The impairment in endothelial function induced by non-esterified fatty acids can be reversed by insulin

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ABSTRACT

Dyslipidaemia, with elevations of circulating triacylglycerols (triglycerides) and non-esterified (free) fatty acids, and hyperinsulinaemia are often found in the same subjects, the so-called ‘insulin resistance syndrome’. The present study aims to investigate how elevated levels of non-esterified fatty acids, hyperinsulinaemia and the combination of these factors affects endothelium-dependent vasodilatation (EDV). Ten volunteers were examined on two occasions. Intralipid® (plus heparin) or saline (0.9% NaCl) was infused for 4 h. During the final 2 h, euglycaemic hyperinsulinaemia (80 ± 4 m-units/l) was imposed. EDV and endothelium-independent vasodilatation were evaluated in the forearm by local intra-arterial infusion of methacholine or sodium nitroprusside at baseline and after 2 and 4 h. Forearm blood flow was measured by venous occlusion plethysmography. Lipid oxidation was determined by measuring plasma malondialdehyde levels. Infusion of Intralipid® plus heparin increased the concentration of non-esterified fatty acids to 2.6 ± 1.2 mmol/l and decreased EDV from 27.6 ± 8.7 to 21.0 ± 5.7 ml·min⁻¹·100 ml⁻¹ tissue (P < 0.01). This effect was completely reversed by hyperinsulinaemia (P < 0.01). Hyperinsulinaemia alone increased EDV (to 30.4 ± 9.5 ml·min⁻¹·100 ml⁻¹ tissue; P < 0.01), while endothelium-independent vasodilatation was unaltered by the interventions. Infusion of Intralipid® plus heparin increased malondialdehyde levels from 0.67 ± 0.22 to 1.2 ± 0.37 μmol/l (P < 0.001), while hyperinsulinaemia did not change the malondialdehyde level. In conclusion, an acute increase in serum levels of non-esterified fatty acids increased lipid oxidation and decreased EDV. The effect on EDV of non-esterified fatty acids could be reversed by hyperinsulinaemia.

INTRODUCTION

More than 10 years ago it was discovered that the endothelium plays an important role not only in regulating vascular tone, but also in leucocyte adhesion, thrombocyte activation and vascular remodelling, due to secretion of substances such as nitric oxide (NO), prostacyclin and endothelin [1–3]. The endothelium is likely, therefore, to play a central role in the development of cardiovascular diseases associated with atherosclerosis plaque evolution and thrombus formation. Consequently, an understanding of factors governing en-

Key words: blood flow, endothelium, fatty acids, insulin, lipids.
Abbreviations: EIDV, endothelium-independent vasodilatation; EDV, endothelium-dependent vasodilatation; FBF, forearm blood flow; MDA, malondialdehyde; NEFA, non-esterified fatty acids; SNP, sodium nitroprusside.
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endothelial function will be important for the prevention of cardiovascular disorders.

In addition to high levels of insulin being regarded as a cardiovascular risk factor, it has been shown that insulin can induce vasodilatation \[4,5\]. Insulin-mediated vasodilatation is attenuated by concomitant infusion of the \(\text{L-arginine analogue N^g-monomethyl-L-arginine, which}
\]

\(\text{inhibits NO synthesis}\ [6,7\), suggesting that insulin-mediated vasodilatation is endothelium-dependent. Insulin infusion has also been found to enhance methacholine-induced endothelium-dependent vasodilatation (EDV) \[6\], providing further evidence that the vasodilatation evoked by insulin is endothelium-mediated.

Insulin is anti-lipolytic, lowering the serum concentration of non-esterified (free) fatty acids (NEFA), an action that is impaired in insulin-resistant states. Oleic acid has been found to attenuate acetylcholine-induced endothelial-dependent relaxation of precontracted rabbit aortic rings \[8\], and infusion with Intralipid\(^\text{G}\) attenuated EDV in humans \[9\]. Thus a decrease in serum NEFA induced by insulin might also be involved in the potentiation of EDV seen during hyperinsulinaemia.

