Utilization of very low density lipoprotein by rat heart: the effect of endotoxin

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Bennett, Mark J., David Hauton, David G. Hole, and Rhys D. Evans. Utilization of very low density lipoprotein by rat heart: the effect of endotoxin. Am J Physiol Endocrinol Metab 278: E802–E810, 2000.—The effect of endotoxin on myocardial utilization of very low density lipoprotein (VLDL) triacylglycerol (TAG) was studied. VLDL was prepared by rat liver perfusion and tested as substrate in the isolated working rat heart. Both liver and heart donor rats were pretreated in vivo with endotoxin or vehicle (control). VLDL-TAG synthesized by endotoxin-pretreated livers was assimilated and oxidized at an increased rate by hearts compared with control VLDL-TAG, regardless of the cardiac endotoxic status, with increased cardiac mechanical performance (cardiac output, hydraulic work). There was no change in incorporation of labeled VLDL lipids into myocardial tissue lipids. Lipoprotein lipase (LPL) activity was increased in endotoxin-pretreated hearts, and after perfusion with “endotoxic” VLDL, there was a tendency for translocation of LPL from tissue-residual to heparin-releasable compartments, but these changes were modest. Analysis of the VLDL composition showed that endotoxin-pretreated livers produced apolipoprotein (apo)-B48 VLDL with decreased particle size (and hence TAG content), but apo-B100 VLDL was unchanged. Oleate content of VLDL was increased, but there was no difference in apo-C or apo-E content. These results suggest that VLDL-TAG produced during sepsis/endotoxinemia may be destined for utilization by the heart as energy substrate. However, the mechanism for its increased efficacy is uncertain.

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cardiac metabolism (31). However, endotoxin can cause myocardial depression directly (34) or via TNF-α (34), but myofilament Ca2⁺ responsiveness of rat ventricular myocytes remains intact (29), and IL-1 released by endotoxin itself attenuates the endotoxin depression of cardiac contractility (40). The heart is a quantitatively important consumer of TAG fatty acid as energetic substrate with high LPL activity (30), and incorporation of fatty acid into intracellular TAG (and phospholipids) is increased after endotoxin treatment in dog myocytes (23); therefore, the heart is a putative destination of fatty acid into intracellular TAG (and phospholipids) is increased after endotoxin treatment in dog myocytes (23); therefore, the heart is a putativedestination of fatty acid into intracellular TAG (and phospholipids) is increased after endotoxin treatment in dog myocytes (23); therefore, the heart is a putative destination of fatty acid into intracellular TAG (and phospholipids) is increased after endotoxin treatment in dog myocytes (23); therefore, the heart is a putative destination of fatty acid into intracellular TAG (and phospholipids) is increased after endotoxin treatment in dog myocytes (23); therefore, the heart is a putative destination of fatty acid into intracellular TAG (and phospholipids) is increased after endotoxin treatment in dog myocytes (23); 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therefore, the heart is a putative destination of fatty acid into intracellular TAG (and phospholipids) is increased after endotoxin treatment in dog myocytes (23); therefore, the heart is a putative destination of fatty acid into intracellular TAG (and phospholipid
2.5% (wt/vol) endotoxin- and fatty acid-free BSA was filtered through a 5-µm cellulose nitrate filter (Millipore, Bedford, MA) and gassed with 95% O2:5% CO2 at 37°C. The first 50 ml of coronary effluent were discarded to free the circuit of blood cells. Afterload was maintained at 100 cm H2O and preload (atrial filling pressure) at 15 cm H2O. After an initial 5-min stabilization period, VLDL was added slowly (2 min) to the reservoir (at t = 0). Peak systolic pressure (PSP) and heart rate (HR) were measured by calibrated pressure transducer (Druck, Groby, Leicestershire, UK) connected to a side arm of the aortic cannula. Aortic flow rate (AFR) was measured by a timed collection of perfusate ejected through the aortic line, and coronary flow rate (CFR) was measured by a timed collection of perfusate effluent dripping from the heart. Measurements were made at t = 0 and at 10-min intervals for 60 min. CO was calculated as (CFR + AFR). Rate-pressure product (RPP) was calculated as (HR × PSP). Hydraulic work (HW) was calculated as (CO × mean aortic pressure ÷ heart rate wt). After the final measurements at 60 min, 5 IU/ml heparin (Leo Laboratories, Princes Risborough, Buckinghamshire, UK) were added to the perfusate, and after a further 2 min, the heart was rapidly excised, freeze-clamped in light alloy tongs cooled in liquid nitrogen, and weighed. A duplicate sample of the postheparin perfusate was also frozen in liquid nitrogen.

