Triglycerides impair postischemic recovery in isolated hearts: roles of endothelin-1 and trimetazidine

LUCILLA D. MONTI,1 SONIA ALLIBARDI,5 PIER MARCO PIATTI,2 GIANPIETRO VALSECHI,1 SABRINA COSTA,1 GUIDO POZZA,3 SERGIO CHIERCHIA,4 AND MICHELE SAMAJA5

1Divisione di Medicina, 2Unita’ di Malattie Metaboliche, Divisione di Medicina, 3Cattedra di Clinica Medica Generale e Terapia Medica, Universita’ Vita-Salute, 4Dipartimento di Cardiologia, Istituto di Ricovero e Cura a Carattere Scientifico, Hospital San Raffaele, 20132 Milan; and 5Dipartimento di Medicina, Chirurgia ed Odontoiatria-DiMCO, Ospedale San Paolo, University of Milan, Milan I-20090, Italy

Received 29 December 2000; accepted in final form 8 May 2001

Monti, Lucilla D., Sonia Allibardi, Pier Marco Piatti, Gianpietro Valsechi, Sabrina Costa, Guido Pozza, Sergio Chierchia, and Michele Samaja. Triglycerides impair postischemic recovery in isolated hearts: roles of endothelin-1 and trimetazidine. Am J Physiol Heart Circ Physiol 281: H1122–H1130, 2001.—There is growing evidence that hypertriglyceridemia exacerbates ischemic injury. We tested the hypothesis that triglycerides impair myocardial recovery from low-flow ischemia in an ex vivo model and that such an effect is related to endothelin-1. Hyperglycemic (glucose concentration = 12 mmol/l) and hyperinsulinemic (insulin concentration = 1.2 μmol/l) isolated rat hearts were perfused with Krebs-Henseleit buffer (PO2 = 670 mmHg, pH 7.4, 37°C) added with increasing triglycerides (0, 1,000, 2,000, and 4,000 mg/dl, n = 6–9 rats/group). Hearts were exposed to 60 min of low-flow ischemia (10% of basal coronary flow), followed by 30 min of reperfusion. We found that increasing triglycerides impaired both the diastolic (P < 0.005) and systolic (P < 0.02) recovery. The release of endothelin-1 during reperfusion increased linearly with triglyceride concentration (P = 0.0009). Elevated triglycerides also increased the release of nitrite and nitrate (NOx), the end products of nitric oxide, up to 6 μmol/min. Trimetazidine (1 μmol) further increased NOx release, blunted endothelin-1 release, and protected myocardial function during reperfusion. We conclude that high triglyceride levels impair myocardial recovery after low-flow ischemia in association with endothelin-1 release. The endothelium-mediated effect of triglycerides on both contractile recovery and endothelin-1 release is prevented by 1 μM trimetazidine.

nitric oxide

ACCUMULATING EPIDEMIOLOGICAL EVIDENCE suggests that the situation characterized by elevated plasma triglycerides (TG) is associated with increased cardiovascular risk independent of factors such as hyperglycemia and elevated plasma cholesterol (3). Hypertriglyceridemia is also a critical risk factor for coronary heart disease (CHD) mortality in subjects with impaired glucose tolerance or diabetes (18). Furthermore, hypertriglyceridemia is a common finding in survivors of acute myocardial infarction (27). This epidemiological evidence suggests that TG influence myocardial performance after ischemia-reperfusion independently of atherosclerosis progression.

Although its role in acute ischemia-reperfusion is controversial, endothelin-1 (ET-1) is known to exacerbate injury, likely via activation of ET type A receptors (9). Studies in isolated hearts showed that ET-1 release increases on early reperfusion after ischemia, thereby contributing to injury (7), and that ET-1 is a major factor that depresses cardiac function (6) and causes cell necrosis (8). These findings are consistent with other studies (21, 23, 30) demonstrating a relationship between ET-1 and the pathogenesis of myocardial ischemia. In humans, acute hypertriglyceridemia stimulates ET-1 release in normal subjects (36). In addition, hypertriglyceridemia is related with elevated plasma levels of ET-1 in glucose-intolerant and type II diabetic patients with insulin resistance syndrome (37). However, on an experimental ground, a link among hypertriglyceridemia, ET-1, and the outcome of the ischemia-reperfusion injury is still lacking. The purpose of this study is to provide experimental evidence of that link by testing the hypothesis that TG exacerbate the injury driven by ischemia-reperfusion and that this phenomenon is linked to ET-1.

