Abstract. The bolus intravenous injection of a novel medium-chain triglyceride:fish oil emulsion (MCT:FO) was recently proposed as a tool to provoke a rapid and sustained increase of cell phospholipid content in long-chain polyunsaturated ω3 fatty acids, for instance in selected subjects prior to anesthesia and surgery. In this study, therefore, the possible protective effect of MCT:FO upon aortic endothelial function was investigated in both normal and diabetic rats. The animals were injected intravenously 20 h before sacrifice with 1.0 ml of either saline, MCT:FO or a control medium-chain triglyceride:long-chain triglyceride emulsion. The vasomotor response of isolated aortic rings was then explored by assessing the relaxation provoked by increasing concentrations of acetylcholine in rings contracted with phenylephrine. Such measurements were performed before and after exposure of the aortic rings to suitable concentrations of oxidized LDL. In both normal and diabetic rats, the prior injection of the MCT:FO emulsion protected the aortic rings against the deleterious effect of oxidized LDL. In the diabetic rats, a beneficial effect of the MCT:FO emulsion was even observed prior to exposure of the aortic rings to oxidized LDL. These findings support the view that this novel procedure is indeed appropriate to protect endothelial function against oxidative stress.

Introduction

We have recently reported that the bolus intravenous injection of a novel medium-chain triglyceride-fish oil emulsion (8:2, MCT:FO) to both rats and human subjects increases within 60 min and for at least 24-48 h the phospholipid content in long-chain polyunsaturated ω3 fatty acids (ω3) in circulating leucocytes or platelets (1) and even in extravascular cells such as hepatocytes (2) or skeletal myocytes (3). It was considered, therefore, that such a procedure may rapidly restore a sufficient amount of ω3 in cell phospholipids and, for instance, protect selected patients against the risk of cardiac arrhythmia when undergoing anesthesia and/or surgery (1).

As a first step towards assessing the validity of the latter proposal, the present study aimed mainly at investigating the possible protective effects of the intravenous injection of the MCT:FO emulsion to both normal and streptozotocin-induced diabetic rats against the deleterious action of oxidized low-density lipoproteins on the vasomotor behaviour of isolated aortic rings.

Materials and methods

Emulsions. The MCT:FO emulsion consists of a 20% (w/v) mixture of 80% medium-chain triacylglycerols and 20% fish oil. It also contains 2.5% (w/v) glycerol, 1.2% (w/v) egg-derived phospholipids as emulsifier and 0.4% (w/v) all-rac-tocopherol. The control MCT:LCT emulsion (Medialipid®, B. Braun), currently used for parenteral nutrition consists of a 20% (w/v) mixture of equal amounts of MCT lipids and soybean LCT (LCT emulsion).

Animals and treatments. All animals procedures were approved by our university ethics committee. Adult male Wistar rats were housed in a controlled environment and given free access to standard rat chow and tap water. They weighed between 300-350 g on the day of sacrifice. When 8 weeks old, some rats received a single i.p. injection of streptozotocin (STZ 75 mg/kg, prepared in saline and citrate buffer pH 4.5). After a 5-week period, with twice a week blood glucose level monitoring using an accutrend sensor (Roche Diagnostics), these rats were used in the experimental setting.

Pilot experiments conducted in normal rats aimed at optimizing the experimental setting, with emphasis on the concentration-response relationship for the effects of oxidized LDL on the vasomotor responses.
In further experiments, three groups of rats were usually examined. In the first group, the animals received intravenously 1.0 ml of the MCT-FO emulsion by catheterization of the saphenous vein under chloral hydrate (400 mg/kg) anesthesia. In the second group, the rats were injected with 1.0 ml of the LCT emulsion. The third group served as control, the rats receiving an isotonic saline injection. All animals were treated with a single dose of carprofen (4.0 μg/g s.c.; Rimadyl®, Pfizer, NY, USA) 3 h before the anesthesia. After the intravenous administrations, the rats were housed for a 20-h period and then eventually sacrificed under carbon dioxide. Their thoracic aorta was removed, cleaned of adhering fat and cut into rings of 4-mm length. During the dissection, utmost care was taken to protect the endothelial lining.