Measurement of lipid oxidation rate. TAG oxidation rate was estimated by measuring 3H2O production in the perfusate from [3H]triolein, as described (11); at 10-min intervals, aliquots of perfusate (1.0 ml) were removed and subjected to Folch lipid extraction with chloroform-methanol (2:1, vol/vol) and water (37). An aliquot of the water phase was removed and counted for radioactivity.

TAG utilization rate (disappearance from the perfusate) was measured by assay of TAG in the organic infranatant phase of the Folch extracts of the timed perfusate aliquots after evaporation of the chloroform and resolubilization with ethanol by means of an enzymatic colorimetric assay test kit (see above).

Incorporation of exogenous lipid into myocardial lipid. Myocardial 3H-labeled lipid content was estimated by grinding frozen myocardium to powder under liquid N2 and extracting this with lipid from an aliquot with chloroform-methanol (Folch). After repeated washing, the lipids were resolubilized in chloroform and were separated by TLC by use of a hexane-diethyl ether-acetic acid system (16) with standards co-run. 3H radioactivity was measured in the various lipid bands after visualization with rhodamine 6G under ultraviolet light.

LPL activity. LPL activity was estimated in duplicate samples by using a 3H-labeled triolein substrate emulsion containing starved rat serum as a source of apolipoprotein-CII to maximize LPL detection (25). The serum was pre-treated by heating to 56°C to inactivate nonspecific plasma lipases. Radioactivity in evolved fatty acids was counted after extraction in methanol-chloroform-heptane. Heparin-releasable LPL activity was measured by adding postheparin perfusate taken at 62 min directly in the above assay system without modification (expressed as nmol fatty acid released·min⁻¹·g wet wt of heart⁻¹). Tissue residual LPL activity was measured in ace tone-diethyl ether-dried tissue powders ground from the working hearts frozen in liquid nitrogen. A duplicate sample of frozen heart tissue was weighed, dried down with acetone-ether in parallel with the samples, and reweighed to correct expression of activity (from nmol fatty acid released·min⁻¹·mg of acetonatedried powder⁻¹ to nmol fatty acid released·min⁻¹·g wet wt of heart⁻¹). Total LPL activity in these experiments was heparin-releasable + residual LPL activities (nmol fatty acid released·min⁻¹·g wet wt of heart⁻¹).

Statistics. Results are expressed as mean values ± SE. Statistical analysis was performed by one-way ANOVA for repeated measurements and Tukey’s test, or by Student’s t-test with Bonferroni correction for multiple comparisons where appropriate. Statistical significance was set at P < 0.05.

RESULTS

Uptake of TAG by perfused working hearts was significantly greater from “endotoxic” VLDL (derived from endotoxin-exposed rat livers) than from control VLDL, regardless of previous exposure of the heart itself to endotoxin (Fig. 1). In the course of the 60-min experiment, this represented removal of up to ~1 mg of VLDL-TAG from the perfusate, ~3% of the total available circulating TAG. The possibility that endotoxin itself bound to the VLDL particle might be responsible for the effects of “endotoxic” VLDL was investigated by preincubating control VLDL with endotoxin. When added to control hearts, a similar TAG uptake to control VLDL was observed (Fig. 1), suggesting that particle-associated endotoxin was not involved.