The isolated crystallloid-perfused heart may be a suitable model to test this issue in three steps: 1) evaluate the direct acute effect of high TG on postischemic recovery, 2) assess the link between the reperfusion injury and ET-1 release, and 3) evaluate the protection afforded by the piperazine drug trimetazidine (TMZ). No attempt is made to investigate the mechanism underlying ET-1 recognition by cardiac myocytes, because it is known to involve ET type A...
receptors (9, 10, 19, 43). However, by testing the effect of TMZ, a recognized anti-anginal and anti-ischemic agent (13), one may understand the site of action of TG. Indeed, TMZ inhibits the activity of 3-ketoacyl coenzyme A (CoA) thiolase, the key enzyme of fatty acid β-oxidation, thereby increasing myocardial oxidative glucose metabolism (29), and the inability to utilize glucose for the oxidative metabolism increases cardiovascular risk in the presence of excess fatty acids (34). Citrate release is a useful index of the flux through the β-oxidation pathway (50). In addition, because inactivation of nitric oxide (NO) may play a prominent role in cardiovascular disease (15), we measured the release of nitrite and nitrate (NO$_x$), the end products of NO metabolism, during the reperfusion as a probe to assess the viability of the endothelial cells.

To mimic the metabolic situation occurring in type II diabetic patients during the postprandial period, we selected hyperglycemic and hyperinsulinemic conditions. Indeed, hyperinsulinemia increases plasma ET-1 in humans (36) because insulin stimulates ET-1 secretion from human endothelial (17) and vascular smooth muscle cells (2). In this study, hearts perfused in the presence of increasing TG concentration ([TG]), as well as 10$^{-6}$ M TMZ, are exposed to low-flow ischemia and reperfused. Data will show that the postischemic injury is proportional to [TG] and ET-1 release and that the deleterious effects of elevated TG are prevented by TMZ.

**MATERIALS AND METHODS**

**Heart perfusion.** Male Sprague-Dawley rats (250–280 g body wt) fed ad libitum were anesthetized with heparinized thiopental sodium (10 mg/100 g body wt). Hearts were excised, immersed in isotonic saline solution (20°C) and mounted on the perfusion system as described previously (44). The time required for these operations never exceeded 45 s and was typically in the 15- to 30-s range. Langendorff perfusion started immediately with the media described before (44). The preheater, were connected to a 1,760-W external water apparatus, including the heart chamber, the oxygenator, and the preheater, were connected to a 1,760-W external water bath (Endocal, Neslab Instruments; Newington, NH) kept at 37.5 ± 0.5°C. A latex balloon in the left ventricle was connected to a pressure transducer (model 52-9966, Harvard Apparatus; Natick, MA) to monitor myocardial performance (see Experimental protocol). An additional transducer connected to the aortic cannula provided the coronary perfusion pressure (CPP). A cannula was inserted into the pulmonary artery to collect the venous return and to monitor venous Po$_2$ by an O$_2$-sensing electrode (model 5300 Oxygen Monitor, Yellow Springs Instruments; Yellow Springs, OH). The investigation conforms to the guidelines in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Publication No. 85-23, Revised 1985).

The perfusion media consisted of a Krebs-Henseleit buffer with 2.0 mmol/l free Ca$^{2+}$, 12 mmol/l glucose, and 20 mU/l human recombinant insulin (Actrapid HM, Novo Nordisk; Rome, Italy) added with variable amounts (22.5–90 ml/l perfusion medium) of Intralipid 20% (Fresenius Kabi; Vienna, Austria). The medium composition was not changed during the protocol. Before dilution, Intralipid 20% contained 200 g/l TG, 12 g/l phospholipids, 25 g/l glycerol, and 257–280 mg/l cholesterol. Linoleic acid is the main fatty acid in TG (18 carbons, 2 cis double bonds), with linolenic, oleic, palmitic, and stearic acids accounting for <50% of the total. Vitamin E present in the mixture partly inhibits oxidation of unsaturated double bonds (G. Arcuri and F. Kabi, unpublished communication).