Preparation of LDL. LDL fraction was isolated from human plasma and collected in 1 mM EDTA by sequential ultracentrifugation, with density adjustments by potassium bromide (4), and oxidized on CuSO₄ for 24 h as previously described (5). The final total concentration of LDL used in the bath was 3.0 mg/l, different oxidation levels being achieved by variations in the proportion of oxidized LDL to total LDL. Pilot experiments were performed using a standardized solution of oxidized LDL from commercial kits (Mercodia™).

Studies of vasomotor responses. All rings were mounted under 2 g resting tension on stainless steel hooks in 20 ml organ baths. These organ chambers were filled with Krebs-Henseleit solution of the following composition (in mM): NaCl 118.1, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, and D-glucose 5, equilibrated with a mixture of 95% O₂/5% CO₂ and kept at 37˚C. Tension was measured isometrically with a force transducer (Grass FTO3C; Grass Instruments, Quincy, MA, USA) and continuously recorded with a transducer amplifier (Janssen Scientific Instruments) and a pen recorder. After 60 min equilibration, the rings were stretched progressively until the optimal point of the length-tension relation was reached, as previously described (5,6). A concentration-response curve to acetylcholine (Ach, 10⁻⁸ to 10⁻⁴ M) was first constructed on phenylephrine pre-constricted rings (10⁻⁵ M). As illustrated in Fig. 1, the tension decreased, as a function of increasing concentrations of oxidized LDL (20 μU/l: closed triangles and solid line; 40 μU/l: closed circles and dotted line; 80 μU/l: open circles and dashed line; 160 μU/l: open triangles and solid line). Mean values (± SEM) are expressed as the percent relaxation relative to either the reference value found in the sole presence of phenylephrine (left and middle panels) or the paired maximal relaxation caused by phenylephrine in the same ring during the first test (right panel), and refer to 6 (left panel) or 1-2 (middle and right panels) rings.

All experiments were performed in the absence of cyclooxygenase inhibitor, because indomethacin does not affect endothelium-dependent relaxations in rat aorta (4,6).

Presentation of results and statistical analysis. All results are expressed as mean (± SEM) together with the number of separate determinations (n) or degree of freedom (d.f.). The relaxations are expressed in cm on the pen recorder, corresponding to 1.0 g for 4 cm. Statistical analysis was performed using Student's t-test.

Results

Response to oxidized LDL. Fig. 1 summarizes the results of a first set of experiments designed to assess the effect of oxidized LDL (from Mercodia) on the response to phenylephrine and acetylcholine in aortic rings obtained from a normal rat. A first relaxation test documented the effect of cumulative doses of acetylcholine (final concentration in the baths: 10⁻⁴ to 10⁻² M) on phenylephrine pre-constricted rings (10⁻⁵ M). As illustrated in Fig. 1 (left panel), the tension decreased, as a function of the concentration of acetylcholine from a control value of 5.70 ± 0.62 cm to a minimal value of 1.12±0.23 cm (n=6 in both cases), suggesting a functional endothelium. The aortic rings were then exposed for 30 min to increasing concentrations of oxidized LDL (20-160 μU/liter final concentration). Under these conditions, the relaxing effect of acetylcholine, as assessed during a second test, was obviously impaired (Fig. 1, middle panel). Essentially comparable information was obtained, when the relaxation found after
The concentration of oxidized LDL. The middle panel of Fig. 3 illustrates the results obtained in aortic rings from rats in group B, incubated for 30 min in the absence or presence of oxidized LDL (1/16 oxidized/total LDL ratio). This second effect of oxidized LDL was not identical in group A and B. As illustrated in Fig. 3 (right panel), which refers to aortic rings exposed for 30 min to a low concentration of oxidized LDL (1/16 oxidized/total ratio), the difference between group A and B, in terms of the relative extent of the relaxation caused by acetylcholine averaged, in the range 10^{-7} to 10^{-4} M range, 22.8±6.0% (d.f.=16; p<0.005) of the maximal tension recorded in the absence of the cholinergic agent. Taken as a whole, these findings emphasize the need of comparing in aortic rings obtained from the same animals the response to phenylephrine and acetylcholine before and after exposure to oxidized LDL.