Metabolic fate of assimilated [3H]TAG was examined by measuring oxidation and deposition of tissue 3H-labeled lipids. As with TAG uptake (Fig. 1), TAG oxidation rate was greater from endotoxic VLDL than from control VLDL (with or without preincubation with endotoxin), again regardless of heart endotoxic status. This was significant in the case of endotoxic VLDL perfusing hearts from animals previously injected with endotoxin (Fig. 2). However, there was no significant difference in accumulation of 3H-labeled tissue lipids (in any class examined by TLC) from 3H-labeled VLDL
during the course of the experiment in any group studied (Table 1). Utilization of TAG expressed as TAG oxidation plus total tissue \(^3\)H-labeled lipid accumulation (assuming no other metabolic fate of TAG taken up from the perfusate by the heart) showed a similar pattern to TAG oxidation, i.e., increased TAG utilization from endotoxic VLDL, significant in the case of hearts from endotoxic animals (data not shown), a reflection on the expected relatively greater contribution of oxidation (>70%; Fig. 2) compared with tissue pool deposition in heart in the working state. The proportion of VLDL-TAG oxidized was greater from endotoxic VLDL than from control VLDL (Fig. 2). Perfusion of the heart with endotoxic VLDL (VLDL-E; Fig. 4) was associated with a significant translocation of LPL activity from tissue-residual to heparin-releasable (endothelial) compartments (P < 0.05), an effect not observed in hearts perfused with control VLDL or NEFA.

Table 1. Effect of endotoxin administration on accumulation of \(^3\)H-labeled lipids in rat heart perfused with VLDL

<table>
<thead>
<tr>
<th>Heart</th>
<th>VLDL</th>
<th>N</th>
<th>Myocardial (^3)H Tissue Lipid, µmol/g wet wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phospholipid</td>
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<tr>
<td>Control</td>
<td>Control</td>
<td>9</td>
<td>0.0436 ± 0.0023</td>
</tr>
<tr>
<td>Control</td>
<td>Endotoxin</td>
<td>7</td>
<td>0.0405 ± 0.0071</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>Control</td>
<td>7</td>
<td>0.0445 ± 0.0174</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>Endotoxin</td>
<td>12</td>
<td>0.0591 ± 0.0071</td>
</tr>
<tr>
<td>Control</td>
<td>Control + endotoxin</td>
<td>7</td>
<td>0.0777 ± 0.0097</td>
</tr>
</tbody>
</table>

Rat hearts were perfused with \(^{3}\)H-triolein-labeled very low density lipoprotein (VLDL) prepared by rat liver perfusion; animals were pretreated in vivo with endotoxin or saline (control). Lipids were extracted from heart tissue after 60 min of perfusion. Control + endotoxin VLDL was VLDL from control liver perfusions incubated with endotoxin in vitro before heart perfusion. For further details see text. Results are means ± SE; no significant differences between groups. NEFA, nonesterified fatty acid.
were expressed as a proportion of combined apo-B100 + apo-B48 to correct for particle numbers (Fig. 6). Apolipoprotein-H was present in relatively high copy number in liver perfusion-derived VLDL compared with native VLDL, regardless of endotoxin exposure, whereas endotoxin exposure tended to decrease VLDL content of apo-E and apo-C (the latter representing combined apo-CII and CIII), regardless of VLDL origin. This was significant in the case of apo-C in endotoxic native VLDL compared with control native VLDL (Fig. 6).

VLDL lipid content was expressed as a proportion of total lipid content; as expected, TAG was found to be the predominant lipid class present (Fig. 7). Cholesterol content was increased in VLDL from endotoxic liver perfusions but was not found in VLDL from endotoxic rat plasma, and the increased cholesterol content of native plasma was not seen in liver perfusion VLDL (Fig. 7). When fatty acid content of VLDL lipids was examined, liver perfusion-derived VLDL contained more 18:1 (oleate) in TAG than native VLDL (livers perfused with oleic acid), and this was further increased by endotoxin exposure, but no other differences in fatty acid distribution were noted (Fig. 8).

**DISCUSSION**

A striking feature of sepsis and endotoxinemia is the associated hypertriglyceridemia resulting from increased circulating VLDL. Because this occurs by two distinct mechanisms, increased (hepatic) production (7, 14, 21) and decreased peripheral uptake by certain tissues (20), and because both these effects are mediated by inflammatory mediators of the host immune response (9, 13), a teleological function seems likely. Heart is a candidate tissue destination, because cardiac work is increased in the early hyperdynamic phase of sepsis/endotoxinemia (26), and lipids are favored metabolic fuels for the myocardium (24, 30).