In control hearts (TG$_0$ group, n = 9), no Intralipid was added to the Krebs-Henseleit buffer. In the TG$_{0,000}$ (n = 9), TG$_{2,000}$ (n = 7), and TG$_{4,000}$ (n = 6) groups, Intralipid was added to the medium to yield [TG] ~1,000, 2,000, and 4,000 mg/dl. The TG$_{4,000}$ + TMZ (n = 5) group was similar to TG$_{4,000}$ group, but with 10$^{-6}$ M freshly prepared TMZ (Servier Laboratories; Courbevoie, France). The medium was equilibrated at Po$_2$ = 670 ± 6 mmHg (means ± SE) and Pco$_2$ = 36 ± 1 mmHg in membrane oxygenators (45). The resulting pH was 7.38 ± 0.01 at 37°C.

Myocardial performance was monitored by a LabView system (National Instruments, Austin, TX) running on Macintosh Quadra 700 (Apple; Cupertino, CA). Measurements included the heart rate (HR), the end-diastolic pressure (PED), the peak systolic pressure (PSP), the maximal rates of pressure development (+dP/dt max) and relaxation (–dP/dt max), and the coronary perfusion pressure (CPP). From these parameters, we derived the left ventricular developed pressure (LVDP = PSP – EDP) and LVDP-HR, which represents the myocardial contractile work. The resistance was calculated as (CPP – EDP)/(flow rate)/(ventricle weight) (11). The O$_2$ uptake was calculated from the arteriovenous Po$_2$ difference and flow rate.

**Experimental protocol.** All hearts were stabilized for 20 min at a flow rate of 15 ml/min for baseline measurements. During this period, the volume of the intraventricular balloon was adjusted to yield an EDP of 10 ± 1 mmHg and was kept constant afterward. Hearts were then subjected to low-flow ischemia for 60 min by reducing the flow to 1.5 ml/min. After ischemia, hearts were reperfused for 30 min with the same flow rate used during baseline. The recovery of postischemic myocardial performance was evaluated at the end of the reperfusion either as an increase of EDP and CPP above baseline values (AEEDP and ACPP, respectively) or as a percentage of HR, LVDP, +dP/dt max, –dP/dt max, and LVDP-HR.

**Measurements in the coronary effluent.** Glucose was measured by a glucose-oxidase analyzer (Yellow Springs Instruments). Insulin was measured in a single assay (within-assay coefficient of variance (CV)-3.0%, between-assay CV-5.0%) with a microparticle enzyme immunoassay (sensitivity = 6 pmol/l, cross-reactivity with proinsulin <2%; IMX, Abbott Laboratories; Abbott Park, IL). Free fatty acid, TG, citrate, and lactate were measured by automated enzymatic spectrofluorimetric methods adapted to COBAS FARA II (within-assay CV-3.0%, between-assay CV-3.0%; Hoffman-La Roche; Basel, Switzerland).

To measure ET-1, the coronary effluent was collected every 10 min for 30 min during the reperfusion, and the samples were extracted on SepPack C18 minicolumn (Amprep, Amersham International; Buckinghamshire, UK). The eluate was evaporated in a Speed Vac (model SC110, Savant Instruments; Farmingdale, NY). Samples were then reconstituted with 250 μl radioimmunoassay buffer and assayed by a radioimmunoassay kit (Endothelin-1, High-sensitivity Assay System; Amersham International). The antiserum was a rabbit anti-ET-1 antibody, and the tracer was 125I-labeled ET-1. The assay sensitivity was 1.25 pg/ml, with a typical within- and between-assay CV = 3.0% and 11.9%, respectiv
the TG1,000 group, resistance was not further altered by 10.220.32.247 on May 28, 2017 http://ajpheart.physiology.org/ Downloaded from