Normal rats. Aortic rings were obtained from five rats injected with the LCT emulsion and six rats injected with the MCT:FO emulsion. During a first relaxation test, neither the absolute value for the tension caused by phenylephrine (10^{-4} M) nor the relative extent of the relaxation caused by acetylcholine (also 10^{-3} M) were significantly different (p>0.4) in the two groups of rats, with overall mean respective values of 4.62±0.32 cm and 79.1±2.3% (n=29 in both cases).

The aortic rings were then incubated for 30 min in the absence or presence of oxidized LDL (1/16 oxidized/total LDL ratio). In the aortic rings obtained from the animals injected with the LCT emulsion and exposed to oxidized LDL, the maximal tension recorded during the second relaxation test in the sole presence of phenylephrine was lower (n=8; p<0.03) than the paired value found at the same time in aortic rings obtained from the same rats but not exposed to oxidized LDL. Such was not the case, however, in the aortic rings from rats injected with the MCT:FO emulsion (n=8). Such paired ratios averaged, in the rats injected with the LCT emulsion,
not significantly affected (p>0.8) by the concentration of (Fig. 4). The difference between the two sets of results was those recorded in the rats injected with the LCT emulsion were always higher than (Table I). Under these conditions, the results obtained in rats exposed to the same concentration of the cholinergic agent rats not preincubated with oxidized LDL and eventually corresponding value found in rings from the same type of oxidized LDL (Table I) were expressed relative to the mean LDL, the relaxing action of acetylcholine in rings exposed to the MCT:FO emulsion against the deleterious effect of oxidized order to assess specifically the protective action of the exposed to oxidized LDL than in the rings from rats injected oxidized LDL in the rings from rats injected with the LCT emulsion only achieved statistical significance (p<0.05 to p<0.005; d.f.=11) at 10^{-8}, 10^{-7} and 10^{-6} M, when judged by the data listed in Table I. However, when compared within each animal, the relaxing action of acetylcholine (10^{-5} and 10^{-4} M) remained significantly lower (p<0.02) in the rings exposed to oxidized LDL than in the control rings not exposed to oxidized LDL. For instance, at 10^{-4} M acetylcholine, the results recorded in the aortic rings exposed to oxidized LDL averaged 76.0±7.8% (n=8; p<0.02) of the paired control value (no oxidized LDL) found in the same animal(s).

The MCT:FO emulsion also protected against the effect of the oxidized LDL upon the response to acetylcholine. The mean relative extent of the relaxing action of acetylcholine, as listed in Table I, was always higher, at all concentrations of the cholinergic agent (10^{-4} to 10^{-4} M), in the aortic rings obtained from rats injected with the MCT:FO emulsion and exposed to oxidized LDL than in the rings from rats injected with the LCT emulsion and also exposed to oxidized LDL. In order to assess specifically the protective action of the MCT:FO emulsion against the deleterious effect of oxidized LDL, the relaxing action of acetylcholine in rings exposed to oxidized LDL (Table I) were expressed relative to the mean corresponding value found in rings from the same type of rats not preincubated with oxidized LDL and eventually exposed to the same concentration of the cholinergic agent (Table I). Under these conditions, the results obtained in rats injected with the MCT:FO emulsion were always higher than those recorded in the rats injected with the LCT emulsion (Fig. 4). The difference between the two sets of results was not significantly affected (p>0.8) by the concentration of acetylcholine, with an overall mean value of 20.5±6.0% (d.f.=80; p<0.001). The results illustrated in Fig. 4 also document that, as expected from prior results, the deleterious effect of oxidized LDL was, in relative terms, most marked at the lowest concentration(s) of acetylcholine.

These findings were confirmed in further experiments conducted in normal rats injected with either the MCT:FO or control LCT emulsion. During the first relaxation test, the tension caused by phenylephrine (10^{-4} M) was not significantly different (p>0.55) in the rats injected with the MCT:FO (6.50±0.95 cm; n=9) or LCT (7.03±0.36 m; n=10) emulsion. Likewise, the relative magnitude of the relaxing action of acetylcholine during this first test failed to differ significantly

<table>
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*The results are expressed relative to the percent relaxation caused by 10^{-5} M acetylcholine in the same aortic ring during a first relaxation test.

