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Effects of *L*-Carnitine and its Derivatives – Studies on Isolated Hearts

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Summary. The metabolism in the heart prefers long-chain fatty acids to other substrates. *L*-Carnitine, a co-factor of coenzyme A, plays an essential role in the transport of long-chain fatty acids through the inner mitochondrial membrane. Without carnitine, metabolisation of long-chain fatty acids in the mitochondria is not possible. In addition, acyl groups from acyl-CoA compounds can be transferred to *L*-carnitine, thus influencing the enzymatic activities of important mitochondrial enzymes.

The isolated heart model developed by *Langendorff* was used to investigate the effects of *L*-carnitine on the heart. During aerobic perfusion, the hemodynamic parameters of isolated hearts reacted in a very sensitive way to alterations in the external conditions (temperature, preload, composition of the perfusion solution). During postischemic perfusion, recovery of the hearts was also influenced by the composition of the perfusion. The hemodynamic parameters of the reperfused hearts increased markedly if there was a sufficiently high supply of long-chain fatty acids and/or glucose. The insufficient recovery of hearts perfused without glucose and at low fatty acid concentrations could be improved by adding *L*-carnitine. Determination of carnitine levels in heart tissue found that the heart loses about 30% of its carnitine content during ischemia, and that exogenous carnitine is taken up by the heart during reperfusion. There it effects the restoration of sufficient concentrations of creatine phosphate and *ATP*, a fact that was confirmed by ^{31}P NMR spectroscopy. NMR spectroscopy also established that *L*-carnitine lessens the harmful effects of ischemia-induced metabolic acidosis.

The favourable influence of *L*-carnitine on the heart in the reperfusion period could be due to a reduction in oxygen radicals (lowering of *MDA* concentrations during reperfusion, raising of GP_x and SOD activities).

The findings of these experiments on isolated hearts as well as the favourable results of two placebo-controlled and double-blind clinical studies (investigating the effects of carnitine in cardiomyopathy patients and the effects of *L*-carnitine in hemodialysis patients) demonstrate that *L*-carnitine produces positive therapeutic effects, particularly in heart and circulatory diseases.

Keywords. Intermediary metabolism; *Langendorff*-heart; Ischemia, reperfusion; MDA and carnitine concentrations.

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Introduction

Because of the central role of *L*-carnitine in intermediary metabolism, researchers assumed at a very early stage that *L*-carnitine might be able to influence the functions of a number of organs [1]. These assumptions were confirmed when *Engel* and *Angelini* discovered the existence of carnitine deficiencies [2]. It could be established that the heart was particularly susceptible to carnitine deficiencies because children with an inborn systemic carnitine deficiency often develop a cardiomyopathy even in the first few months of their lives. For many of these children, this cardiomyopathy may be fatal if no carnitine substitution is undertaken [3]. Disturbances of the carnitine metabolism are often found to be associated with cardiac and circulatory diseases, and in most cases an improvement can be achieved by supplying the patients with external carnitine [4].

Within the last decades there has been an enormous increase in the number of publications on the application of *L*-carnitine and its short-chain esters acetyl- and propionyl-*L*-carnitine in cardiac and circulatory diseases. There is a wide range of applications for *L*-carnitine in connection with these diseases: from ischemic

Table 1. Experimental possibilities of the *Langendorff*-heart

Experimental Designs
Varieties of Ischemia Duration (15–60 min), intensity (low-flow or no-flow ischemia, full flow with N_2), and type (intermittent, total, partial)
Perfusion Variations Constant pressure (defined by column height), constant flow (defined by perfusion pump), recirculation of the perfusate or disposal after a single passage through the heart
Perfusion Substrates With or without glucose resp. fatty acids, with or without addition of various substances (substances to be tested or medicaments), such as *L*(−)- or *D*(+)-carnitine, its *O*-acylesters, or carnitine carboxyl esters
Hearts Normal, healthy hearts or hearts with mechanical (ligation of coronary vessels), chemical (adriamycin), or disease-related (diabetes mellitus, myocarditis, *etc*.) damage
Measurable Parameters
Determination of Hemodynamic Parameters Left-ventricular pressure (LVP), coronary flow (CF), heart rate (HR), maximum rate of pressure increase ($+dp/dt_{max}$) resp. maximum rate of pressure decrease ($-dp/dt_{max}$) and their quotients, pressure-rate product (PRP), contractility index (*CI*), and tension-time-index (*TTI*)
Biophysical Parameters Oxygen consumption, changes in *pH* values, recording of ECG
Biochemical Investigations Formation of radicals (by simultaneous ESR measuring), formation of H_2O_2 (*e.g.*, by measuring chemiluminescence in the perfusate), determination of *ATP* and *CrP* (by ^{31}P NMR spectroscopy of the beating heart), measuring of enzyme activities in tissue (*e.g.*, SOD, GPx, *etc*.), measuring of freed metabolites (catecholamines, malondialdehyde, *etc*.)

cardiac diseases, such as angina pectoris and myocardial infarction, to arrhythmias, hypertrophy, myocarditis, cardiac failure, cardiogenic shock, and vasculopathies [5].

In order to solve a number of fundamental questions we employed the isolated-heart method introduced by *Langendorff* [6] as a model for studying the whole organism. It is, of course, true that the results obtained by studying isolated hearts are not completely comparable to those found for the whole organism, as there are a number of limitations. One of the most severe problems is the fact that oxygen is physically dissolved and not bound to erythrocytes; therefore any exchange of gases can only take place by diffusion. In addition, the metabolism is influenced by the absence of hormones such as insulin which plays an important role in the utilization of glucose.

Although hearts contained in the intact organism are only comparable to a certain degree to isolated hearts, which are completely cut off from the nerval and hormonal interference that is typical in the whole organism, studies using isolated hearts are a valuable complement to research carried out on the whole body. They also offer an additional advantage: Studies with isolated hearts can be conducted with a free choice of perfusion conditions and perfusion solutions, ischemias of varying length and type can be installed at any given time, followed by perfusion periods of freely selectable duration. In addition, a large number of hemodynamic parameters, biochemical values and physical data can be determined in isolated hearts (Table 1).

Materials and Methods

The Heart Models Used by Langendorff and Neely

In 1895, *Oscar Langendorff* established that it was possible to keep a mammal's heart alive after its excision from the body by perfusing it retrogradely, *i.e.*, *via* an aortic catheter. The *Langendorff*-heart method is based on the principle that the perfusion of the excised animal heart must be restarted as quickly as possible (<1 minute of preparation ischemia), using an aortic catheter and a suitable perfusion solution, which has to guarantee that the isolated hearts will keep beating (*e.g.*, an electrolytic Tyrode solution or *Krebs-Henseleit*-buffer) [7]. Working with a constant pressure (usually 110 cm $H_2O = 81$ mm Hg), the perfusion solution closes the aortic valves and is pumped through the coronary arteries and the capillary flow area; then it leaves the coronary vessels *via* the right atrium. Due to the closed aortic valves, the ventricles are not filled. The perfusion solution trickles off the heart and keeps it warm and moist. In addition, it can be re-infused after filtration (Fig. 1).

Since the ventricles in the *Langendorff*-heart remain empty, no volume work is done by it, *i.e.*, no liquid is transported. A modification was introduced by *Neely* [8]: After mounting the heart on a *Langendorff* apparatus, a catheter is inserted into the left atrium whereas all other veins are ligated. Then the heart is antegradely perfused *via* the left atrium. Thus, the left ventricle is filled and the heart performs volume work by expelling the perfusion solution *via* the aorta (aortic catheter). This way, both left-ventricular pressure (LVP) and cardiac output