Table 1. Myocardial performance during baseline

<table>
<thead>
<tr>
<th></th>
<th>TG0 (n = 9)</th>
<th>TG1,000 (n = 9)</th>
<th>TG2,000 (n = 7)</th>
<th>TG4,000 (n = 6)</th>
<th>P</th>
<th>TG4,000 + TMZ</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides, mg/dl</td>
<td>2 ± 1</td>
<td>952 ± 69</td>
<td>2,083 ± 177</td>
<td>3,882 ± 150</td>
<td>NA*</td>
<td>3,809 ± 76</td>
<td>NS†</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>252 ± 10</td>
<td>258 ± 7</td>
<td>232 ± 15</td>
<td>245 ± 9</td>
<td>NS*</td>
<td>244 ± 9</td>
<td>NS†</td>
</tr>
<tr>
<td>Developed pressure, mmHg</td>
<td>115 ± 7</td>
<td>93 ± 8</td>
<td>124 ± 12</td>
<td>138 ± 16</td>
<td>NS*</td>
<td>164 ± 7</td>
<td>NS†</td>
</tr>
<tr>
<td>LVDP-HR, mmHg·1,000·min⁻¹</td>
<td>28.8 ± 1.6</td>
<td>23.6 ± 1.9</td>
<td>31.3 ± 3.4</td>
<td>33.6 ± 3.8</td>
<td>NS*</td>
<td>40.1 ± 2.0</td>
<td>NS†</td>
</tr>
<tr>
<td>End-diastolic pressure, mmHg</td>
<td>9.9 ± 0.2</td>
<td>9.1 ± 0.4</td>
<td>10.3 ± 0.4</td>
<td>11.8 ± 0.7</td>
<td>NS*</td>
<td>10.4 ± 0.6</td>
<td>NS†</td>
</tr>
<tr>
<td>Perfusion pressure, mmHg</td>
<td>82 ± 9</td>
<td>120 ± 11</td>
<td>121 ± 12</td>
<td>113 ± 11</td>
<td>0.05*</td>
<td>96 ± 16</td>
<td>NS†</td>
</tr>
<tr>
<td>Resistance, mmHg·ml⁻¹·min⁻¹·g⁻¹</td>
<td>4.79 ± 0.57</td>
<td>7.39 ± 0.71</td>
<td>7.37 ± 0.79</td>
<td>6.76 ± 0.75</td>
<td>NS*</td>
<td>5.75 ± 1.07</td>
<td>NS†</td>
</tr>
<tr>
<td>+dP/dt max, mmHg/s</td>
<td>3,201 ± 151</td>
<td>2,542 ± 196</td>
<td>3,496 ± 434</td>
<td>3,487 ± 287</td>
<td>NS*</td>
<td>3,912 ± 160</td>
<td>NS†</td>
</tr>
<tr>
<td>−dP/dt min, mmHg/s</td>
<td>2,110 ± 148</td>
<td>1,842 ± 180</td>
<td>2,263 ± 239</td>
<td>2,217 ± 223</td>
<td>NS*</td>
<td>2,714 ± 137</td>
<td>NS†</td>
</tr>
<tr>
<td>Venous [lactate], mmol/l</td>
<td>0.12 ± 0.04</td>
<td>0.16 ± 0.07</td>
<td>0.21 ± 0.08</td>
<td>0.17 ± 0.02</td>
<td>NS*</td>
<td>0.21 ± 0.03</td>
<td>NS†</td>
</tr>
<tr>
<td>Venous PO2, mmHg</td>
<td>205 ± 32</td>
<td>208 ± 36</td>
<td>193 ± 43</td>
<td>259 ± 24</td>
<td>NS*</td>
<td>163 ± 11</td>
<td>0.01†</td>
</tr>
<tr>
<td>O2 uptake, μmol/min</td>
<td>9.8 ± 0.7</td>
<td>9.7 ± 0.8</td>
<td>10.0 ± 0.9</td>
<td>8.6 ± 0.5</td>
<td>NS*</td>
<td>10.6 ± 0.2</td>
<td>0.01†</td>
</tr>
<tr>
<td>O2 uptake/LVDP-HR, μmol·mmHg⁻¹·1,000</td>
<td>0.34 ± 0.02</td>
<td>0.42 ± 0.03</td>
<td>0.34 ± 0.03</td>
<td>0.27 ± 0.02</td>
<td>NS*</td>
<td>0.31 ± 0.01</td>
<td>NS†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. TG, triglycerides; TMZ, trimetazidine; LVDP-HR, left ventricular developed pressure × (HR); +dP/dt max, maximal rate of pressure development; −dP/dt min, minimal rate of pressure development; NA, not applicable; NS, not significant.