Figure 4. Relaxing action of acetylcholine (10^{-8} to 10^{-4} M) in aortic rings obtained from rats injected with an LCT or MCT:FO emulsion (5-6 rats in each group). The results obtained in rings exposed to oxidized LDL (40 μU/l) are expressed relative to the mean corresponding values found in control rings not exposed to LDL (see Table I for primary data). The data collected in the rats injected with the LCT emulsion are illustrated below the ‘zero’ horizontal line. The data recorded in rats injected with the MCT:FO emulsion are illustrated above the ‘zero’ horizontal line, after subtraction of the results obtained in the rats injected with the LCL emulsion. Mean values (± SEM) refer to 8 (LCT rats) and 10 (MCT:FO rats) rings.
in the two groups of rats (Fig. 5, left panel). Such was also
the case during the second relaxation test in aortic rings not
exposed to oxidized LDL (Fig. 5, middle panel). In both cases,
the trend was towards a greater relative relaxing action of
acetylcholine in the rings obtained from rats injected with the
MCT:FO, as distinct from LCT, emulsion.

Similar to previous experiments, the tension measured in
the sole presence of phenylephrine (10^-6 M) during the second
relaxation test progressively decreased as the oxidized/total
LDL ratio increased from 1/16 to 1/1, the values reached in the
latter case representing 66.0±11.4% (n=4; p<0.06) of the paired
value found in the same rat(s) in the former case.

The exposure to oxidized LDL also impaired the relaxing
action of acetylcholine. For instance, in the aortic rings exposed
to oxidized LDL at an oxidized/total ratio of 1/16, the maximal
response to the cholinergic agent (10^-4 M) was decreased
from 92.7±6.4% (n=3) to 69.0±2.0% (n=2) in the rats injected
with the MCT:FO emulsion and from 92.0±3.8% (n=4) to
42.0±7.0% (n=2) in the animals injected with the control
LCT emulsion. This alteration of the functional response to
acetylcholine is further documented by comparison of the
results illustrated in the middle and right panels of Fig. 5.

As documented in Fig. 5 (right panel), in the aortic rings
exposed to the low concentration of oxidized LDL (oxidized/
total LDL ratio to 1/16) the mean relative magnitude of the
relaxing action of acetylcholine (10^-9 to 10^-4 M) was always
higher in the rings obtained from rats injected with the
MCT:FO emulsion than in those obtained from animals
injected with the LCT emulsion. Such a difference achieved
statistical significance (p≤0.05) in the 10^-7 to 10^-5 M range
of acetylcholine concentrations. Between 10^-7 and 10^-4 M
acetylcholine, the relative extent of the relaxing action of the
cholinergic agent averaged in the rat injected with the LCT
emulsion only 50.5±4.8% (n=8; p<0.001) of the corresponding
mean value recorded at the same concentration of acetyl-
choline in the aortic rings from rats injected with the MCT:FO
emulsion (100.0±1.7%; n=8). The protective effect of the
MCT:FO emulsion faded out, however, at a higher
concentration of oxidized LDL (oxidized/total LDL ratio of
1/4 and 1/1). Thus, in this case, the relative magnitude of the
relaxing action of acetylcholine (10^-7 to 10^-4 M) recorded in
the aortic rings from rats injected with the MCT:FO emulsion
averaged no more than 106.0±16.3% (n=16; p>0.7) of the
corresponding value found at the same concentration of the
cholinergic agent and within the same experiment in rings
obtained from animals injected with the LCT emulsion.

Taken as a whole, the experiments conducted in normal
rats injected with the MCT:FO emulsion thus indicate that
it failed to affect significantly the functional response to
phenylephrine and acetylcholine in aortic rings not exposed
to oxidized LDL but exerted a significant protective action
in rings exposed to a relatively low concentration of oxidized
LDL.

**STZ rats.** The mean body weights of the STZ rats injected
with either saline, the LCT emulsion or the MCT:FO emulsion
were not significantly different from one another (p≥0.3),
with an overall mean value of 288±12 g (n=25). Likewise,
the mean glycemia was not significantly different (p≥0.19) in
the three groups of STZ rats, with an overall mean value of
16.44±1.70 mM (n=24).