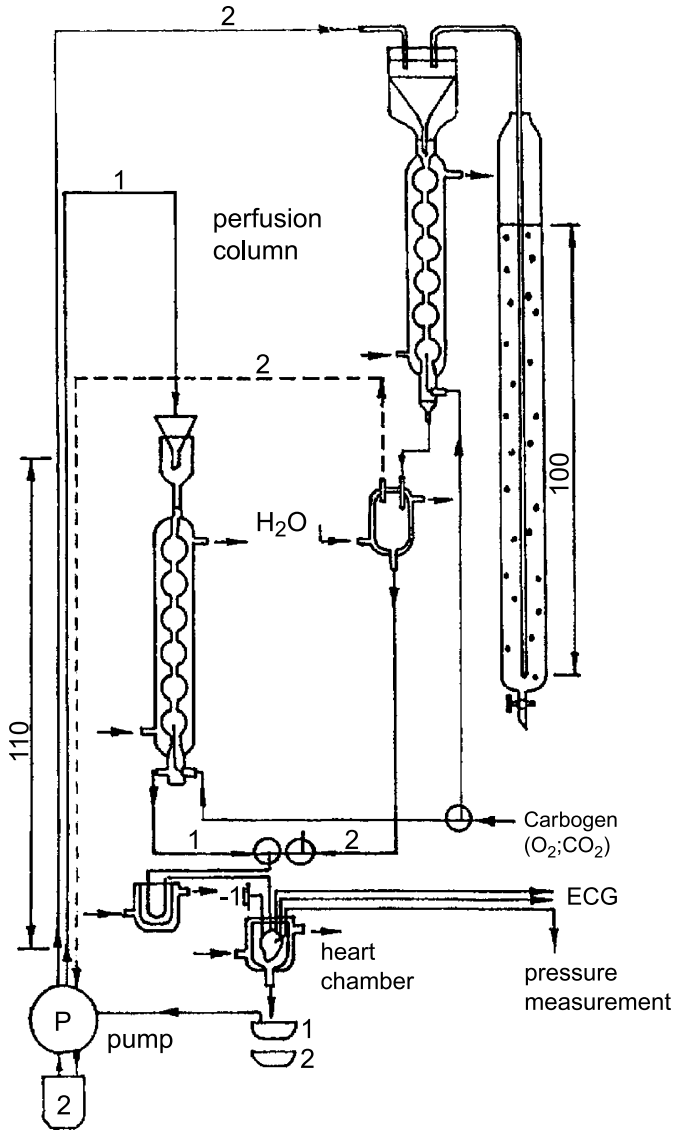

Fig. 1. Scheme of the *Langendorff* apparatus

can be determined directly. It is also possible to increase and preset the afterload by raising the catheter. The *Neely*-heart is a more physiological model, but apart from the above-mentioned advantages there are also some disadvantages: The preparation is difficult and much more time-consuming, which may cause mechanical and ischemic damage to the heart [9]. Since the heart has to work, it cannot tolerate as much stress as the *Langendorff*-heart. Therefore, the results obtained with these two models are not directly comparable.

The central part of both apparatuses consists of a temperature-controlled (37°C) and sealable glass vessel that contains the isolated beating heart, one or several gas-exchange columns for gassing various perfusion solutions with carbogen (95% O_2, 5% CO_2), which can be switched over whenever necessary, and a pump to fill the

columns. In order to measure the left-ventricular pressure in the *Langendorff*-heart, a catheter with a water-filled balloon is inserted into the left ventricle *via* the atrium. During the ventricle's contraction the resulting pressure in the balloon is measured as LVP by means of an electromechanical pressure transducer, then the signal is amplified and recorded with a biomonitor (there are also ultra-miniaturised catheters with a tiny quartz crystal attached to the tip of an amplifier cord, which measure LVP directly in the left ventricle [10]). In order to obtain an bipolar electrogram, two electrodes are fastened to the auricles and another one to the tip of the heart, which separately register the excitation of atrium and ventricle. Hemodynamic parameters are recorded either electronically or graphically and evaluated with suitable software (Hugo Sachs-Electronics, March-Hugstetten, Germany).

Animals and Preparation

The animals used were female Wistar rats (AB Jena) with a mean weight of 245 g. After anaesthetising the animals with sodium pentobarbital (60 mg/kg body weight), their hearts were excised and immediately mounted and perfused on the *Langendorff* apparatus with a standard Tyrode solution. With the exception of three animals that were given *L*-carnitine infusions *via* the *v. jugularis externa* in the days before the experiment, the rats were not pre-treated.

Hemodynamic and Energetic Parameters

Left-ventricular pressure was measured at an initial pressure of 10 mm Hg, which was maintained throughout the whole test. Other values that were measured and recorded included heart rate (HR), maximum rate of pressure increase ($+dp/dt_{max}$) respectively maximum rate of pressure decrease ($-dp/dt_{max}$) as indicators of myocardial contractility and relaxation, coronary flow (cm^3/min), and the electrogram. Based on contractility and relaxation, the quotients were calculated ($Q_{CR} = -dp/dt_{max}/+dp/dt_{max}$), which react to positive or negative inotropic substances in a very sensitive way. As it was not possible to determine the cardiac output directly, the comparable pressure rate product was calculated from LVP and HR. The contractility index (*CI*) is expressed by the (dp/dt/LVP) ratio, and the tension-time-index is calculated as follows: HR × LVP/100.

Each experimental design was used for a number of experiments of the same type (*vide infra*). For comparison reasons, in each group of experiments the values measured immediately before the onset of ischemia (at −20 minutes) were taken to be 100%, irrespective of their absolute values. Thus it was possible to compare the hemodynamic parameters measured during reperfusion in the same experimental group.

^{31}P NMR Assays

^{31}P NMR spectra (Fig. 2) can be used to establish the concentrations of *ATP*, creatine phosphate (*CrP*), and inorganic phosphate (P_i) in a non-invasive, non-destructive, and continuous way and without influencing the activity of the heart; at the same time it is possible to calculate the *pH* value in the beating heart [11].

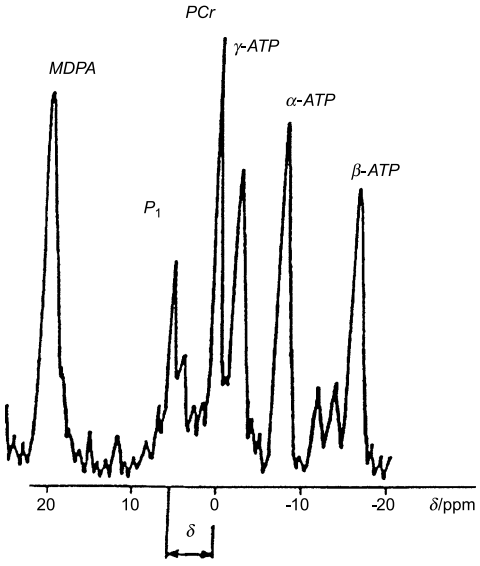

Fig. 2. ^{31}P NMR spectrum with the signals of *CrP*, *ATP*, and inorganic phosphate

The ^{31}P NMR spectra were measured using a magnetising force of 2.1 Tesla and an electromagnetic field of 36.53 MHz; for each spectrum 150 separate signals were accumulated at a repetition rate of 2 seconds and with a 60° excitation. Based on this scheme, the spectra of *ATP*, *CrP*, and P_i were recorded at five-minute intervals, which made it possible to determine their concentrations within these periods. Metabolite concentrations were determined from the area below the NMR signals, and *pH* values from the chemical shifts of the $H_2PO_4^-$ and HPO_4^{2-} signals according to Eq. (1).

$$pH = 6.75 + \log\left[(\delta_i - 3.35)/(5.60 - \delta_i)\right]. \quad (1)$$

Based on the *ATP*, *CrP*, and P_i values measured, the NMR-energy index was calculated, which represents the energy status of the tissue (Eq. (2)).

$$[ATP] + [CrP]/[ATP] + [CrP] + [P_i] \quad (2)$$

The effects of acetyl-*L*-carnitine and propionyl-*L*-carnitine were also investigated in the course of the ^{31}P NMR studies (1.5 mmol/dm^3). In order to separate the effects of glucose and fatty acids, fatty-acid-free perfusion solutions were used for these assays.

Perfusion Solutions

A modified Tyrode solution was used as the standard perfusion solution in the assays: 2.1 $MgSO_4$, 0.03 KH_2PO_4, 118.0 NaCl, 24.7 $NaHCO_3$, 4.7 KCl, 0.6 *EDTA*, 1.5 $CaCl_2$, and 11.0 glucose (all data in mmol/dm^3).

This standard perfusion solution was supplemented with the perfusion solutions that were to be tested (whenever necessary, glucose was removed): 0.4 resp. 1.2 mmol/dm^3 sodium palmitate, bound to 2% bovine serum albumin (fatty acid-free), and – depending on the perfusion protocol – 5 mmol/dm^3 *L*-carnitine. Before being used, all perfusion solutions were adjusted to a *pH* of 7.4.

Table 2. Experimental groups according to glucose and carnitine application

0.4 mmol/dm^3 Na-palmitate	1.2 mmol/dm^3 Na-palmitate
(1) without glucose: without *L*-carnitine ($n = 6$)	(5) without glucose: without *L*-carnitine ($n = 6$)
(2) with *L*-carnitine ($n = 7$)	(6) with *L*-carnitine ($n = 6$)
(3) with glucose: without *L*-carnitine ($n = 6$)	(7) with glucose: without *L*-carnitine ($n = 6$)
(4) with *L*-carnitine ($n = 5$)	(8) with *L*-carnitine ($n = 6$)

A total of eight test groups were formed, and the hearts perfused with glucose were used as controls (Table 2).

Experiments

After the hearts had been removed and mounted, they were perfused with a standard Tyrode solution until all hemodynamic parameters remained constant for a period of approximately 30 minutes. After switching over to the solution to be investigated, it was found that the hemodynamic parameters often displayed considerable changes [12]. Perfusion was continued until the hemodynamic parameters remained constant for a longer period (15–40 minutes). Then a no-flow ischemia was installed by switching off perfusion and gassing, during which nearly all hemodynamic parameters approached 0. After 20 minutes of ischemia, perfusion was restarted and a 60 minute reperfusion period installed, and all hemodynamic parameters were recorded as before [13].