Myocardial performance was assessed during baseline perfusion with Krebs-Henseleit medium (2 mmol/l free Ca²⁺, 12 mmol/l glucose, 20 mM/l insulin, and variable amounts of TG). In control hearts (TG0 group), no TG was added. In the TG1,000, TG2,000, and TG4,000 groups, Intralipid was added to the medium to yield a TG concentration of 1,000, 2,000, and 4,000 mg/dl, respectively. In the TG4,000 group, 10⁻⁴ M of freshly prepared TMZ was added (TG4,000 + TMZ group). Media were equilibrated at PO2 = 670 ± 6 mmHg and PCO2 = 38 ± 1 mmHg; resulting pH was 7.38 ± 0.01 at 37°C. Flow was 15 ml/min. *P < 0.05, analysis of variance test vs. TG; †P > 0.05, Student’s t-test vs. TG4,000 group.

**RESULTS**

The total release of ET-1 during the reperfusion was calculated by taking the area under the curves representing ET-1 versus time by the trapezoidal rule and by considering, as a basal value, the ET-1 level measured at the end of ischemia.

NOx was measured by enzymatic catalysis coupled with the Griess reaction (49). As for ET-1, total NOx release during reperfusion was measured by calculating the area under the curves representing NOx versus time by the trapezoidal rule, after taking the NOx level measured at the end of ischemia as the basal value.

**Statistics.** Data are expressed as means ± SE. To assess the effect of increasing TG, we performed a two-way factorial analysis of variance test (StatView, Abacus Concepts; Berkeley, CA). To assess the effects of TMZ at constant TG, we used Student’s t-test. Simple regression analysis was performed using the indices of myocardial performance at recovery as the dependent variables and TG or ET-1 levels as the independent variables.

**RESULTS**

The concentration of glucose and insulin in the media were 12.5 ± 0.5 mmol/l and 1.22 ± 0.03 μmol/l, respectively. The level of free fatty acids was <0.2 mmol/l in both the arterial inflow and venous effluent. Because all hearts kept contracting through the ischemia-reperfusion protocol, all data were available for analysis. Table 1 shows myocardial performance during baseline. All parameters (except for CPP) were not altered by the increase of TG. Although increased in the TG1,000 group, resistance was not further altered for fourfold Intralipid increases. There was no effect of TMZ during baseline except for the higher O2 uptake in the TG4,000 + TMZ group, which reflects the slightly improved, albeit nonsignificant, performance in TMZ hearts. Despite some intergroup differences in the O2 uptake-to-LVDP-HR ratio, which helps to address the relative contribution of carbohydrates and lipids to energy production, there is no [TG]-associated trend or significant effects of TMZ.

**AJP-Heart Circ Physiol • VOL 281 • SEPTEMBER 2001 • www.ajpheart.org**
The exposure of hearts to 60-min low-flow ischemia, followed by 30-min reperfusion, impaired myocardial performance. Increasing [TG] further impaired recovery. ΔEDP increased with increasing [TG] (P = 0.005) up to 38.5 ± 10.7 mmHg (Fig. 1A). ΔCPP tended to increase, although nonsignificantly (P = 0.08). The presence of 10−6 M TMZ in the medium blunted (P = 0.05) the increase in EDP; ΔCPP tended to decrease, although nonsignificantly (P = 0.07) (Fig. 1B). Figure 2 shows other parameters of the ventricular function. HR was not affected by TG, but TMZ increased HR at the end of the postischemic recovery (P = 0.03). The recovery of LVDP was progressively impaired by the increase of [TG] to 55 ± 5% of baseline (P = 0.0008). However, 10−6 M TMZ significantly (P = 0.02) protected hearts and increased the recovery of developed pressure to 81 ± 7% baseline. The same trend as that described for LVDP was also observed for +dP/dt_{max}, −dP/dt_{max}, and LVDP-HR.

