under control conditions (buffer *a*) CPP slightly increased, whereas during oxfenicine (buffer *b*) it slightly decreased, until the fourth minute. The addition of hexanoate (buffer *c*) reversed the effect of oxfenicine on CPP response. The values of CPP during oxfenicine were significantly different from those observed during the perfusion of other buffers starting from the second minute of CF increase.

It is noteworthy that the variations in $MVO_2$ and developed LVP paralleled the changes in CPP (Table 1). In particular, oxygen consumption of the heart increased more under control conditions (+40 %) and during hexanoate (+45 %) than during oxfenicine (+20 %).

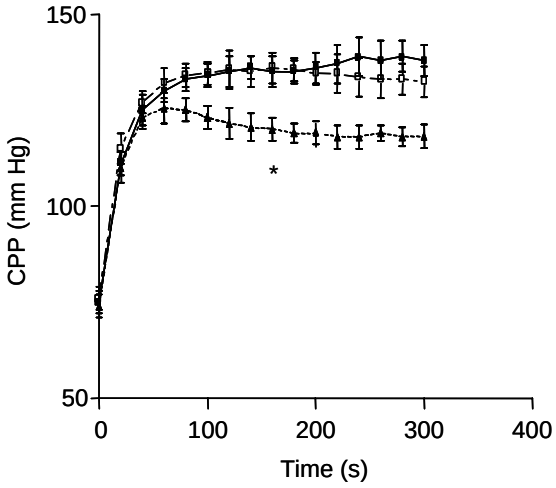


**Fig. 1.** Time course of increase in coronary perfusion pressure (CPP) following a swift step increase in coronary flow (+50 %). * Starting from this time point $p<0.05$ vs. buffers a and c. —■— Tyrode (buffer a); ---▲--- Oxfenicine (buffer b); – – □ – – Hexanoate (buffer c)

## Discussion

This study performed in *ex vivo* preparations demonstrates for the first time that the fuel utilized by the heart tissue influences the coronary tone response to increased flow.

In control conditions (buffer *a*), the calculated vessel radius decreased in response to an increase in flow. In such a condition, the heart derives a part of the energy from the oxidation of endogenous reserves of LCFA (Morgan *et al.* 1984, Saddik and Lopaschuk 1991). On the contrary, when CPT-I was inhibited by oxfenicine (buffer *b*) and the heart was forced to utilize glucose as the only fuel (Saddik and Lopashuk 1991) the increase in flow induced an increase in vessel radius. The addition to the solution (buffer *c*) of hexanoic acid reversed the effects of oxfenicine. Hexanoic acid is a medium chain fatty acid which can enter into the mitochondria and then be metabolized by-passing CPT-I inhibition (Madden *et al.* 1995), Therefore, the possibility of the heart to utilize fatty acids and glucose simultaneously (buffers *a* and *c*) led to vasoconstriction with a marked increase in CPP and shear stress in response to an increase in flow. On the other hand, the forced glucose utilization resulted in flow-induced vasodilatation with a lesser increase in CPP and shear stress.

On the basis of existing reports of tissue metabolism and function, the different sensitivity of vessels to mechanical stimulation allows to hypothesize on the type of tissue involved in this vessel response. It is likely that the endothelium is the tissue mainly involved in the response. Physical stimuli are sensed by vessels and lead to enhanced synthesis and release of endothelium-derived autacoids (Rubanyi 1991, Lüscher *et al.* 1992, Busse and Fleming 1998). These autacoids may contribute to an increase or decrease in vessels radius depending on the prevalence of vasodilator or vasoconstrictor factors, respectively. When a new steady state is reached, the mean vessel radius and shear are set at new levels depending on the resulting balance of physical force and autacoids (vasodilators and vasoconstrictors) which act on the vessels.

In the present study, the mutual relationship between the action of vasodilatory and vasoconstrictory factors was shifted to one side starting from 60-80 seconds after the step increase in flow, when it was possible to note a difference in CPP under the two experimental conditions (availability of fatty acids vs. forced glucose utilization). The delayed reduction in vessel radius during glucose utilization is in agreement with the recent study of Sun *et al.* (2001) performed *in vitro* conditions, in which glucose was the only fuel supplied. They report that deformation of endothelial cells by changes in transmural vessel pressure elicits a delayed release of nitric oxide (NO). From the above findings (Sun *et al.* 2001) and the results of the present study, we can speculate that substrate used by the vessel tissues may affect NO release in response to mechanical stimuli. Unfortunately, we cannot easily test this hypothesis in our experimental preparation as NO

inhibition not only strongly reduces coronary vessel radius (Kostic and Schader 1992, Kostic *et al.* 1996, Rastaldo *et al.* 2001), but also alters substrate metabolism (Bernstein *et al.* 1996, Recchia *et al.* 1998, 1999), thus affecting the studied parameters. Finally, it has been reported that endothelial cells can use both glucose and triglycerides as fuel and that they down-regulate their energy expenditure by reducing protein synthesis when only fatty acids are utilized (Culic *et al.* 1999). It may be argued that during altered metabolism such as forced glucose oxidation, endothelial cells may also reduce the synthesis of peptides, including endothelins. It remains to be elucidated whether the reduced increase in CPP during the increase in flow is either due to an increase in the production of vasodilators (e.g. NO) or due to a reduced release of vasoconstrictors (e.g. endothelins) or both events.