After reperfusion the hearts were washed with a carnitine-free physiological NaCl solution and shock-frozen, then tissue *L*-carnitine [14] and malondialdehyde [15] were determined.

The significance of the differences found between the hearts, which were perfused with or without *L*-carnitine respectively acetyl- or propionyl-*L*-carnitine, was determined using *Mann* and *Whitney*'s U-test.

Experimental protocol:

Preparation	Tyrode I	Tyrode II (tested solution)	Ischemia 20 min	Reperfusion 60 min

Results

Reaction of the Langendorff-Hearts at the Switch-over from Perfusion Solution I to Perfusion Solution II

Because of the vast number of results, the following part will deal in detail with the systolic peak pressure only. The other hemodynamic parameters such as pressure rate product, coronary flow, and the myocardial relaxation usually reacted in the same way, with the myocardial relaxation showing the most sensitive reaction. In most experiments, however, the quotient of myocardial relaxation and myocardial contractility showed hardly any changes.

A comparison of perfusion parameters under low (0.4 mmol/dm^3) and high (1.2 mmol/dm^3) palmitate concentrations, and with glucose present, found that

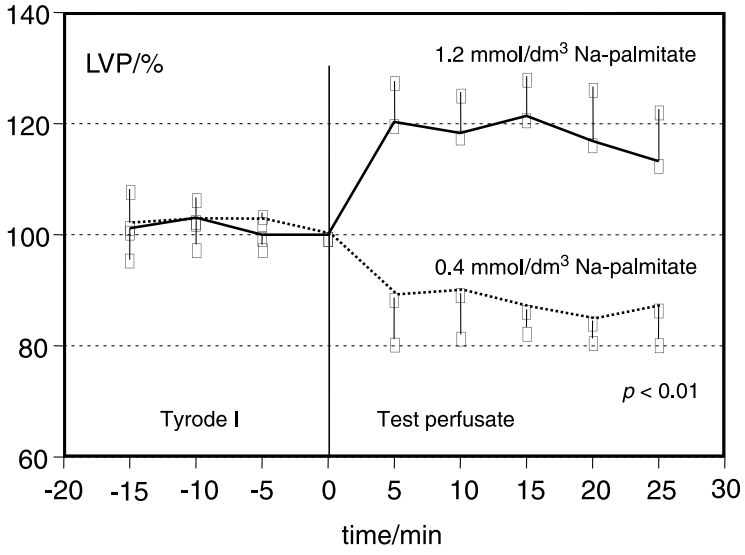

Fig. 3. Left ventricular pressure after switching from Tyrode I to Tyrode II in the presence of glucose and the influence of palmitate concentrations

switching from Tyrode solution I to Tyrode solution II effected a significant ($p<0.01$) increase in LVP of about 20% at high palmitate concentrations, whereas a decrease of 10–15% was found with the lower palmitate concentration (Fig. 3). The fact that low fatty acid concentrations and glucose produced worse results than high palmitate levels with glucose means that, in the former case, the heart muscle is not optimally supplied with energy.

L-Carnitine supplementation not only made up for this decrease in pressure but also increased LVP by about 8% (Fig. 4). The other hemodynamic parameters

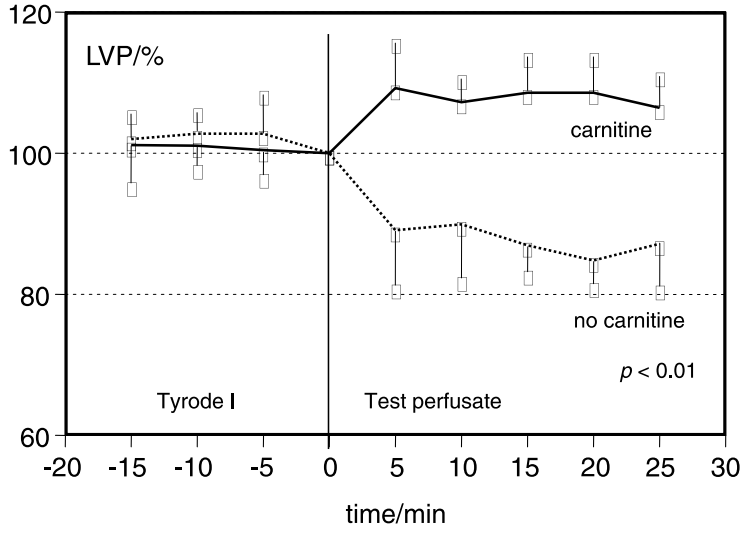

Fig. 4. Left ventricular pressure after switching from Tyrode I to Tyrode II at low concentrations of fatty acids (0.4 mmol/dm^3 Na-palmitate) and the influence of carnitine administration

reacted similarly in this series of experiments as well. In comparison with the controls, *L*-carnitine improved systolic peak pressure and relaxation speed at low fatty-acid concentrations and with glucose.

Glucose plays a quite important role in this. Removing glucose from the perfusion solutions containing high fatty-acid concentrations (1.2 mmol/dm^3) produced a decrease in pressure of nearly 10% upon switch-over, whereas an increase of 20% was effected if a perfusion solution containing glucose was used (Fig. 5). The other hemodynamic parameters (pressure rate product, myocardial relaxation) developed in a similar way, whereas no changes were found in the quotient, and the coronary

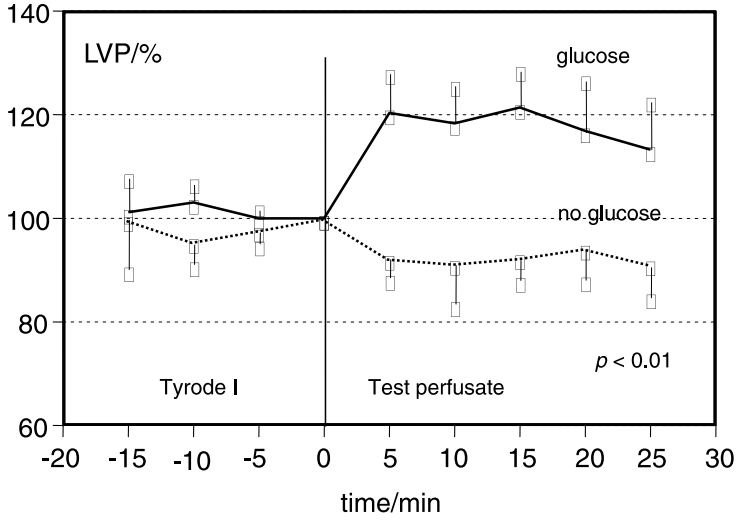

Fig. 5. Left ventricular pressure after switching from Tyrode I to Tyrode II at high concentrations of fatty acids (1.2 mmol/dm^3 Na-palmitate) and the effect of glucose administration

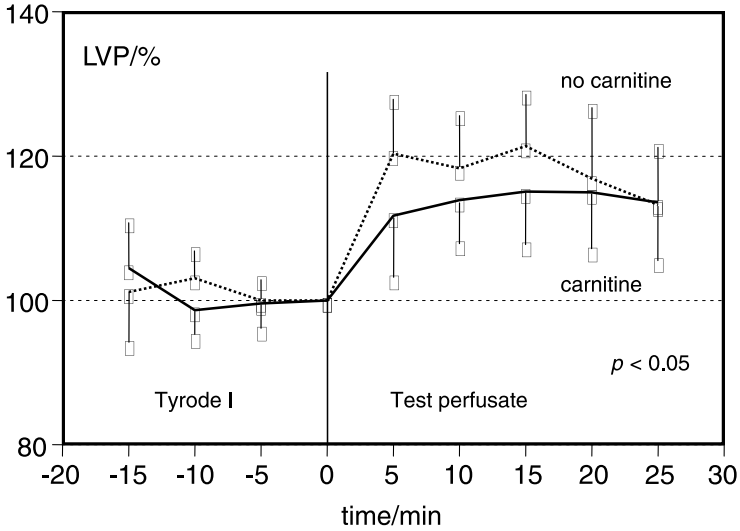

Fig. 6. Left ventricular pressure after switching from Tyrode I to Tyrode II at high concentrations of fatty acids (1.2 mmol/dm^3 Na-palmitate) and the effect of carnitine administration

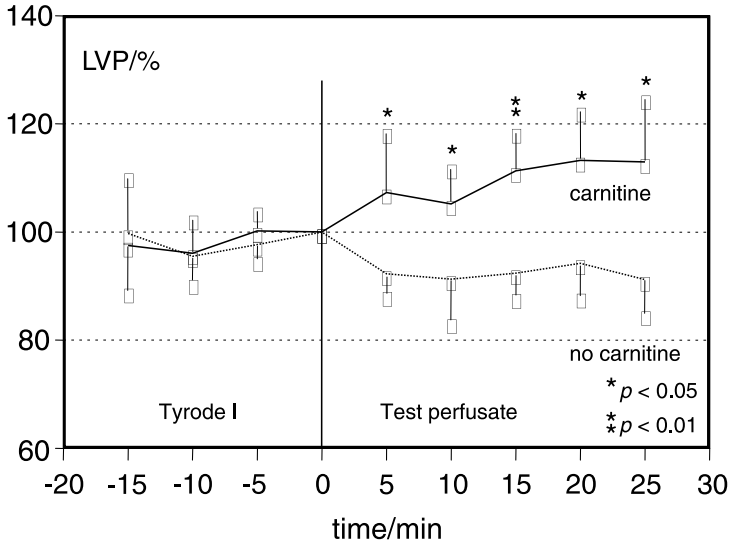

Fig. 7. Left ventricular pressure after switching from Tyrode I to Tyrode II at high fatty acid concentrations without glucose and the effect of carnitine administration

flow increased with both solutions. High levels of fatty acids and glucose produced considerably higher values than perfusion solutions without glucose. In combination, both substrates effected a markedly higher pressure development, myocardial relaxation, and pressure rate product.