A method of producing radiolabeled rat VLDL by perfusing rat liver was necessarily developed to generate sufficient VLDL for subsequent rat whole heart perfusions; even with the increased circulating VLDL in sepsis/endotoxinemia, isolation of native VLDL from rats in vivo for subsequent heart perfusions was quantitatively impractical. After 8 h of perfusion, livers remained functional with perfusate flow $\geq 10$ ml/min, bile flow $\geq 1.0$ ml/h, and perfusate pH $>7.26$ (if no $\text{HCO}_3^-$ additions were made), with $K^+$ $< 6$ mM and an unchanged lactate-to-pyruvate ratio (data not shown). VLDL-TAG production was linear, with control livers producing $13 \pm 6$ mg TAG/h ($n = 8$) and endotoxic livers producing $8 \pm 1$ mg TAG/h ($n = 6$; not significant). Total recovery of $[\text{3H}]$oleate (oxidized to $\text{3H}_2\text{O}$, $\text{3H}$-labeled ketogenesis, deposition as tissue $\text{3H}$-labeled lipids, and VLDL-$\text{3H}$-labeled lipid production) was $> 90\%$ with $64\%$ of $[\text{3H}]$oleate recovery in VLDL-$[\text{3H}]$TAG (data not shown). The VLDL produced by liver perfusion was examined for lipid and apolipoprotein composition; it contained more TAG per particle (i.e., per apo-B, hence was larger) than native VLDL. This effect has been observed by others in liver perfusion experiments with unsaturated fatty acids (33, 38) and is presumably a reflection of ongoing hydrolysis of the native VLDL particle in vivo; relative distribution of lipid classes in the VLDL particle agrees well with this work (38). The fatty acid composition of VLDL from the two sources examined differed markedly. As expected, the predominant fatty acid species ($> 50\%$) in VLDL-TAG from liver perfusions was 18:1 (i.e., oleate), derived from the perfusate; a smaller amount of fatty acid (16:0, 18:2) was derived from endogenous hepatic stores. Fatty acid species composition in native VLDL-TAG reflected the wider range of fatty acids available for VLDL synthesis in vivo with chow diet. VLDL produced by control liver perfusions appeared to have a normal complement of apo-E and -C compared with control native VLDL (relative to apo-B). However, in both liver...
perfusion-derived VLDL and native VLDL, previous exposure of donor animal to endotoxin in vivo produced a modest but significant change in composition and/or size of the apo-B48 VLDL subtype (smaller particles) with a resulting decrease in overall VLDL particle. Therefore the (smaller) apo-B48 VLDL particles produced after endotoxin treatment carried less TAG. Endotoxin treatment also altered the fatty acid distribution profile of the VLDL: endotoxic VLDL contained significantly more (perfusate-derived) oleate than control VLDL (Fig. 8). The mechanism for this effect was not pursued but could be due to increased uptake of exogenous fatty acid relative to endogenous fatty acid utilization by the endotoxic liver, or decreased oxidation of the perfusate fatty acid compared with intrahepatic sources. Endotoxin is known to affect hepatic lipogenesis and VLDL synthesis in vivo (14), but contributions of specific fatty acids to this process have not been previously investigated. Endotoxin also stimulates hepatic glycogenolysis in perfused rat liver in vitro as a paracrine effect involving eicosanoids (4), but previous endotoxin treatment of rats in vivo did not stimulate TAG output or ketogenesis in subsequent liver perfusion experiments (5).

Endotoxic VLDL was found to be a better substrate for isolated working hearts than control VLDL in that it was oxidized more rapidly and supported greater cardiac work; however, the reasons for this were not immediately apparent. Furthermore, despite taking up more endotoxic VLDL than control VLDL, control hearts (Fig. 1) did not oxidize (Fig. 2) or incorporate into tissue lipids (Table 1) a similarly greater proportion of label. The fate of this \[^{3}H\]TAG is uncertain. It is possible that some water-soluble products (e.g., ketone bodies) were produced (unlikely, given that the heart is not a ketogenic organ), and endotoxin (and/or LPL) may have

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**Fig. 4.** Effect of endotoxin on lipoprotein lipase (LPL) activity in isolated working rat hearts perfused with rat VLDL. LPL was measured in postheparin perfusate (heparin-releasable) and in myocardial tissue (tissue-residual). Nonesterified fatty acid (NEFA) group was perfused with 1.1 mM oleate. For further details see text. VLDL-C, control VLDL; VLDL-E, endotoxic VLDL prepared from rat pretreated with endotoxin in vivo; VLDL-C + LPS, control VLDL preincubated with endotoxin in vitro. Results are means ± SE for 6–10 experiments. *P < 0.05, significant differences between hearts from endotoxin-pretreated rats and control hearts under similar perfusion conditions; **P < 0.05, significant differences between heparin-releasable and tissue-residual enzyme activities.