It is unlikely that the effects observed during oxfenicine may be attributed to a reduced strength of smooth muscle contraction (i.e. to an altered myogenic response) because long-chain fatty acids are minor contributors to substrate oxidation (approximately 5 %) in vascular smooth muscle (Allen and Hardin 2001). Moreover, under the three buffer perfusion conditions used in the present study, the endothelium-independent vasodilator, diazoxide, induced a similar vasodilator response (personal unpublished observations). Since diazoxide acts on $K^+$ channels of smooth muscle membrane, these results suggest that the smooth muscle function is substrate-insensitive, and further supports the idea that the opposed flow-induced response (vasodilatation during fatty acid inhibition vs. vasoconstriction during fatty acid availability) may reflect different endothelial function. Unfortunately, diazoxide also acts on mitochondrial $K^+$ channels (Pagliaro *et al.* 2001) and it is not the most appropriate drug for this purpose.

It can be excluded that the differences in extra-vascular compression may play a role in the observed opposite effects on vascular radius (increase vs. decrease). In fact, in response to the increase in flow developed LVP increased (Gregg effect) both during the inhibition of fatty acid utilization and during fatty acid availability. Moreover, the increase in CF induced a smaller increase in $MVO_2$ during the forced glucose utilization than during fatty acid utilization. These results are in agreement with the observation of Dijkman *et al.* (1996) who reported that variations of $MVO_2$ and developed LVP are influenced not only by changes in coronary flow, but also by concomitant changes in coronary pressure and shear stress. The reduced $MVO_2$ augmentation during forced glucose utilization implies a reduced release of vasodilator metabolite (e.g. adenosine) by the myocardium. This further supports the idea that the delayed increase in vessel radius during forced glucose utilization was vessel-dependent rather than myocardial-dependent.

In the heart perfused with crystalloids, coronary vessels may dilate in response to either mechanical, pharmacological or ischemic stimuli (Kostic and Schrader 1992, Kostic *et al.* 1996, Rastaldo *et al.* 2001). This fact rends the model suitable for studying the effects of mechanical stimuli on vasomotor tone. However, in many *in vitro* studies with isolated organ or isolated vessels glucose is the only supplied fuel. Moreover, *in vivo* setting the heart metabolism can be influenced by changes of substrates availability (Nuutila *et al.* 1994), changes in NO production (Bernstein *et al.* 1996) or by pathological conditions (Stanley *et al.* 1997). For these reasons it is important to stress that the vessel response to physical stimuli (i.e. shear stress) may be greatly influenced by the nature of the substrate utilized. Interestingly, during exercise the increased NO synthesis may favor fatty acid consumption (Bernstein *et al.* 1996). This last finding is only in apparent contrast with our results: fatty acid inhibition favors vasodilatation. It is quite possible that a feedback exists under physiological conditions. Thus we can speculate that the increasing NO stimulates fatty acid consumption (Bernstein *et al.* 1996) which in turn may limit shear-dependent vasodilatation (present study). *Vice versa*, a reduction in fatty acid consumption may favor the release of shear-dependent vasodilator(s) (i.e. NO which in turn may stimulate the enzymes involved in fatty acid metabolism (Recchia *et al.* 1998, 1999)).

Finally, it may be argued that in patients in which the fatty acid oxidation is pharmacologically inhibited, the beneficial effects can be ascribed to the increased efficiency of the myocardium (Burkhoff *et al.* 1991, Korvald *et al.* 2000) and to limitation of shear stress. In fact, this limitation is beneficial for the vessels as a chronic increase may lead to endothelial dysfunction and atherosclerosis (Nomura *et al.* 2001).

In conclusions, we found that in isolated hearts during inhibition of fatty acid utilization an increase in flow elicits a reduced increase in shear stress as a result of delayed coronary dilation. Our findings suggest that alterations in substrate metabolism may influence arteriolar diameter and may contribute to the endothelium-dependent regulation of vascular resistance

and consequently to local regulation of blood flow. Further studies are required to understand the mechanism(s) of this delayed coronary dilation.

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