Figure 3, A and B, shows that increasing [TG] augments ET-1 release during reperfusion from 0.9 ± 0.2 ng/min in the absence of TG to 6.3 ± 1.6 ng/min in the presence of 4,000 mg/dl TG (P = 0.001). The presence of 10−6 M TMZ completely blunted the release of ET-1 (P = 0.008). Figure 3, C and D, shows that increasing [TG] slightly increased the release of NOx. The release of NOx appeared to be blunted when [TG] = 2,000 mg/dl because a further [TG] increase did not augment NOx release. However, in the presence of 10−6 M TMZ, the release of NOx was augmented threefold with respect to the same [TG] in the absence of TMZ. Figure 3E shows that the release of citrate increases linearly with [TG] and is blunted in the presence of 10−6 M TMZ.
Figure 4, A and B, shows the correlation existing between ET-1 release and EDP or the recovery of LVDP measured at the end of reperfusion. The correlation is statistically significant ($P < 0.0002$ and $P < 0.01$, respectively). Similarly, a significant correlation is found when substituting EDP or LVDP with either LVDP ($P = 0.002$), $+\frac{dp}{dt_{\text{max}}}$ ($P = 0.005$), or $-\frac{dp}{dt_{\text{max}}}$ ($P = 0.004$) but not CPP ($P$ not significant).

Figure 5 shows the changes in the $O_2$ uptake-to-LVDP-HR ratio in the various groups. Low-flow ischemia significantly decreased that ratio in all groups with respect to baseline ($P < 0.007$). On reperfusion, the $O_2$ uptake-to-LVDP-HR ratio recovered to near-normal values, with the exception of the TG4,000 group, for which that ratio increased from $0.27 \pm 0.02$ to $0.55 \pm 0.05 \, \mu \text{mol} \cdot \text{mmHg}^{-1} \cdot \text{1,000}$ ($P = 0.003$). Venous lactate concentration ([lactate]) at the end of ischemia ranged from 1.6 to 2.3 mmol/l in all groups without differences among the groups.

**DISCUSSION**

In this hyperglycemic, hyperinsulinemic model, elevated [TG] in the medium progressively impairs the postsischemic recovery of both systolic and diastolic functions. The impairment is associated with increased ET-1 release. The addition of $10^{-6}$ M TMZ protects hearts from the deleterious effect of high TG. The protection is associated with blunted ET-1 release and increased NOx release.

**Critique of the model.** By reducing to a reasonable minimum the number of the involved variables, the isolated crystalloid-perfused heart model appears suitable for studying the effects of TG in the ischemic-reperfused cardiac muscle. The animals were not pretreated; thus the observations relate to acute metabolic effects. Any interference by neurohormonal factors is excluded because isolated hearts are denervated. Perfusion with blood cell-free media excludes the disturbing effect of neutrophil accumulation and thrombin-induced platelet aggregation. Temperature is strictly controlled ($\pm 0.5^\circ\text{C}$). A constant balloon volume rules out differences in loading conditions. The duration and severity of ischemia are the same in all groups. The changes in vascular resistance are monitored as $\Delta \text{CPP}$ because the flows were the same in all the groups. Although the selected experimental conditions with relatively short ischemia-reperfusion times do not allow for appreciable no reflow, the slight, nonsignificant increase of vascular resistance shown in Fig. 1 indicate that this phenomenon might have occurred on a longer time basis. The changes in diastolic contracture are reflected by $\Delta \text{EDP}$ because the balloon volume is fixed at the start of the experiment and kept constant afterward.

Hearts were perfused with media containing glucose and lipids as oxidizable substrates. The experiments reported here were not designed to address the question of their relative contribution to energy production, but the changes in the $O_2$ uptake-to-LVDP-HR ratio reflect different substrate contribution to the rate of ATP synthesis. The fall of that ratio at the end of low-flow ischemia is a consequence of increased substrate-level phosphorylation, as it is evident from the increased venous [lactate]. It is well known that ATP generated from glycolysis represents a substantial portion of total energy production under low-flow ischemia (5, 16); this feature was also verified in the same experimental model as that used in this study (45). However, the $O_2$ uptake-to-LVDP-HR ratio returned to near-normal values at the end of reperfusion except for the TG4,000 group. Thus these hearts shifted from carbohydrate to lipid metabolism, a well-known $O_2$ waste effect (32), to a greater extent than those in the other groups. Interestingly, in the TG4,000 + TMZ group, the ratio was normal, supporting the role of TMZ in shift-
ing heart metabolism toward the use of glucose rather than lipids (29).