Addition of *L*-carnitine to high fatty acid concentrations, however, produced only a slight increase in systolic pressures in comparison with carnitine-free solutions. This shows that high levels of fatty acids in combination with glucose superimpose themselves on the pressure-increasing properties of *L*-carnitine (Fig. 6). However, if glucose is removed from the perfusion solution, the presence of *L*-carnitine effects an increase in LVP again (Fig. 7). *L*-Carnitine produced an increase in pressure, pressure rate product, and maximum rates of myocardial relaxation with perfusion solutions containing high levels of fatty acids but no glucose.

Maintaining a sufficient coronary flow of 8–12 cm^3/min was important in all experiments. Changing the perfusion solution to Tyrode solution II effected an increase in coronary flow in all experimental series. This increase is probably also caused by the presence of albumin, which was added to the perfusion solution to facilitate the transport of palmitate.

Langendorff-Hearts during Ischemia and Reperfusion

The main topic of the assays was the reaction of isolated hearts during reperfusion after 20 minutes of ischemia. During reperfusion, the *Langendorff*-hearts reacted in a very sensitive way to the concentrations of fatty acids contained in the perfusion solutions. At low sodium palmitate levels (0.4 $mmol/dm^3$) only a limited restoration of left ventricular pressure and related values was possible, although glucose was available. LVP could be restored to only 50% of its pre-ischemic value (Fig. 8). At high palmitate concentrations, however, an LVP of 70% was found after only 30 min; thus it can be said that a much quicker and more pronounced restoration of

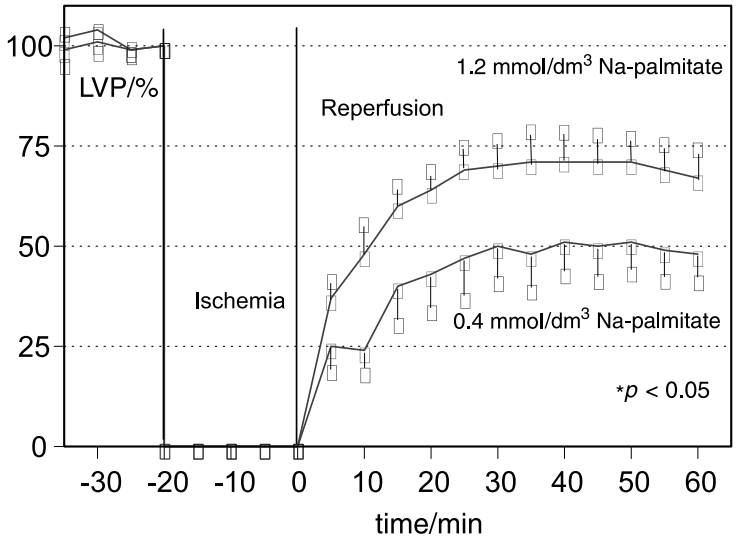

Fig. 8. Recovery of left ventricular pressure of isolated *Langendorff*-hearts during reperfusion at various concentrations of fatty acids

LVP takes place if a high supply of fatty acids is available. A similar reaction was found for pressure rate product, maximum rate of myocardial relaxation, and quotient Q_{CR}. Although high concentrations of fatty acids are often blamed for increasing the stress on hearts by supporting the formation of radicals and arrhythmias, it appears that high levels of fatty acids are necessary for the production of energy during aerobic perfusion.

Just like switching over from perfusion solution I to perfusion solution II, supplementation with *L*-carnitine produced a very strong reaction: With 0.4 mmol/dm^3 sodium palmitate, the LVPs of isolated hearts exceeded their pre-ischemic values

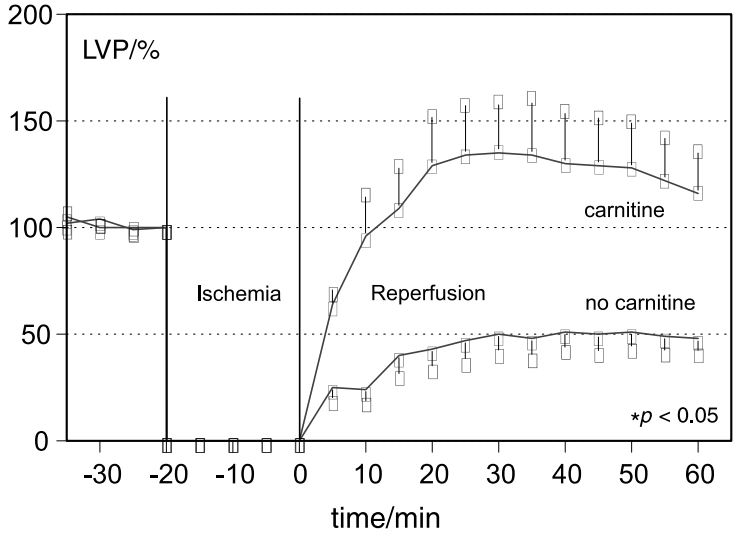

Fig. 9. Recovery of left ventricular pressure of isolated *Langendorff*-hearts during reperfusion with 0.4 mmol/dm^3 Na-palmitate and glucose and the effect of adding *L*-carnitine

after only 10 minutes of reperfusion, reaching impressive peaks of more than 130%, whereas controls without *L*-carnitine reached only about 50% of their pre-ischemic values (Fig. 9). The high LVPs were held up to the 35th minute, then they began to decrease slowly. However, until the end of the reperfusion period they remained higher than the pre-ischemic values. Here, too, the other hemodynamic parameters behaved in a similar way.

At high fatty acid levels (1.2 mmol/dm^3 Na-palmitate), the LVP during reperfusion is also influenced by the glucose level (of course, this is also the case with low fatty acid levels, but those hearts do not produce any sufficient LVP). Glucose supplementation during reperfusion improved both left-ventricular pressure and maximum relaxation rate. Without glucose there was only a very slow development of LVP from 0 to 60% within 60 minutes, whereas an addition of glucose produced 70% of the pre-ischemic values after only 30 minutes (Fig. 10).

L-Carnitine supplementation effected a further increase in LVP. In spite of the presence of glucose, *L*-carnitine boosted LVPs to 90% of their pre-ischemic values in the reperfusion period, compared to 70% without *L*-carnitine (Fig. 11).

As expected, LVP decreased without glucose. There was a slow but steady increase to a maximum of 60% over 60 minutes. Once again, addition of *L*-carnitine increased LVP to nearly 90% of the pre-ischemic pressure (Fig. 12). In the assays with 1.2 mmol/dm^3 Na-palmitate, the other hemodynamic parameters improved in the same way as described before.

It should also be noted that, in most cases, there was an increase in coronary flow under *L*-carnitine. Particularly in the experiments with 0.4 mmol/dm^3 Na-palmitate and glucose as well as in the assays with 1.2 mmol/dm^3 Na-palmitate but without glucose, *L*-carnitine effected a significant increase of about 25% in the coronary flow rates. This result should be of particular importance with regard to ischemic cardiac diseases.