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**Fig. 5.** Effect of endotoxin on apolipoprotein composition of rat VLDL. VLDL was prepared from perfused rat livers or isolated from plasma of 24-h-starved rats (native). Animals were pretreated with endotoxin or vehicle (control). Proteins derived from 100 µg TAG-VLDL were separated by denaturing SDS-PAGE and quantified against an albumin standard with correction for variable chromogenicity. For further details see text. Results are means ± SE for 3–5 experiments. *P < 0.05, significant differences between endotoxic liver perfusion VLDL and control liver perfusion VLDL; **P < 0.05, significant differences between endotoxic native VLDL and control native VLDL; ***P < 0.05, significant differences between liver perfusion VLDL and native VLDL for apo-B100 + apo-B48 group.

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**Fig. 6.** Effect of endotoxin on ratio of apolipoproteins to apolipoprotein-B in rat VLDL. Amounts of apolipoproteins were expressed as proportion of total apo-B (apo-B48 + apo-B100). For further details see legend to Fig. 5. Results are means ± SE for 3–5 experiments. *P < 0.05, significant differences between endotoxic and control VLDL.
mediated lipoprotein binding/sequestration to the perfusion glassware, but the most plausible explanation is that the $\text{H}_2\text{O}$ technique underestimates the oxidation rate, especially at higher rates (11). The efficacy of VLDL as substrate for working myocardium is poorly documented, because a methodology for generating sufficient lipoprotein for testing in whole organ systems has not been available. Using a system similar to the present study, we recently attempted to quantify cardiac utilization of VLDL (37). VLDL-triolein was utilized at a rate comparable to chylomicron-triolein but did not support cardiac mechanical function as efficiently and was oxidized at only about one-half the rate of NEFA (36, 37). However, utilization of VLDL-TAG is not insignificant, because a physiological suppression of VLDL-TAG utilization and oxidation occurs during lactation, when demand for circulating TAG by the mammary gland is very high, a mechanism mediated by suppression of cardiac LPL activity and thus also of chylomicron-TAG utilization, and VLDL-TAG utilization increases again on litter weaning (37). In the present experiments, differences in VLDL-TAG utilization and oxidation occur during lactation, when demand for circulating TAG by the mammary gland is very high, a mechanism mediated by suppression of cardiac LPL activity and thus also of chylomicron-TAG utilization, and VLDL-TAG utilization increases again on litter weaning (37). In the present experiments, differences in VLDL-TAG utilization were accompanied by only minor changes in cardiac LPL activity. The regulation of cardiac LPL is not completely understood, and alterations in the enzyme activity in this site are modest compared with changes observed elsewhere (e.g., white adipose tissue and mammary gland) after pathophysiological/endocrine signaling (2). We and others previously found increased LPL activity in rat hearts after endotoxin treatment (36), in polymicrobial sepsis (32), and in guinea pig hearts after burn injury (1); however, decreased heart LPL activity in vivo has been reported after high-dose ($\geq 30$ mg/kg) endotoxin treatment for relatively prolonged periods ($\leq 24$ h) without change in heart LPL mRNA (17). Endotoxin administered to perfused rat hearts in vitro decreases myocardial LPL activity, but only in protein-free perfusate; addition of albumin to the perfusate abolished the inhibitory effect of LPS (18). Experimental Gram-negative sepsis decreases myocardial LPL activity in fasted but not fed rats (21). It is likely that nutritional status, heart work, and/or VLDL itself may be important physiological regulators of LPL activity in heart in vivo, but the mechanism for this is unknown. The finding that perfusion with endotoxic VLDL was associated with increased translocation of enzyme from inactive tissue-residual depot to the physiologically active endothelial (heparin-releasable) site may partly explain the increased TAG assimilation (and hence oxidation) observed in endotoxic VLDL compared with control VLDL, but the changes are very modest. LPL was measured only at the end of

Fig. 7. Effect of endotoxin on lipid composition of rat VLDL. VLDL was prepared from perfused rat livers or isolated from plasma of 24-h-starved rats (native). Animals were pretreated with endotoxin or vehicle (control). Lipids were analyzed and expressed as percentage of total lipid detected. For further details see text. PL, phospholipid; CE, cholesterol ester. Results are means $\pm$ SE for 3–10 experiments. *$P < 0.05$, **$P < 0.01$, significant differences between endotoxic and control VLDL, respectively.