Dyslipidaemia, with elevated serum NEFA and triacylglycerols, often co-exists with insulin resistance and hyperinsulinaemia in subjects with cardiovascular disorders \[10–13\]. In order to understand in more detail how NEFA and insulin affect EDV under normal conditions and how these factors act together on the endothelium, acute changes in these metabolic variables were induced in young, healthy volunteers. Based on previous data on the action of NEFA and insulin on EDV, we tested whether insulin could counteract the expected negative effect of NEFA on EDV, at least in part. As it has been suggested that the impairment in EDV induced by certain NEFA \(\text{in vitro}\) could be due to lipid oxidation \[8\], we also measured the circulating levels of plasma malondialdehyde (MDA), a marker of lipid oxidation, during the studies.

**MATERIALS AND METHODS**

Ten young, healthy volunteers \(\text{[mean age 23 years (range 20–26 years); six males and four females; mean body mass index 23.2 ± 2.4 kg/m}^2\text{, without any known metabolic or cardiovascular abnormalities, were randomly allocated to either of two investigations with a 2–3-week interval. All studies began in the morning after an overnight fast.}}\)

During the experiments, an intravenous infusion of Intralipid\(^\text{G}\) (200 mg/ml; Pharmacia & Upjohn, Uppsala, Sweden) plus heparin or of saline (0.9% NaCl) was administered for 4 h (Figure 1). During the final 2 h of the infusions a euglycaemic hyperinsulinaemic clamp was added. EDV and endothelium-independent vasodilatation (EIDV) were examined before the start of the infusions and after 2 h and 4 h of infusion. The protocol was repeated after 2–3 weeks, with the exception that saline was given if Intralipid\(^\text{G}\) had been given on the first occasion, and vice versa. Intralipid\(^\text{G}\) was given as a bolus injection of 0.5 ml/kg over 10 min and thereafter as a continuous intravenous infusion at a rate of 90 ml/h. Saline was infused in a similar manner. For the Intralipid\(^\text{G}\) infusions, intermittent injections of heparin (Pharmacia & Upjohn, Uppsala, Sweden) were also given intravenously every 5 min, at a dose corresponding to a rate of 150 units/h.

The euglycaemic hyperinsulinaemic clamp was performed according to DeFronzo et al. \[14\]. After a priming dose given over 10 min, insulin (Actrapid Human\(^\text{G}\) Novo Nordisk, Bagsvaerd, Denmark) was infused at a rate given over 10 min, insulin (Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Human\(^\text{G}\) Actrapid Hum...}]

During the studies, the subjects rested supine in a room at constant temperature (20–22 °C). An arterial catheter (1.0 mm; Ohmeda, Swindon, U.K.) was inserted into the brachial artery for regional infusion of methacholine or sodium nitroprusside (SNP).

Resting forearm blood flow (FBF) was measured before the two separate infusions (methacholine and SNP). Both infusions were given in two dosage steps, of 5 min for each step, with a 20 min wash-out period between the two different vasodilators. The infused doses were 2 and 4 μg/min for methacholine (for evaluation of EDV) and 5 and 10 μg/min for SNP (for evaluation of EIDV). The two infusions were given in a random order and at a rate of 1 ml/min. Previous control studies have shown that these infusion rates do not induce any

**Figure 1** Experimental protocol

The protocol was carried out twice in each individual: once with Intralipid\(^\text{G}\) plus heparin and once with saline. The thin horizontal arrows indicate the different infusions. The vertical arrows indicate the time points at which EDV and EIDV were measured.
alterations in systemic haemodynamics, or in blood flow in the contra-lateral arm [15].

We have recently found, in a pilot study in healthy volunteers, that methacholine (4 µg/min) induced a significant increase in forearm venous plasma nitrate and nitrite concentrations (L. Lind and A. Larsson, unpublished work). When plasma nitrite and nitrate concentrations were measured in both arterial blood and forearm venous blood samples, the forearm release of these two breakdown products of NO increased by more than 8-fold, indicating that the vasodilatory capacity of methacholine is mediated by an increase in NO production.