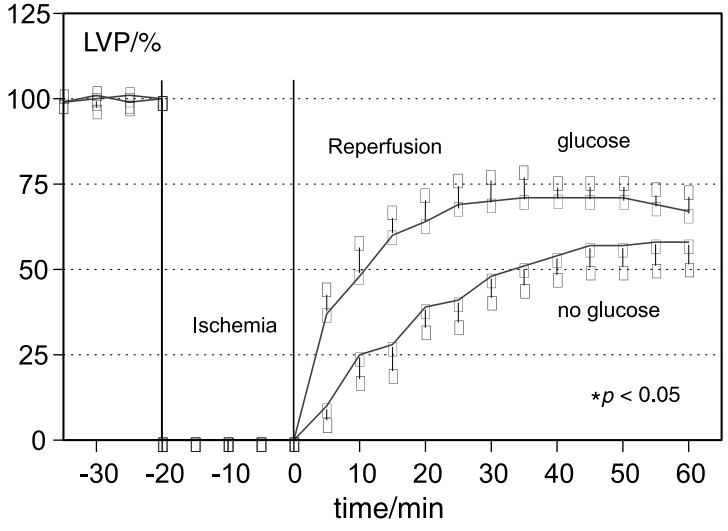

Fig. 10. Recovery of left ventricular pressure of isolated *Langendorff*-hearts during reperfusion with 1.2 mmol/dm^3 Na-palmitate and the effect of adding glucose

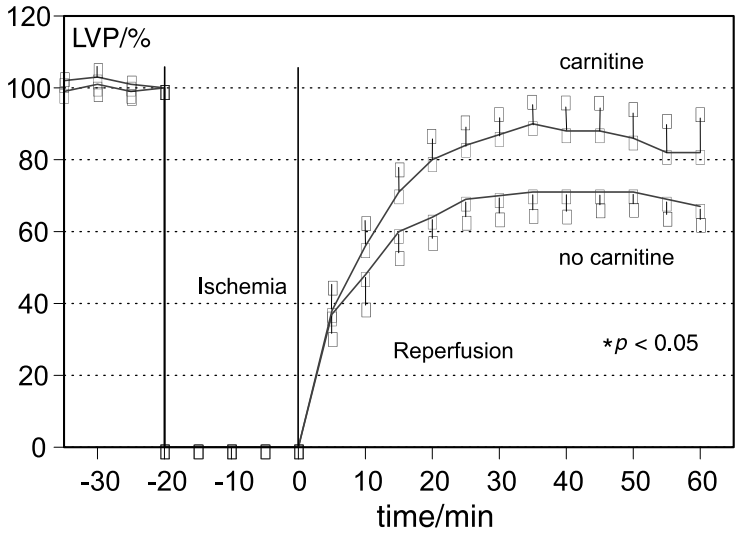

Fig. 11. Recovery of left ventricular pressure of isolated *Langendorff*-hearts during reperfusion with 1.2 mmol/dm^3 Na-palmitate and glucose and the effect of adding *L*-carnitine

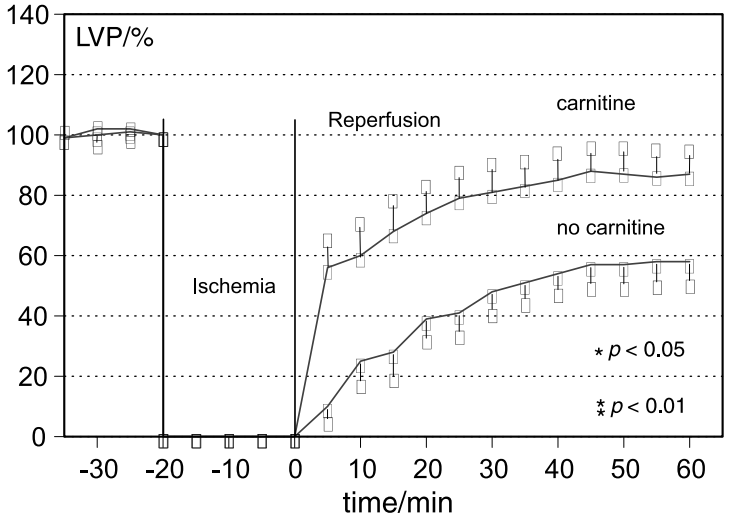

Fig. 12. Recovery of left ventricular pressure of isolated *Langendorff*-hearts during reperfusion with 1.2 mmol/dm^3 Na-palmitate without glucose and the effect of adding *L*-carnitine

Intracellular Concentrations of L-Carnitine and Malondialdehyde

These findings lead to the question whether ischemia effects any long-term alterations in tissue carnitine levels and whether the changes in LVP might also be due to lowered endogenous *L*-carnitine concentrations. Therefore, after measuring hemodynamic parameters, total carnitine (T-*Cn*) and free carnitine (F-*Cn*) were determined in cardiac tissue and put in relation to non-collagen-protein (NCP) concentrations (Fig. 13). NCP concentrations were nearly identical in all groups:

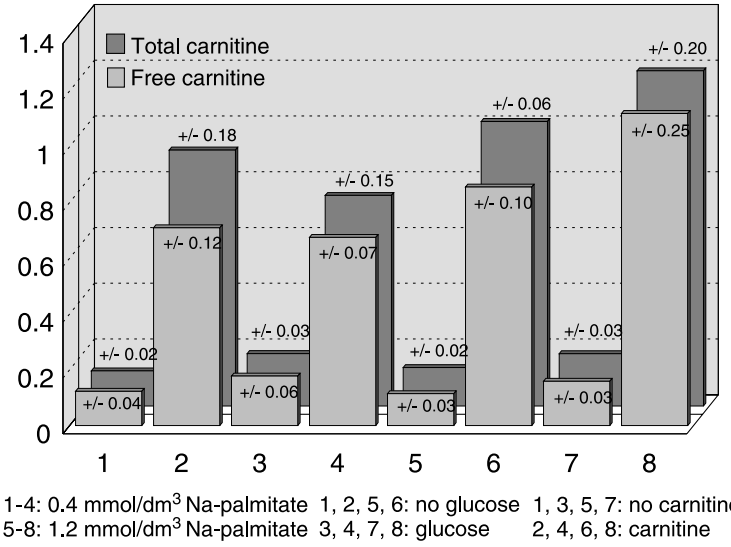

Fig. 13. Concentrations of *L*-carnitine in rat heart tissue under various perfusion conditions [μmol/dm^3 g NCP]

about 17.0 ± 3.2 g/dm^3. It was found that cellular carnitine levels during ischemia were reduced to approximately 30% of their pre-ischemic values [from 6.86 ± 0.99 (T-*Cn*) and 6.26 ± 0.78 μmol/g NCP (F-*Cn*) to 2.81 ± 0.46 (T-*Cn*) and 1.99 ± 0.37 μmol/g NCP (F-*Cn*)] if hearts were perfused with just fatty acids (no glucose or carnitine). Thus it must be assumed that a considerable loss of carnitine occurs during ischemia. During perfusion with 1.2 mmol/dm^3 Na-palmitate (groups 5–8 *vs*. groups 1–4) there were no significant differences in F-*Cn* compared to perfusion with 0.4 mmol/dm^3. T-*Cn*, however, was lowered ($p < 0.005$), which means that there must be an additional loss of carnitine under these conditions.

Additional perfusion with glucose was able to minimise this loss of carnitine but could not prevent it completely (groups 3, 4, 7, 8 *vs*. 1, 2, 5, 6). In rat hearts perfused with 0.4 mmol/dm^3 Na-palmitate, F-*Cn* amounted to 44% and T-*Cn* to 55% of their normal values; hearts perfused with 1.2 mmol/dm^3 Na-palmitate contained 42% of the normal F-*Cn* values and 49% of normal total carnitine ($p < 0.01$).

Carnitine levels that considerably exceeded those found in healthy rat hearts were measured in hearts perfused with endogenous *L*-carnitine (groups 2, 4, 6, 8 vs. 1, 3, 5, 7). Obviously, supplemented *L*-carnitine was actively taken up into the heart cells in the experimental period because some of the LVPs found in the various experimental groups differed quite considerably and the direct determination of the tissue content of *L*-carnitine and its esters produced the same differences.

What is remarkable is the fact that, within the short experimental period of about three hours, exogenous *L*-carnitine was converted into carnitine esters. The percentage of acylcarnitines in the perfusion solutions with carnitine was higher than it should have been during the perfusion with exogenous, free *L*-carnitine; therefore, an active carnitine metabolism must be assumed (Table 3).

Table 3. Concentrations of *L*-carnitine and acylcarnitines in rat heart tissue

Perfusion solution	Free carnitine [μmol]/total NCP [g]	Total carnitine [μmol]/total NCP [g]	Acylcarnitines [μmol]/total NCP [g]	Amount of acylcarnitines [%]
1	1.99 ± 0.37	2.81 ± 0.46	0.82	29.18
2	12.37 ± 2.12	16.91 ± 3.09	4.54	26.85
3	2.73 ± 0.37	3.80 ± 1.11	1.07	28.16
4	12.33 ± 1.59	15.08 ± 3.02	2.75	18.24
5	1.94 ± 0.57	2.13 ± 0.55	0.19	8.92
6	16.19 ± 1.58	19.32 ± 0.73	3.13	16.20
7	2.63 ± 0.47	3.04 ± 0.39	0.41	13.49
8	18.76 ± 4.20	21.64 ± 4.07	2.88	13.31