**TG-associated injury.** The deleterious effect of plasma free fatty acids on myocardial postischemic injury in vivo is well known (34). However, the level of free fatty acids in the perfusion media employed in this study was always <0.2 mmol/l. Because of the relatively high flow, free fatty acids were undetectable in the venous effluent. However, it is likely that TG were in part hydrolyzed in the vascular compartment, with release of fatty acids into the cytoplasm. Measurement of the citrate release rate provided evidence of this mechanism because this rate reflects the mitochondrial efflux of citrate and is an index of the concentration of substrates feeding acetyl-CoA and oxaloacetate for the citrate synthase reaction (50). The essentially linear relationship between the citrate release rate and [TG] supports the view that TG are partly hydrolyzed into fatty acids, and fatty acids are uptaken into the myocytes. Intracellular fatty acids are known to depress myocardial recovery from ischemia (28), possibly through increased β-oxidation and decreased glycolysis and/or glucose oxidation. Measurement of citrate release during reperfusion rules out the potentially masking effects of intracellular citrate concentration peaks that might have occurred during ischemia (24).

The blunted citrate release in TMZ-perfused hearts reinforces the hypothesis that the protection afforded by TMZ is exerted through inhibition of β-oxidation and stimulation of glycolysis and/or glucose oxidation (29). Glycolysis is important in restoring postischemic Ca\(^{2+}\) homeostasis and myocardial function (26), as well as in maintaining membrane integrity (5). This study, however, shows that high TG may impair the postischemic recovery also through increased release of ET-1. Although this hypothesis requires more mechanistic information on how high TG increases ET-1 formation or expression, the present data demonstrates that in this model progressively increased [TG] results in a dose-dependent increase in ET-1 release and that increased ET-1 is highly related to the ischemia-induced performance dysfunction (Fig. 4).

Intralipid is a pool of different types of triglycerides, with fatty acids with variable chain lengths and numbers of cis double bonds. Thus the mechanism of action of TG in the isolated perfused heart requires further work to be elucidated. Previous work (48) pointed out that Intralipid administration during reperfusion is protective, with linoleic acid and phospholipids having complementary actions. However, this situation is different from that in the present study, with Intralipid administered throughout the ischemia-reperfusion.

**Fig. 4.** Correlation between ET-1 release and end-diastolic pressure (A) or the recovery of LVDP-HR (B) at the end of reperfusion. P = 0.0002 and 0.01, respectively.
protocol. Regardless of the involved mechanism, it was already demonstrated (42) that under normal conditions, the contribution of phospholipids, cholesterol esters, monoacyl glycerols, and diacyl glycerols to myocardial oxidative metabolism in the presence of TG is 5% of the total. Although this work deals with ischemia-reperfusion and not normal conditions, we believe that the effects observed here are to be attributed mainly to TG.

**NO.** Decreased NO availability plays a significant role in the reperfusion injury even in the absence of blood components, especially at the level of the diastolic function (35). Indeed, supplementation with sodium nitroprusside during hypoxia improves left ventricle relaxation (14) and NO donors inhibit reoxygenation-induced hypercontracture (46). The present data shows that TG increases NOx release, probably by a mechanism analogous to that described in small rabbit arteries, by which NO-mediated, shear-induced dilatation opposes the vasoconstriction elicited by increased pressure (38). Indeed, ischemia and reperfusion cause injury to the vascular endothelium, expressed as a reduction in NO release (47). However, it appears from the data shown Fig. 3 that the increase in NOx is blunted at [TG] = 2,000 mg/dl, thereby reducing the possible cardioprotective effect of NO, which is restored by 10^{-6} M TMZ. It was shown (31) that at low doses NO may exert a positive inotropic effect on cardiac function, whereas a relaxation-hardening effect of NO becomes apparent while the dose of NO is increased. Therefore, it remains to be established whether the NOx release found in the presence of TMZ falls within the protective NO dose range. If we assume that the release of NOx was constant over time during the 30 min of reperfusion, then the value of 1,200 μmol/l divided by 30 min yields 40 μmol·l^{-1}·min^{-1} release, which is apparently beneficial to protect hearts after 60-min low-flow ischemia.

**TMZ.** We explored the effect of TMZ at concentrations (10^{-6} M) that were previously found efficient with regard to ischemic protection (4). This concentration is within the therapeutic range because it compares with the blood levels obtained in ischemic patients receiving oral treatment (40). In this study, 10^{-6} M TMZ inhibits ET-1 secretion, increases the release of NOx, and reduces the deleterious effect of high TG.