FBF was measured by venous occlusion plethysmography. A mercury-in-silastic strain gauge was placed on the upper third of the forearm, which rested comfortably slightly above the level of the heart. The strain gauge was coupled to a calibrated plethysmograph. Venous occlusion was achieved by a blood pressure cuff applied proximal to the elbow and inflated to 40 mmHg by a rapid cuff inflator. FBF was calculated from the mean of at least five consecutive recordings.

The reproducibility of this test of EDV and EIDV was evaluated in the present study. Both after 2 h of saline infusion and when repeated after 3 weeks during baseline evaluated in the present study. Both after 2 h of saline infusion with Intralipid* plus heparin induced a significant increase in NO levels (0.43 ± 0.25 to 2.6 ± 1.2 mmol/l (P < 0.001). Serum triacylglycerols increased similarly, while the plasma levels of insulin did not change. Euglycaemic hyperinsulinaemia alone increased plasma insulin to 80 ± 4 m-units/l, while serum NEFA (P < 0.001), but not serum triacylglycerols, were significantly suppressed. The combination of hyperinsulinaemia and Intralipid* infusion did not result in significantly different levels of serum NEFA and triacylglycerols when compared with Intralipid* infusion alone. Similarly, the combination did not produce significantly different levels of plasma insulin compared with those induced by hyperinsulinaemia alone (Table 1).

After 2 h of saline infusion, no significant changes were seen in resting FBF or in the vasodilation produced by methacholine or SNP (Table 2). In contrast, 2 h of infusion of Intralipid* plus heparin induced a significant increase in resting FBF, and also produced a significant decline in the vasodilatation induced by the two different doses of methacholine (P < 0.01), but not in that induced by SNP. Hyperinsulinaemia alone also increased resting FBF, but, in contrast with the response to Intralipid infusion, FBF was significantly enhanced at both doses of methacholine (P < 0.01). Hyperinsulinaemia had no significant effects on the vasodilatation induced by SNP (Table 2).

When infusion of Intralipid* plus heparin was combined with euglycaemic hyperinsulinaemia, the impaired methacholine-induced vasodilatation induced by the acute elevation of NEFA was completely reversed at both doses of methacholine (P < 0.01) (Figure 2). No significant effects on SNP-induced vasodilatation were seen when Intralipid* infusion was combined with hyperinsulinaemia (Table 2).

When blood flow was expressed as the endothelial function index, rather than as absolute FBF values, during methacholine and SNP infusions, no change was induced by saline infusion (from 1.09 ± 0.17 at baseline to

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Table 1 Serum triacylglycerols, serum NEFA and plasma insulin at baseline, during saline infusion and during the different metabolic conditions
Values are means ± S.D. Significance of differences: **P < 0.01 compared with baseline and saline infusion at 120 min; †††P < 0.001 compared with hyperlipidaemia at 120 min.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Time (min)</th>
<th>Serum triacylglycerols (mmol/l)</th>
<th>Serum NEFA (mmol/l)</th>
<th>Plasma insulin (m-units/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0</td>
<td>1.03 ± 0.27</td>
<td>0.43 ± 0.25</td>
<td>9.9 ± 3.8</td>
</tr>
<tr>
<td>Saline</td>
<td>120</td>
<td>0.93 ± 0.29</td>
<td>0.45 ± 0.11</td>
<td>8.6 ± 4.2</td>
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<tr>
<td>Hyperlipidaemia</td>
<td>120</td>
<td>3.9 ± 0.29***</td>
<td>2.6 ± 1.2***</td>
<td>9.6 ± 4.6</td>
</tr>
<tr>
<td>Hyperinsulinaemia</td>
<td>240</td>
<td>0.76 ± 0.28</td>
<td>0.05 ± 0.08***</td>
<td>80 ± 4***</td>
</tr>
<tr>
<td>Hyperlipidaemia + hyperinsulinaemia</td>
<td>240</td>
<td>4.7 ± 2.0***</td>
<td>2.6 ± 1.5***</td>
<td>84 ± 29***†††</td>
</tr>
</tbody>
</table>