Table 4. Concentrations of malondialdehyde in rat heart tissue

Perfusion conditions	*MDA* [μmol/g NCP]
(1) 0.4 mmol/dm^3 Na-palmitate, no glucose, no *L*-carnitine	8.18 ± 1.24
(2) 0.4 mmol/dm^3 Na-palmitate, no glucose, 5 mmol/dm^3 *L*-carnitine	6.31 ± 0.75
(3) 0.4 mmol/dm^3 Na-palmitate, 11 mmol/dm^3 glucose	10.17 ± 0.92
(4) 0.4 mmol/dm^3 Na-palmitate, 11 mmol/dm^3 glucose, 5 mmol/dm^3 *L*-carnitine	5.72 ± 1.39
(5) 1.2 mmol/dm^3 Na-palmitate, no glucose, no *L*-carnitine	6.17 ± 0.14
(6) 1.2 mmol/dm^3 Na-palmitate, no glucose, 5 mmol/dm^3 *L*-carnitine	6.13 ± 0.29
(7) 1.2 mmol/dm^3 Na-palmitate, 11 mmol/dm^3 glucose, no *L*-carnitine	7.59 ± 0.80
(8) 1.2 mmol/dm^3 Na-palmitate, 11 mmol/dm^3 glucose, 5 mmol/dm^3 *L*-carnitine	3.99 ± 0.67

The cellular concentrations of malondialdehyde (*MDA*), which are markers for oxidative cell damage, were also influenced by the perfusion conditions (Table 4). In group 1 (no glucose, no *L*-carnitine), *MDA* concentration amounted to 8.18 μmol/g NCP. By simply raising the Na-palmitate concentration to 1.2 mmol/dm^3 (group 5), this value could be reduced by about 25% to 6.17 μmol/g NCP ($p < 0.01$). Contrary to expectations, glucose provided no additional protective effect. Quite the opposite was the case. Glucose significantly increased the *MDA* concentrations in the group with 0.4 mmol/dm^3 Na-palmitate (group 3) from 8.18 to 10.17 μmol/g NCP, and in the group with 1.2 mmol/dm^3 Na-palmitate (group 7) from 6.17 to 7.59 μmol/g NCP ($p < 0.005$).

Only when *L*-carnitine was added (groups 2, 4, 6, 8), a significant decrease in malondialdehyde concentrations in the rat hearts became evident ($p < 0.05$). Surprisingly, this effect could even be strengthened by combining glucose and *L*-carnitine. In the group perfused with 0.4 mmol/dm^3 Na-palmitate, *L*-carnitine lowered *MDA* concentrations from 8.18 to 6.31 μmol/dm^3; a combination of glucose and *L*-carnitine even reduced *MDA* to 5.72 μmol/dm^3, whereas glucose alone increased *MDA* to 10.17 μmol/dm^3. Hearts perfused with 1.2 mmol/dm^3 Na-palmitate reacted in a similar, although not that pronounced way. *MDA*

concentrations were reduced from 6.17 to 6.13 μmol/dm^3 by *L*-carnitine, and to 3.99 μmol/dm^3 by *L*-carnitine combined with glucose, whereas they were increased to 7.59 μmol/dm^3 by glucose alone.

Thus, there was an additional protective effect which reduced reperfusion damage to isolated hearts. Increased Na-palmitate concentrations, an addition of *L*-carnitine to the perfusion solution, respectively a combination of *L*-carnitine and glucose in it – they all exert a favourable influence on the heart during reperfusion, which is also expressed by a more rapid and higher return of LVP in the reperfusion period.

ATP and Creatine Phosphate in Cardiac Tissue

Since the reaction of hemodynamic parameters during reperfusion ultimately depends on the restoration of sufficient *ATP* and creatine phosphate (*CrP*) levels, these were measured by means of ^{31}P NMR spectroscopy.

The evaluation of the spectra obtained (*CrP*, *ATP*, P_i) made it clear that, as expected, even changes in the composition of the perfusion solution were sufficient to influence the energy supply in the hearts. A dramatic increase in the CrP/P_i ratio to approximately 300%, for example, can be achieved by switching from a perfusion solution containing only glucose to one that contains glucose and 1.2 mmol/dm^3 Na-palmitate bound to albumin. Hemodynamically, a decrease in heart rate is found if the left-ventricular pressure increases by about 20% (see above). These findings agree with the fact that hearts obtain between 65 to 95% of their energy from the metabolisation of long-chain fatty acids; consequently the *CrP* level has to increase when the perfusion of a *Langendorff*-heart is switched from glucose to Na-palmitate.

The restoration of sufficient *ATP* and *CrP* concentrations during reperfusion after 20 minutes of no-flow ischemia is strongly influenced by *L*-carnitine supplementation. With *L*-carnitine, higher concentrations are achieved than in the controls. Particularly the *ATP* concentrations in the controls are markedly lower. On

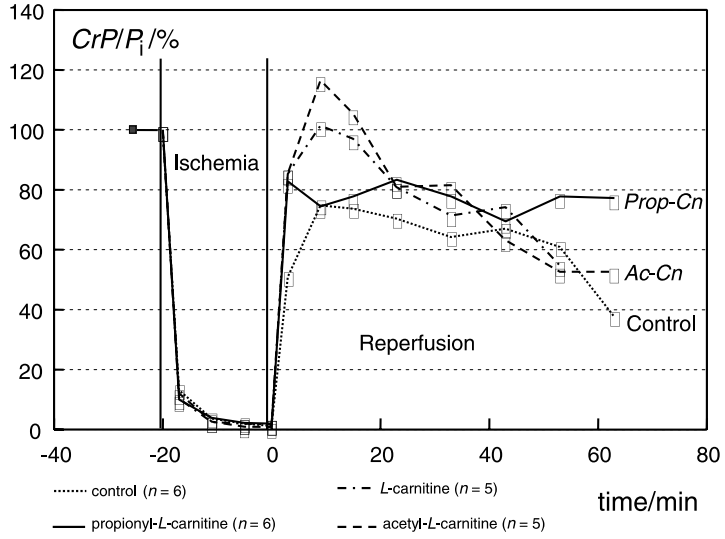

Fig. 14. Influence of *L*-carnitine and its esters on CrP/P_i ratios of isolated rat hearts during ischemia and reperfusion

average, the CrP/P_i ratio in the control hearts was 65% of the pre-ischemic value after 20 minutes of reperfusion, whereas the ratio in *L*-carnitine-treated hearts amounted to more than 85% (Fig. 14).

There is another interesting fact. The short-chain carnitine esters acetyl-*L*-carnitine and propionyl-*L*-carnitine developed a similar but individual effect. In reperfused controls the CrP/P_i ratio remained relatively constant at about 65% as of the tenth minute and decreased to 45% towards the end of the reperfusion period (50th minute). *L*-Carnitine and acetyl-*L*-carnitine, however, caused a strongly overshooting effect in the early reperfusion period (minutes 0–10 of reperfusion), which means the CrP/P_i ratio was higher than the pre-ischemic one. Under acetyl-*L*-carnitine, *Langendorff*-hearts even reached a CrP/P_i ratio of 130%, but began to decrease strongly as of the 15th minute (100% after 20 min, 75% after 40 min, and only 60% towards the end of reperfusion).

Propionyl-*L*-carnitine, on the other hand, does not produce such an overshooting effect. Very soon after ischemia these hearts developed about 80% of their pre-ischemic values, which was maintained (with slight alterations) until the end of the reperfusion period. Therefore, propionyl-*L*-carnitine produces the best effect of the three substances as it helps to develop a constant and high LVP.

Closer investigation of *L*-carnitine-perfused hearts established such a strong protective effect of *L*-carnitine that these hearts were able to withstand several ischemia-reperfusion-periods. Whereas the hearts in the control group came to a standstill after the second ischemia in spite of a sufficient substrate supply, hearts under *L*-carnitine continued to work. At the beginning of each reperfusion period they produced another overshooting CrP/P_i quotient. One experiment was stopped after a fourth ischemia with a CrP/P_i quotient of $<10\%$. At this point, this *Langendorff*-heart had been active for six hours and survived a total load of 80 min of ischemia (Fig. 15).