Fig. 8. Distribution of fatty acids in rat VLDL. VLDL was prepared from perfused rat livers or isolated from plasma of 24-h-starved rats (native). Animals were pretreated with endotoxin or vehicle (control). Fatty acid species (nonesterified or after transesterification of TAGs and cholesterol esters) were analyzed by gas chromatography. For further details see text. Results are means $\pm$ SE for 3 experiments, expressed as fatty acid mass per mg total VLDL apolipoprotein. *$P < 0.05$, significant differences between endotoxic and control VLDL.
the 60-min perfusion period; at the beginning of the perfusion, the control heart groups should have identical LPL status, as should the endotoxic heart groups, but despite this, there is a significant difference in TAG uptake between the control heart groups and the two endotoxic heart groups after the perfusion (Fig. 1). Therefore, if LPL is involved, it must change after VLDL exposure during the perfusion. This is feasible, because lipoproteins bind LPL and can both remove and replenish endothelial enzyme. LPL is a notoriously difficult enzyme to assay reliably, with large interassay variability, and it is possible that subtle but important shifts were underrepresented by the current data despite batching of assay samples. However, some workers would argue that TAG uptake/utilization itself is the most accurate and physiological assessment of functional LPL activity.

Another possibility is that endotoxic VLDL is a better substrate for LPL by virtue of a superior structural composition, i.e., of lipid and/or apolipoprotein. Evidence suggests that large TAG-rich lipoprotein particles are better substrates for LPL than smaller relatively TAG-poor particles; thus chylomicrons outcompete VLDL for TAG hydrolysis by virtue of their larger size and greater TAG mass. In the present study, both control and endotoxic VLDL, as generated by liver perfusion in vitro, were large particles compared with native VLDL; however, endotoxin exposure made apo-B48 particles smaller, each particle carrying less TAG but contributing more to the apolipoprotein pool. The relative efficacy of apo-B48 and apo-B100 VLDL as substrate for cardiac LPL is not known, but it is possible that apo-B100 VLDL is more readily assimilated; the smaller size of the endotoxic apo-B48 VLDL would suggest that it is a poorer substrate for LPL. The relevance of this to the human situation is questionable because human liver lacks apo-B editing function, and human VLDL, therefore, contains apo-B100. Differences in apo-CII/apo-CIII content may also explain different LPL substrate efficacy, because apo-CII is an obligatory cofactor and activator of LPL, and apo-CIII is an inhibitor. In the present studies, endotoxin treatment tended to decrease apo-C content of VLDL. It was not possible to distinguish the apo-C subtypes, because they have similar electrophoretic mobilities and are co-detected, but apo-CII and apo-CIII have reciprocal ratios in disease states such as diabetes (27), and because apo-CIII is the predominant subtype, the decreased apo-C may therefore represent less apo-CIII inhibition of LPL. Endotoxin treatment also increased 18:1 fatty acid content of liver perfusion VLDL, an effect not previously noted. It is possible that a higher oleate-triolein content renders the VLDL a better substrate for the heart, and this mechanism may be distal to the action of LPL hydrolysis (i.e., intracellular). However, the relative efficacy of different TAG-derived fatty acid species as cardiac substrates is unknown. The presence of inflammatory hepatic acute phase proteins or modified (e.g., oxidized) lipids in VLDL after endotoxin exposure was not examined in the present study.

Another putative portal of entry for VLDL-TAG into the cardiac cell is the VLDL/apo-E receptor (19); however, because no significant differences in apo-E were detected between endotoxic and control VLDL, this argues against a role for this route in the present situation.

Therefore, despite the suggestion that the effect of endotoxin on VLDL utilization by the working rat heart is mediated through the VLDL particle rather than through an effect on the myocardium, it has not been possible to define the mechanism. The possibility remains that a complex interplay of several factors may be involved, and these require further elucidation.

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