Table 2 Resting FBF and vasodilatation during local infusions of methacholine or SNP at baseline, during saline infusion and under the different metabolic conditions
Values are means ± S.D. Significance of differences: **P < 0.01 compared with baseline and saline at 120 min; ††P < 0.01 compared with hyperlipidaemia at 120 min.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Time (min)</th>
<th>Resting</th>
<th>+ Methacholine (2 μg/min)</th>
<th>+ Methacholine (4 μg/min)</th>
<th>+ SNP (5 μg/min)</th>
<th>+ SNP (10 μg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0</td>
<td>3.9 ± 1.1</td>
<td>22.4 ± 7.1</td>
<td>27.6 ± 8.7</td>
<td>18.7 ± 4.2</td>
<td>23.7 ± 5.8</td>
</tr>
<tr>
<td>Saline</td>
<td>120</td>
<td>4.2 ± 1.2</td>
<td>21.9 ± 6.9</td>
<td>27.3 ± 8.4</td>
<td>19.3 ± 4.5</td>
<td>23.5 ± 6.7</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>120</td>
<td>5.7 ± 1.6**</td>
<td>17.3 ± 6.7**</td>
<td>21.0 ± 5.7**</td>
<td>14.9 ± 6.5</td>
<td>24.5 ± 7.9</td>
</tr>
<tr>
<td>Hyperinsulinaemia</td>
<td>240</td>
<td>6.3 ± 1.6**</td>
<td>24.2 ± 7.7**</td>
<td>30.4 ± 9.5**</td>
<td>19.8 ± 5.6</td>
<td>24.7 ± 7.7</td>
</tr>
<tr>
<td>Hyperlipidaemia + hyperinsulinaemia</td>
<td>240</td>
<td>8.4 ± 2.9***</td>
<td>23.9 ± 7.3***</td>
<td>28.9 ± 6.7***</td>
<td>21.4 ± 5.8</td>
<td>26.5 ± 5.0</td>
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</table>

1.10 ± 0.16). Intralipid® plus heparin infusion reduced the endothelial function index (from 1.11 ± 0.14 at baseline to 0.90 ± 0.22; P < 0.05), whereas, in contrast, euglycaemic hyperinsulinaemia increased this index of endothelial vasodilatory function (from 1.10 ± 0.16 to 1.18 ± 0.20; P < 0.05). When euglycaemic hyperinsulinaemia was combined with the Intralipid® plus heparin infusion, an improvement in the endothelial function index was seen compared with that with infusion alone (from 0.90 ± 0.22 to 1.09 ± 0.17; P < 0.05), resulting in the same level as was seen at baseline.

Whereas euglycaemic hyperinsulinaemia alone did not affect plasma levels of MDA, infusion of Intralipid® plus heparin induced a significant rise in MDA levels (P < 0.001) (Figure 3). A tendency for a decrease in plasma
MDA levels was seen when hyperinsulinaemia was combined with Intralipid® plus heparin infusion ($P = 0.07$) (Figure 3). A weak, non-significant relationship was seen between the change in MDA levels induced by Intralipid® plus heparin infusion and the change in methacholine-induced vasodilatation ($r = -0.37$).

**DISCUSSION**

The present study shows that an acute elevation in NEFA and serum triacylglycerols, produced by infusion of Intralipid® plus heparin, lowered EDV but not EIDV, in accordance with a previous study [9]. Furthermore, this effect of an acute elevation of NEFA was accompanied by an increase in plasma MDA levels, and was completely reversed by an elevation in plasma insulin.

An acute rise in circulating NEFA impaired EDV in humans *in vivo*, similar to what was shown previously in the rabbit aorta *in vitro* [8]. There are several steps at which EDV could be influenced by NEFA. First, infusion of Intralipid® lowers the circulating levels of most amino acids, including arginine [17], which may render the substrate for formation of NO less readily available and thereby impair NO production. Impaired EDV due to inhibition of NO synthesis by fatty acids is a second alternative [18].