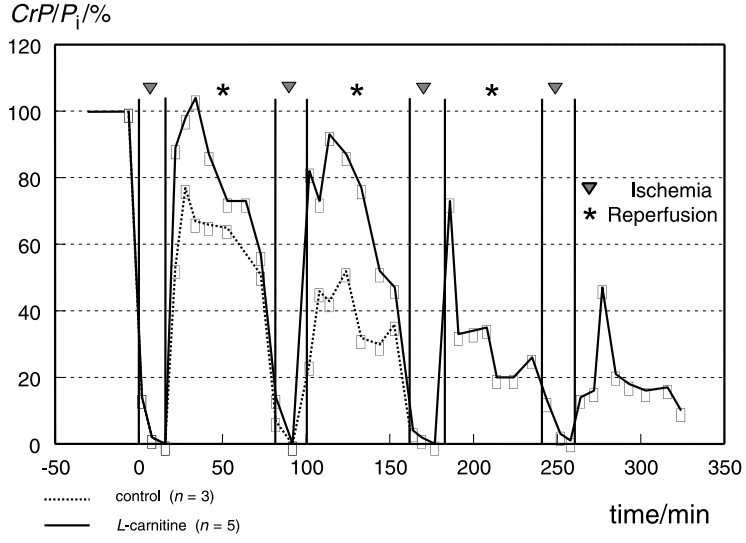

Fig. 15. Influence of *L*-carnitine on CrP/P_i ratios of isolated rat hearts after several ischemia/reperfusion periods

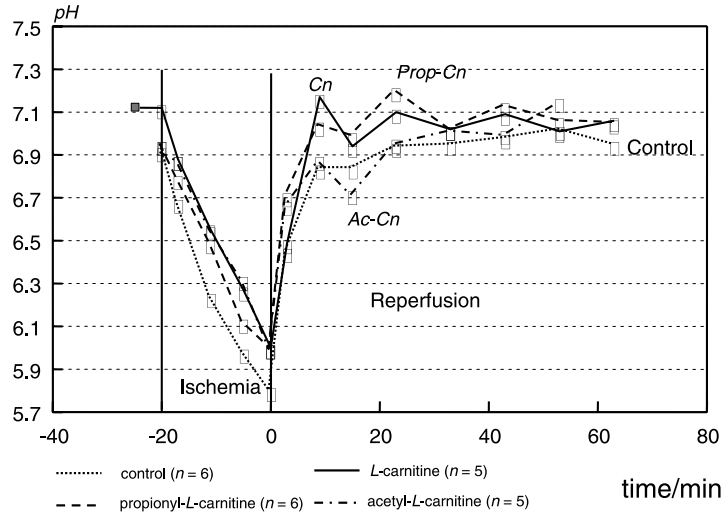

Fig. 16. Influence of *L*-carnitine and its esters on the cytosolic *pH* of isolated rat hearts during ischemia and reperfusion

An analogous behaviour was also found for the energy index. Due to the inclusion of the *ATP* content, values did not decrease to less than 50% and there was also a moderate increase in the early stages of reperfusion.

As it is possible to calculate the intracellular *pH* value from the mean chemical shifts of the $H_2PO_4^-$ and HPO_4^{2-} signals, this method was used to determine the changes in *pH* in the isolated hearts during ischemia and reperfusion. As expected, *pH* values in heart tissue strongly decreased during ischemia, reaching a minimum *pH* of 5.68 in the control hearts (Fig. 16). On the other hand, *pH* values in hearts perfused with *L*-carnitine and its esters did not decrease as strongly; the least decrease, due to its amphiphilic properties, was found for *L*-carnitine ($pH = 6.13$). The difference of $\delta pH = 0.45$ points to a substantial protective effect of *L*-carnitine against tissue acidosis during ischemia.

During repeated perfusion it was acetyl-*L*-carnitine again that effected the strongest recovery at the onset of reperfusion, because it took the *pH* value only 12 minutes to reach its pre-ischemic level. Propionyl-*L*-carnitine, too, restored the *pH* value to the same level during reperfusion, whereas the *pH* value under *L*-carnitine only slowly approached its pre-ischemic value. Throughout the whole reperfusion period, the *pH* values in the hearts of the control group remained below those of hearts perfused with *L*-carnitine or its esters, and it took a very long time until the *pH* values in the control hearts reached their final levels, which were somewhat below the ones achieved with *L*-carnitine and its esters.

Discussion

The main physiological function of *L*-carnitine is to facilitate the transport of medium- and long-chain fatty acids across the inner mitochondrial membrane by means of the enzymes carnitine-palmitoyl-transferase I and II (CPT I and II) and

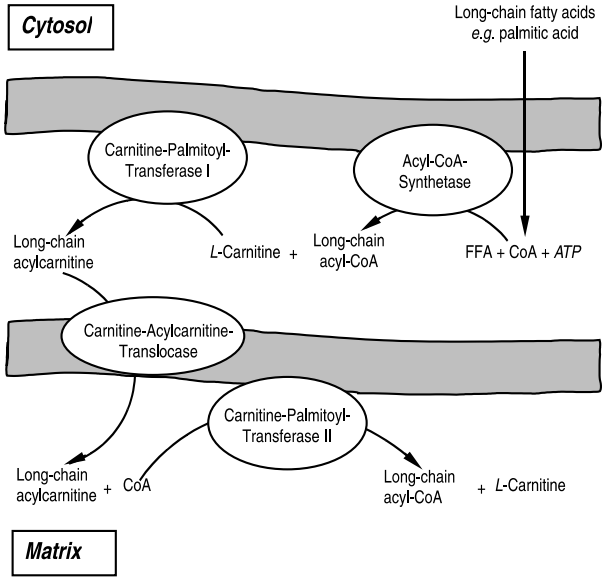

Fig. 17. The carnitine shuttle mechanism

carnitine-acylcarnitine-translocase (CACT) [16] (Fig. 17). Therefore, *L*-carnitine is an essential co-factor of the β-oxidation process. Therefore, a low endogenous carnitine level (primary or secondary carnitine deficiency) or a defect in the enzymes employed (enzymopathies) will lead to a number of severe metabolic disturbances [17, 18].

In addition to this carrier function, *L*-carnitine has a possibly even more important regulatory function: influencing the CoA status [19] (Fig. 18). Since only low activation energies are needed to transfer acyl groups from acyl-CoA to *L*-carnitine, the acyl-CoA/free CoA ratio can easily be altered in favour of free CoA.

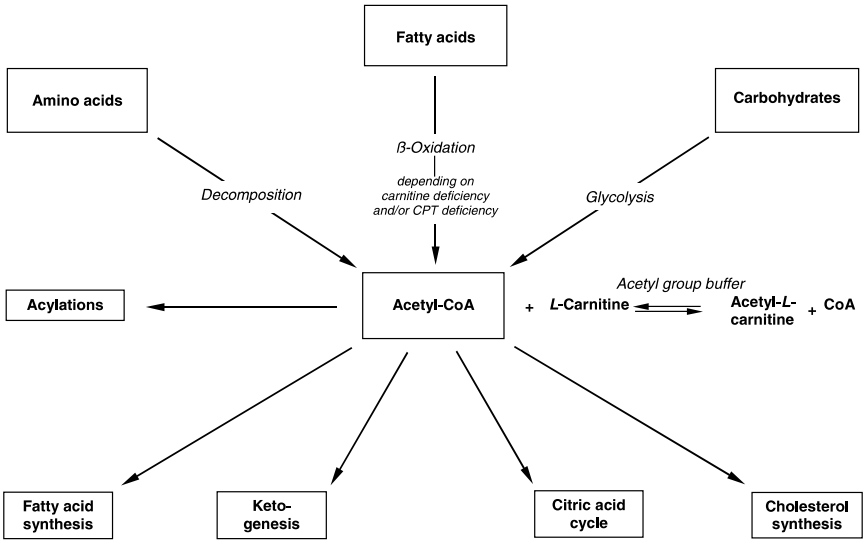

Fig. 18. Influence of *L*-carnitine on the acyl-CoA/CoA ratio

Increased concentrations of long-chain (LC) acyl-CoA compounds inhibit a number of important enzymes of the intermediary metabolism such as acetyl-CoA carboxylase, adenine nucleotide translocase (ANT), citrate synthetase, pyruvate dehydrogenase (PDH) [20], pyruvate carboxylase, and *N*-acetyl glutamate synthetase [21]. If the LC-acyl-CoA/CoA quotient is larger than 1, the aforementioned enzymes are inhibited, which causes severe disturbances of the mitochondrial metabolism.

Because of the central position of *L*-carnitine in the intermediary metabolism, numerous effects of exogenous *L*-carnitine applications in a wide variety of metabolic disorders are under discussion. The majority of disturbances of intermediary metabolism can be corrected by *L*-carnitine application. Because of the oxygen deficiency during ischemia, the metabolisation of long-chain fatty acids comes to a standstill and long-chain fatty acids accumulate both inside and outside the mitochondria [22]. Due to the esterification of CoA and the associated consumption of *ATP*, a strong increase in the long-chain acyl-CoA ester/free CoA ratio develops (LC-acyl-CoA/CoA >1, respectively acetyl-CoA/CoA >1). As the *NAD*H/NAD^+ quotient increases in the same way, a metabolic situation develops in which the increased LC-acyl-CoA concentrations inhibit ANT and PDH activities, thus preventing the efflux of remaining *ATP* from the mitochondria [23, 24].

By stimulating carnitine-acyl-transferase activities, exogenous *L*-carnitine effects a re-esterification of LC-acyl-CoA into LC-acylcarnitines, which increases the concentrations of free CoA and leads to a 10- to 20-fold intramitochondrial decrease in the acyl-CoA/CoA ratio, thus normalising the inhibited enzymatic activities. The inhibition of ANT is lifted, which permits the transport of synthetised *ATP* from the mitochondria [25]. The processes involved in fatty acid oxidation, citric acid cycle and respiratory chain, are more or less normalised, although *L*-carnitine is not able to correct an oxygen deficiency. As demonstrated by NMR experiments, *L*-carnitine and its esters obviously effect a more rapid increase in *ATP* and *CrP* during reperfusion by accelerating the lifting of the blockades of ANT and PDH. It is of importance that the acylcarnitines that were formed after a carnitine application are able to leave the mitochondria and the cell whereas acyl-CoA compounds are retained, thus reducing the acyl-group load on the cell [26].