Several hypotheses, not necessarily exclusive, have been proposed to explain the effect of TMZ. First, by inhibiting the activity of 3-ketoacyl CoA thiolase and the flow through the β-oxidation, TMZ increases oxidative glucose metabolism (29) (see TG-associated injury). Second, by sparing energy during ischemia, TMZ preserves the ATP pool (1). Third, TMZ reduces the intracellular acidosis caused by ischemia (39). Fourth, TMZ enhances mitochondrial function (12). Other studies aimed at assessing the effect of TMZ on Na^+-K^+-ATPase (25) and on mitochondrial Ca^{2+} uptake (22) showed that this effect occurs only for TMZ levels much higher than those that protect the myocardium. In the present study, it is difficult to assess whether blunted ET-1 release is a consequence of the TMZ protective effect on myocardium or if TMZ inhibits ET-1 release by protecting the endothelium. However, the observation that TMZ greatly increases NOx release strongly supports the hypothesis that in this model part of the protection is exerted at the level of the endothelium.
the endothelial cells. Indeed, immunocytochemical studies aimed at localizing NO synthase and ET-1 in the coronary vascular bed showed that both occur in the endothelial cells (41). Furthermore, pressure-induced tone is regulated by NO and ET-1 but no interaction between the two factors was evident because they involve different kinds of receptors, i.e., $\alpha_1$- and $\alpha_2$-adrenoceptors (33).

Study limitation and clinical implications. Although we designed this study to mimic the reduction of coronary blood flow that might occur in atherosclerotic coronary vessels of hyperglycemic, hyperinsulinemic, and hyperlipidemic type II diabetic patients during the postprandial period, extrapolation of our data to the clinical situation is to be made with care. First, responses may be different in normal hearts and hearts from diabetic or hyperlipidemic rats. Second, although uncommon, the situation of TG = 4,000 mg/dl may be found in diabetic hyperlipidemic patients in the postprandial period. This situation is worth studying because the link between hypertriglyceridemia and CHD is best seen in the postprandial period, when patients experience exaggerated lipemia, probably related to the delayed clearance of dietary fats (51). The plasma TG level in the postprandial period is positively correlated (3–4 times) with the fasting TG level (20). Furthermore, normalization of plasma TG is markedly delayed in CHD patients because of the presence of gut-derived plasma lipids.

The use of media with increasing Intralipid contents might in principle alter the vascular tone. However, although resistance increased from TG$_0$ to TG$_{1,000}$, it remained constant up to TG$_{4,000}$, suggesting that increased viscosity would not have significantly altered data. Although we cannot rule out the possibility that the presence of TG induces vasodilation, possibly via increased NO production, viscosity may not be a central problem. Indeed, the mean globule size for Intralipid 340 nm (G. Arcuri and F. Kami, unpublished communication), thus in the same order of magnitude of the size of chilomicrons (75–1,000 nm). However, the selected way to report data and evaluate statistics, i.e., by considering [TG] as a continuous variable, takes this issue into account.

In this study, we did not investigate whether the presence of ET-1 receptor antagonists in the perfusion medium is able to reverse the deleterious effects of high TG on postischemic myocardial function. This important issue clearly deserves further work.

In conclusion, high TG progressively impairs the myocardial recovery from low-flow ischemia. The impairment is significantly related to the release of ET-1, which appears to mediate the mechanism leading to injury. TG also increases the release of NO but not sufficiently to protect the heart from reperfusion injury. By further increasing NO release, 1 \mu mol/l TMZ prevents ET-1 release and reverses the harmful myocardial effect of TG. By providing experimental evidence of a link among elevated TG, ET-1, and myocardial ischemia-reperfusion injury, this study supports the epidemiological evidence that suggests that the situation characterized by elevated plasma TG is associated with increased cardiovascular risk independent of factors such as hyperglycemia and elevated plasma cholesterol.

This study was supported in part by the Ministero dell’ Università e della Ricerca Scientifica e Tecnologica Grant “Molecular mechanisms of the protection of the ischemic heart,” in part by Italian Ministry of Health Grant RF99.52 “Invalidant Complications of Diabetes,” and in part by a grant from the Istituto di Ricoercro and Cura a Carattere Scientifico, Hospital San Raffaele.

REFERENCES


