Non-esterified fatty acids impair endothelium-dependent vasodilation in rat mesenteric resistance vessels

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ABSTRACT

Elevated circulating levels of NEFAs (non-esterified fatty acids) are associated with states of insulin resistance and increased risk of vascular disease. Previous animal and human studies have demonstrated NEFA-induced endothelial dysfunction of large conduit arteries, reversible by the antioxidant ascorbic acid. We therefore investigated the effect of NEFAs on carbachol-induced endothelium-dependent vasodilation of rat resistance arteries in vitro using the technique of wire myography. In addition, we investigated the effect of co-incubation of NEFAs and ascorbic acid. Cumulative concentration–response curves to carbachol (endothelium-dependent vasodilation) and SNAP (S-nitroso-N-acetyl-DL-penicillamine; endothelium-independent vasodilation) were constructed. Those to carbachol were repeated following a 30 min incubation with either oleic acid (10⁻⁴ M) or palmitic acid (10⁻⁴ M), demonstrating significant impairment of endothelium-dependent vasodilation with both \( P < 0.05 \) (comparison of pD₂ values (the negative log concentration of agonist required to effect a 50% response)). A cumulative concentration–response curve to carbachol was repeated following co-incubation with palmitic acid (10⁻⁴ M) and the antioxidant ascorbic acid (10⁻⁵ M), demonstrating an abolition of the previously observed endothelial dysfunction induced by palmitic acid. There was no impairment of vasodilation to SNAP following NEFA incubation. We conclude that NEFAs directly impair endothelial function in rat resistance arteries via an increase in oxidative stress at the vascular endothelium.

INTRODUCTION

Impairment of endothelial function is recognized to be a key aetiological factor in the development of atherosclerotic vascular disease [1]. Elevated levels of plasma triacylglycerol (triglyceride) have been shown to correlate independently with subsequent coronary artery disease [2] and, along with high fasting plasma levels of NEFAs (non-esterified fatty acids), are characteristic of conditions associated with states of insulin resistance [3]. In addition, insulin resistance is associated with impaired insulin-mediated postprandial NEFA suppression [4]. Thus insulin-resistant individuals are continually exposed to elevated plasma concentrations of NEFAs, raising the possibility that this may be a factor in the development of endothelial dysfunction observed in these groups.

Previous investigators have demonstrated that fatty acids directly impair endothelial function in a large artery: the rat femoral artery [5,6]. In vivo data from human...
studies have demonstrated that infusions of fatty acids impair endothelium-dependent vasodilation [7], possibly due to a reduction in NO (nitric oxide) bioavailability [8]. Reversal of fatty acid-induced endothelial dysfunction has been demonstrated previously in the presence of the antioxidant ascorbic acid in vivo [9] and in vitro [5]. In addition, endothelial dysfunction associated with both hyperglycaemia and hypercholesterolaemia is reversible with ascorbic acid [10,11]. These in vivo studies utilized the techniques of forearm plethysmography or leg blood flow measurement using thermodilution. Both techniques assume a change in blood flow to represent altered resistance artery tone. These techniques integrate the response of the whole vascular tree within the limb studied and, therefore, the relative influences of large and resistance arteries cannot be elucidated, for which further in vitro investigation is required. Additionally, controversy exists regarding the reproducibility of these techniques [12,13].

Taken together, the available in vivo evidence suggests oxidative stress as an effecter mechanism for the deleterious effect of NEFAs on endothelial function and NO bioavailability; however, the relative effects on large and resistance arteries cannot be determined from these studies. Previous in vitro work has focussed on conduit arteries [5]. The present study was designed to investigate the effect of NEFAs on isolated resistance vessels. Palmitic and oleic acids were chosen as physiologically relevant saturated and monounsaturated fatty acids. Additionally, the effect of co-incubation with ascorbic acid was investigated.

**METHODS**

**Artery dissection and preparation**

All experiments were performed in accordance with U.K. legislation. Male Wistar rats (12-week old) were killed by stunning and immediate cervical dislocation. The ileum along with mesentery and mesenteric vessels were dissected and transferred immediately to ice-cold PSS (physiological saline solution: 118.4 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4·H2O, 1.2 mM KH2PO4, 24.9 mM Na HCO3, 2.5 mM CaCl2, 11.1 mM glucose and 0.023 mM EDTA, giving a pH of 7.4 when gassed with a 5 % CO2/95 % O2 mixture). Resistance arteries were dissected under a dissecting microscope from the proximal part of the ileum, divided into 2-mm segments, and stored in PSS overnight at 4 °C. Studies were performed on a Mulvany–Halpern four-channel wire myograph (Danish MyoTech). At approx. 24 h after dissection, arterial segments were mounted on stainless steel wires in a four-channel myograph with wires attached to a force transducer and micrometer respectively. The temperature was raised to 37 °C, and a gas mixture (5 % CO2/95 % O2) was bubbled in for the duration of the experiment.

**Fatty acid preparation**

Fatty acid solutions were prepared fresh before each experiment. Briefly, the sodium salt of either palmitic or oleic acid (Sigma) was dissolved in 100 % ethanol to give a 10^{-2} M solution. Sonication for 2 min was required to produce complete solution of the fatty acids. A 50 µl aliquot in the 5 ml myograph bath gave a final concentration of 10^{-4} M, with a maximum concentration of ethanol of 1 %.