Carnitine deficiency does not only cause disturbances in lipid metabolism but also, because of the inhibition of the pyruvate-dehydrogenase complex, in carbohydrate metabolism [27]. Instead of acetyl-CoA, lactate is produced from pyruvate, which lowers the *pH* in the ischemic cell. As *L*-carnitine substitution increases PDH activity and restarts the formation of acetyl-CoA, *L*-carnitine also controls the metabolisation ratio of fatty acids and carbohydrates in various organs and especially in the heart [28].

Depending on load, substrate, and oxygen supply, the heart metabolises more than 60% of fatty acids (20–95%) under aerobic conditions, followed by substrates such as glucose with 20% (15–35%), lactate with 15% (5–60%), ketone bodies with 4% (3–20%), amino acids with 2% (3–5%), and pyruvate with 1% (0.5–2.5%) [29, 30]. If sufficient oxygen is supplied, carnitine facilitates the transport of LC-fatty acids across the inner mitochondrial membrane into the cell, where they are metabolised by β-oxidation and citric acid cycle, and the H equivalents are used for *ATP*-synthesis in the respiratory chain.

These processes are disturbed in the case of

- carnitine, CPT or CACT deficiencies (no fatty-acid transport into the mitochondria),
- oxygen deficiency (no *ATP*-synthesis in the respiratory chain),
- excessive supply of fatty acids (high LC-acyl-CoA concentrations inhibit the above-mentioned enzymes in metabolic or endocrine disturbances such as diabetes mellitus and excessive supply of catecholamines [phaeochromocytoma]).

This results in an excessive mitochondrial supply of LC-acyl-CoA, which cannot be transported into the mitochondria because of carnitine or enzyme deficiency [31]. Intramitochondrially, reduced *ATP*-synthesis leads to an accumulation of substrates that reaches, *via* respiratory chain and citric acid cycle, right back to β-oxidation [32].

In our experiments, myocardial ischemia caused creatine phosphate and *ATP* deficiencies, which limited hemodynamic functions [33, 34]. The underlying biochemical processes are primarily the results of oxygen deficiency, and secondarily an accumulation of long-chain acyl-CoA esters, which make the damage caused by ischemia even more pronounced. This status also influences the hemodynamic parameters measured in the isolated hearts.

Restoration of left-ventricular pressure during reperfusion depends on the duration of the ischemia, its conditions (no-flow, low-flow, or intermittent ischemia), and the composition of the perfusion solution [35]. During reperfusion, left-ventricular pressure and other hemodynamic parameters increased if palmitate concentrations were increased; this means that high palmitate concentrations are necessary for the restoration of a sufficient LVP in the first phase of reperfusion. Because of an increase in fatty-acid oxidation, the ischemically damaged *Langendorff*-heart needs a higher supply of free fatty acids after no-flow ischemia, in spite of the fact that high levels of fatty acids are blamed for the development of arrhythmias [36] and radicals [37].

With glucose the situation is similar. In isolated hearts perfused with high concentrations of fatty acids (1.2 $\mathrm{mmol/dm^3}$) but without glucose, LVP increased only slowly. Addition of glucose, however, effected an impressive increase in the recovery rate. A comparison of reperfusion results showed quite clearly that perfusion with a perfusion solution containing glucose produced a better recovery than perfusion solutions that supplied the metabolic processes in the heart only with free fatty acids but not with glucose. Obviously, glucose also exerts a protective effect in the first minutes of reperfusion, whereas after 60 minutes the values for perfusion with respectively without glucose are getting closer to each other. Thus it can be assumed that post-ischemic oxidation of glucose alone, in spite of its low percentage, is sufficient to improve reperfusion [38]. Apparently, glucose as a rapidly available substrate has a favourable effect on the LVP in hearts in the first minutes of reperfusion. Since *L*-carnitine lowers the intramitochondrial acetyl-CoA/CoA ratio and thus reactivates pyruvate dehydrogenase, *L*-carnitine directly influences glucose metabolism by increasing it [39]. A comparison of perfusion solutions with low fatty-acid content and with respectively without carnitine demonstrates this quite clearly.

During perfusion with low concentrations of fatty acids (0.4 $\mathrm{mmol/dm^3}$), addition of *L*-carnitine to the perfusion solution effected, compared to the control

group, a very strong increase in left-ventricular pressure and coronary flow. Comparison with the respective control group showed that *L*-carnitine favourably influenced reperfusion hemodynamics during reperfusion with high fatty-acid concentrations ($1.2\,\mathrm{mmol/dm^3}$) as well.

The pronounced and mostly significant favourable effect of *L*-carnitine on the isolated heart that is perfused with fatty acids after ischemia confirms similar findings of other authors. Most authors assume that *L*-carnitine protects the myocardium by facilitating the removal of long-chain acyl groups, which lifts the inhibition of mitochondrial enzyme systems and improves the intermediary metabolism in the mitochondria. Fatty-acid oxidation is stimulated by increasing the activity of carnitine-palmitoyl-transferase [40]. In addition to the improvement effected in the oxidation of fatty acids, it is increasingly suggested that improving the oxidative substrate metabolisation of glucose is the decisive protective effect of *L*-carnitine [41, 42].

In order to investigate the effects of glucose and *L*-carnitine separately, non-physiological hearts that were perfused only with fatty acids were used to find out whether *L*-carnitine might effect any post-ischemic protection under these conditions as well. As it turned out, all reperfusion parameters measured showed a significant or even highly significant improvement under *L*-carnitine. In comparison with glucose-free control experiments, *L*-carnitine produced improvements in left-ventricular reperfusion parameters as well as in coronary flow; therefore the effects of carnitine cannot be due to its stimulation of glucose oxidation alone, its influence on mitochondrial fatty-acid metabolism is just as important [43].

As coronary flow was also significantly improved under *L*-carnitine substitution, it can be suggested that this compound probably has an individual effect on the post-ischemic myogenic autodilatation of the coronary arteries, which appears to be dependent on the dose applied [44].

The protective effects of *L*-carnitine must be attributed to a marked increase in the carnitine concentrations in the cells after perfusion with a solution containing carnitine. Exogenous *L*-carnitine permeates the cell's membrane in an extremely short time and can be determined in tissue after the experiments. It should be noted that there is obviously a strongly increased carnitine metabolism, because perfusion with free, non-esterified carnitine produces higher acylcarnitine levels in tissue than perfusion without *L*-carnitine [45].

The concentrations of malondialdehyde (*MDA*), an indicator of oxidative stress, behave in an inversely proportional way. Since perfusion with *L*-carnitine lowers *MDA* levels, the observed favourable effects of *L*-carnitine such as improved cardiac dynamics and a reduced number of arrhythmias can be explained by the decrease in ischemic damage under *L*-carnitine [46].

Investigations of cardiac metabolism have also established that radicals are present during ischemia and reperfusion, which cause, depending on the time, a large number of tissue-damaging processes. Reperfusion, however, does not put an end to the cell-damaging processes induced by ischemia; the sudden supply of oxygen leads to an increased formation of oxygen radicals, which cause even more damage to the cell (oxygen paradoxon) [47].

An interesting point in this respect is the question whether and how *L*-carnitine and its short-chain acyl esters might act as antioxidants or radical scavengers

[48, 49]. There are reports on favourable effects of *L*-carnitine and particularly propionyl-*L*-carnitine on hearts during reperfusion. Such improvements were found both in isolated hearts and in patients [50]. As working on isolated hearts permits free choice of reperfusion conditions, these tests provided a somewhat clearer picture. Irrespective of the disputed question whether or not *L*-carnitine can act as a scavenger of radicals, our experiments have shown that both the momentary *L*-carnitine concentrations in cardiac tissue and the formation of malondialdehyde during reperfusion are influenced by *L*-carnitine supplementation [51]. Older experiments on the restoration of LVP during reperfusion had shown that *L*-carnitine has to be added to the perfusion solution before the ischemic period in order to produce its protective effect.

The ways by which carnitine esters exert their influence have not been fully clarified yet, but it appears that they possess their own special mechanisms. Firstly, their concentrations in the perfusion solution are lower than they should be if their effects are recalculated to the carnitine concentration, and secondly, a chemical effect that corresponds to that of *L*-carnitine should entail the presence of free *L*-carnitine. The suggestion that carnitine esters have their own special mechanisms is also supported by a number of studies in which other authors investigated hemodynamic and electrophysiological parameters in isolated hearts, living animals, and patients [52, 53].

Based on the results mentioned in this paper, numerous clinical studies on carnitine applications, and its proven protective effects, an adjuvant therapy with *L*-carnitine might be a new, purely metabolic approach, which could supplement existing therapies in a number of cardiac diseases.

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