During the first relaxation test conducted in aortic rings
from STZ rats, the tension caused by phenylephrine (10^-5 M)
was not significantly different (p>0.3) in the rats injected
with the LCT emulsion (6.65±0.37 cm; n=33) or MCT:FO emulsion (6.09±0.47 cm; n=30). However, the mean relaxation
tension caused by acetylcholine was, in the 10^-9 to 10^-4 M range, higher
in the latter than former rats. This is illustrated in Fig. 6, which
refers to a representative set of experiments conducted in 4 rats
in each group. In the $10^{-8}$ to $10^{-6}$ M range of acetylcholine concentrations, the shift of the concentration-response relationship in the rats injected with the LCT versus MCT:FO emulsion corresponded to a difference in concentration of the cholinergic agent close to one order of magnitude, e.g. from $10^{-8}$ to $10^{-7}$ M. These findings were confirmed in another set of comparable experiments conducted in 4-5 STZ rats injected with either the LCT or MCT:FO emulsion. The overall mean results are illustrated in Fig. 7. It indicates that, at all concentrations of acetylcholine ($10^{-9}$ to $10^{-4}$ M), the relaxation was significantly higher ($p\leq0.005$) in the rats injected with the MCT:FO emulsion than in those injected with the LCT emulsion. The former results represented between $8.67\pm1.91$ (n=30 at $10^{-9}$ M acetylcholine) and $1.16\pm0.03$ (n=30 at $10^{-4}$ M acetylcholine) times the corresponding mean values found in the rats injected with the LCT emulsion and derived in all cases from 33 measurements in individual aortic rings.

Although the normal and STZ rats were examined in two separate series of experiments, three sets of findings point towards an alteration or aortic endothelium function in the diabetic animals. First, whether in the rats injected with the LCT or MCT:FO emulsion, the tension caused by phenylephrine during the first relaxation test was higher in the STZ rats than in the normal animals. It averaged, in the former rats, $117.4\pm5.5$% (n=63; $p<0.04$) of the mean corresponding value found in normal rats injected with the same emulsion ($100.0\pm6.0$%; n=48). Second, during the second test conducted in aortic rings not exposed to oxidized LDL, the relaxation caused by acetylcholine was higher in the normal rats than in the diabetic animals. For instance, the fractional relaxation caused by $10^{-8}$ M acetylcholine averaged in the STZ rats $58.2\pm13.0$% (n=25; $p<0.04$) of the mean corresponding value found in normal rats injected with the same lipid emulsion ($100.0\pm13.5$%; n=35). Third, when comparing STZ rats injected with the LCT versus MCT:FO emulsion and at variance with the situation found in normal rats, the exposure of the aortic rings to oxidized LDL failed to affect in a significantly different manner the reference tension measured during the second relaxation test in the sole presence of phenylephrine. Such was the case, whether the results were considered in absolute terms or relative to the paired measurement made in the same ring during the first relaxation test. For instance, when the determinations made during the second relaxation test in aortic rings that had been exposed to oxidized LDL were expressed relative to the corresponding value(s) found in aortic ring(s) of the same animal not exposed to oxidized LDL, the readings made in STZ rats injected with saline ~20 h before sacrifice.

Nevertheless, in aortic rings from STZ rats, like in those from normal rats, prior exposure to oxidized LDL (1/16 oxidized/total LDL ratio) altered the response to acetylcholine. For instance, Fig. 8 illustrates the results collected in STZ rats injected with saline and preincubated for 30 min in the absence (closed circles and solid line) or presence (open circles and dashed line) of oxidized LDL (1/16 oxidized/total LDL ratio). Mean values (± SEM) refer to 13-16 rings.
lower in the rings exposed to oxidized LDL than in those not exposed to oxidized LDL. This difference achieved statistical significance (p≤0.05) in the 10⁻⁸ to 10⁻⁴ M range of acetylcholine.

During the second relaxation test, the mean value for the fractional relaxation caused by acetylcholine was always higher in the STZ rats injected with the MCT:FO emulsion compared to LCT emulsion. Mean values (±SEM) refer to 30-34 rings, are ranged on a logarithmic scale, and are shown together with the statistical significance of differences between the two sets of data.

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