**Myography protocol**

After a rest period of 30 min, each artery was stretched at 1-min intervals to determine the passive exponential wall tension/internal circumference (L) relationship. According to the Laplace equation (P = T/r, where P is effective pressure, T is wall tension, and r is internal radius), the equivalent circumference (L_{100}) for a transmural pressure of 100 mm Hg was calculated for each vessel using an iterative method. Each vessel was then set to the normalized internal diameter, L_{1} = 0.9 × L_{100}/π, at which contraction is thought to be optimum. After normalization, vessels were left for an additional hour and then exposed to a high concentration (123 mM) of KPSS (potassium PSS; identical with PSS, except with sodium replaced by potassium on an equimolar basis) for a series of 5-min periods until repeatable maximal contractions were achieved.

**Effect of oleic and palmitic acids on carbachol-induced vasodilation**

Two protocols were followed. Arteries were preconstricted with 10^{-7} M U-46619 (a thromboxane A2 mimetic). Concentration–response curves were constructed with carbachol (10^{-9}–10^{-5} M). In one channel, the curve was repeated after 30 min of incubation with oleic acid (10^{-4} M). A second vessel underwent a similar protocol, with construction of an initial carbachol concentration–response curve, followed by a further curve after 30 min incubation with palmitic acid (10^{-4} M). Finally, in each channel, a concentration–response curve was constructed with SNAP (5-nitroso-N-acetyl-DL-penicillamine; 10^{-9}–10^{-5} M).

**Effect of ascorbic acid on palmitic acid-induced impairment of carbachol-induced vasodilation**

A further protocol was undertaken in vessels preconstricted with 10^{-7} M U-46619. Concentration–response curves were constructed with carbachol, and then repeated following 30 min of incubation with palmitic acid (10^{-4} M), as above. A further curve was then constructed following 30 min of co-incubation with palmitic acid (10^{-4} M) and ascorbic acid (10^{-5} M).
Statistical analysis
Results are expressed as means (S.E.M.). Relaxation responses to carbachol are expressed as a percentage relative to the maximum preconstriction to U-46619. For concentration–response curves generated with carbachol and SNAP, pD2 (the negative log concentration of agonist required to effect a 50% response) was calculated. Comparison of pD2 values was made using Student’s t test.

RESULTS

Resistance arteries
The mean (S.E.M.) internal diameter of the 80 dissected resistance arteries was 381 (6.3) \( \mu m \). There were no significant differences in internal diameters of arteries in any experimental group. There were no differences in maximal contractile response to KPSS in either group.

Endothelium-dependent vasodilation

Effect of ethanol (vehicle) on carbachol-induced vasodilation
No significant impairment of endothelium-dependent vasodilation (comparison of pD2 values) of arteries preincubated with ethanol (1% in bath) was detected (Figure 1).

Effect of palmitic and oleic acids on carbachol-induced vasodilation
A significant impairment of endothelium-dependent vasodilation (comparison of pD2 values) of arteries preincubated with either oleic acid (10\(^{-4}\) M; Figure 2) or palmitic acid (10\(^{-4}\) M; Figure 3) was detected.

Effect of palmitic and ascorbic acid co-incubation on carbachol-induced vasodilation
No significant impairment of endothelium-dependent vasodilation (comparison of pD2 values) of arteries co-}

Figure 1 Effect of 30 min preincubation with ethanol (1%) on carbachol-mediated vasodilation
\( n = 6; \ P > 0.05 \).

Figure 2 Effect of 30 min preincubation with oleic acid (10\(^{-4}\) M) on carbachol-induced vasodilation
\( n = 10; \ P < 0.05 \).

Figure 3 Effect of 30 min pre-incubation with palmitic acid (10\(^{-4}\) M) on carbachol-induced vasodilation
\( n = 10; \ P < 0.05 \).

Endothelium-independent vasodilation

Effect of palmitic and oleic acids on SNAP-induced vasodilation
No significant impairment of endothelium-independent vasodilation (comparison of pD2 values) of arteries preincubated with either oleic acid (10\(^{-4}\) M) or palmitic acid (10\(^{-4}\) M) was detected.

DISCUSSION
Previous in vivo studies have demonstrated that NEFAs inhibit endothelial function in large arteries. In vitro studies, investigating the relative effects of NEFAs on large and small vessels have, to date, concentrated on large conduit arteries. Lundman et al. [5] reported a deleterious
Effect of 30 min preincubation with ascorbic acid (10^{-3} M) and palmitic acid (10^{-4} M) on carbachol-induced vasodilation

\[ n = 6; P = \text{not significant.} \]

The relatively short time course over which NEFAs exerted an effect on endothelial function is in keeping with the proposed mechanism via oxidative stress. One potential weakness of our present study lies in the preparation of fatty acids used in the myograph baths. Much previous work using NEFAs has used albumin binding to aid solution. Our use of ethanol as a solvent may have rendered fatty acids less bioavailable, and may partly explain the smaller effect observed in these vessels compared with conduit arteries. There is scope for much further experimental investigation, including investigation of the effect of other fatty acids (e.g. polyunsaturated fatty acids), and additional characterization of the effect on NO bioavailability and ROS generation.

In summary, the present study demonstrates a direct effect of oleic and palmitic acids to impair carbachol-induced endothelium-mediated vasodilation, reversed by co-incubation with the antioxidant ascorbic acid. These data are consistent with a mechanism mediated by increased oxidative stress, possibly via activation of NAD(P)H and consequent induction of ROS production. They are the first in vitro data relating to NEFAs and resistance artery endothelium and suggest that the mechanism underlying endothelial dysfunction in resistance arteries may differ from that identified in larger arteries. This finding is paralleled in other conditions associated with resistance artery endothelial dysfunction, possibly representing a difference in the dominant mechanisms coupling endothelium and smooth muscle in conduit and resistance vessels.
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