In both *in vitro* experiments [19] and human studies [20], formation of oxygen free radicals has been linked to exposure to fatty acids. This heterogeneous group of highly reactive compounds interacts with NO and thereby inhibits vasodilatation caused by NO. This is thought to be one mechanism by which oleic acid impairs endothelium-dependent relaxation, as endothelial dysfunction in the presence of oleic acid was reversed by the addition of the oxygen free radical scavenger superoxide dismutase [8]. In the present study, plasma levels of MDA, formed as a product of lipid oxidation, increased substantially during administration of Intralipid®, suggesting that an interaction between NO and oxygen free radicals could be the cause of the impaired EDV. When the change in MDA levels was related to the change in EDV during administration of Intralipid® plus heparin, however, only a non-significant inverse correlation was seen. This might be due to the fact either that lipid oxidation, as measured by MDA levels, only plays a minor role in the impairment of EDV induced by an acute rise in NEFA, or that the sample size of the present study was too small to detect a significant relationship.

EDV is impaired in subjects with hypercholesterolaemia [21,22]. In experimental studies this has been attributed to the presence of oxidized LDL [23,24], and other studies have shown oleic acid to be a major component of the oxidized LDL fraction [8]. Taken together, these results suggest some common mechanisms by which high levels of NEFA and hypercholesterolaemia might impair EDV. It seems unlikely that the elevation in serum triacylglycerols induced by infusion of Intralipid® plus heparin would result in impaired EDV, since infusion with Intralipid® without heparin, which results in an elevation of serum triacylglycerols without a change in NEFA levels, did not impair EDV [9].

The present study corroborates previous findings which showed that hyperinsulinaemia enhanced EDV produced by methacholine or acetylcholine [6]. Furthermore, hyperinsulinaemia reversed the adverse effects of elevated NEFA levels on EDV. This is most likely to be due to a stimulation of NO synthase activity, since the vasodilatory effect of insulin seems to be mediated by NO [6,7]. The possibility that insulin enhances EDV through its anti-lipolytic effect [9] is unlikely, since serum NEFA levels were unaltered when hyperinsulinaemia restored the EDV during Intralipid® infusion. Furthermore, circulating MDA levels were only lowered to a small extent during hyperinsulinaemia, suggesting that the improvement in EDV induced by the addition of insulin was not solely due to a decrease in the formation of oxidation products.

The two metabolic abnormalities (elevated levels of NEFA and hyperinsulinaemia) affected endothelial function in different ways in this sample of young, healthy individuals. Insulin-resistant subjects are also resistant to the vasodilatory action of insulin [25], and in these subjects the effect of NEFA on EDV might be dominant. It remains to be shown, however, how insulin-resistant subjects respond to elevations in NEFA and insulin. Furthermore, in the present study, acute major alterations within the physiological range of NEFA and insulin levels were induced in order to explore the physiological effects of these metabolic conditions on endothelial function. It must be remembered, therefore, that similar effects on endothelial function might not be found in insulin-resistant subjects, in whom more modest and chronic elevations of NEFA and insulin are seen.

In the present study, Intralipid® plus heparin, as well as hyperinsulinaemia alone or in combination with elevated serum NEFA levels, all increased basal FBF. We have data indicating that the evaluation of endothelial function is not related to basal FBF [15]. In the present study the effect of SNP was virtually unaffected by the different metabolic conditions, despite the fact that basal FBF changed. This observation suggests that changes following acute intervention should be evaluated by the absolute levels of FBF and not in relation to basal FBF.

Furthermore, we also used the endothelial function index in the evaluation of the effects of NEFA and insulin. This index was defined as the ratio between FBF during infusion of methacholine and FBF during infusion of SNP. As SNP induces vasodilatation that is independent of the endothelium, this index expresses the contribution of the endothelium to the vasodilatory
process. This index has the advantage that effects on endothelial vasodilatory function could be evaluated independently of changes in basal FBF. Similar conclusions regarding the effects of NEFA and insulin were obtained using this index of endothelial function as when the absolute levels of FBF during methacholine and SNP treatments were used.

In conclusion, the present study shows that an acute elevation of NEFA induced by intravenous infusion of Intralipid® plus heparin decreased EDV and increased lipid oxidation in young, healthy subjects. This effect on EDV was fully reversed by hyperinsulinemia under euglycaemic conditions.

REFERENCES


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