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Effect of simvastatin on vascular tone in porcine coronary artery: Potential role of the mitochondria

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ABSTRACT

Statins induce acute vasorelaxation which may contribute to the overall benefits of statins in the treatment of cardiovascular disease. The mechanism underlying this relaxation is unknown. As statins have been shown to alter mitochondrial function, in this study we investigated the role of mitochondria in the relaxation to simvastatin.
Relaxation of porcine coronary artery segments by statins was measured using isolated tissue baths. Mitochondrial activity was determined by measuring changes in rhodamine 123 fluorescence. Changes in intracellular calcium levels were determined in freshly isolated smooth muscle cells with Fluo-4 using standard epifluorescent imaging techniques.
Simvastatin, but not pravastatin, produced a slow relaxation of the coronary artery, which was independent of the endothelium. The relaxation was attenuated by the mitochondrial complex I inhibitor rotenone (10 μM) and the complex III inhibitor myxothiazol (10 μM), or a combination of the two. The complex III inhibitor antimycin A (10 μM) produced a similar time-dependent relaxation of the porcine coronary artery, which was attenuated by rotenone. Changes in rhodamine 123 fluorescence showed that simvastatin (10 μM) depolarized the membrane potential of mitochondria in both isolated mitochondria and intact blood vessels. Simvastatin and antimycin A both inhibited calcium-induced contractions in isolated blood vessels and calcium influx in smooth muscle cells and this inhibition was prevented by rotenone.
In conclusion, simvastatin produces an endothelium-independent relaxation of the porcine coronary artery which is dependent, in part, upon effects on the mitochondria. The effects on the mitochondria may lead to a reduction in calcium influx and hence relaxation of the blood vessel.

1. Introduction

An HMG-CoA reductase inhibitor, or statin, is the drug of first choice for treatment of hypercholesterolemia. Treatment with statins reduces cardiovascular risk and, more specifically, has a beneficial effect on coronary artery disease. However, these beneficial effects cannot be explained fully through reductions in plasma cholesterol levels. A number of studies have demonstrated an effect of statins on vascular tone and these direct effects on vascular smooth muscle tone are thought to underlie some of the improvements in cardiovascular outcomes in patients on statins. A number of different mechanisms have been proposed for the effect of statins on vascular tone. In some blood vessels, statins induce an endothelium-dependent relaxation. For example, simvastatin, atorvastatin, pravastatin and cerivastatin have been shown to exert an endothelium-dependent vasorelaxation, suggested to be mediated by nitric oxide and prostanoids (Alvarez De Sotomayor et al., 2000; Sonmez Uydes-Dogan et al., 2005; Ghaffari et al., 2011). Treatment with statins has also been shown to improve endothelial function in disease states (Dupuis et al., 1999; Alvarez De Sotomayor et al., 2000; Tiefenbacher et al., 2004). In other studies, endothelium-independent relaxations have been described, which may be may be due to inhibition of the mechanism of contraction. For example, lovastatin has been shown to inhibit calcium influx through L-type calcium channels in rat basilar artery (Bergdahl et al., 2003), although it is not clear how this inhibition occurs. Rosuvastatin has been proposed to improve Ca^{2+}-activated K^+ channel function (Miller et al., 2004) and rosuvastatin-induced relaxation of rat aorta may be due to opening of Ca^{2+}-activated K^+ channels (Lopez et al., 2008), which in turn could lead to reduced calcium influx However, in contrast to this, simvastatin has been shown to inhibit Ca^{2+}-activated K^+ channels in coronary

Abbreviations: Rh123, Rhodamine 123; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; ROS, reactive oxygen species; DMSO, dimethylsulphoxide; FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; DETCA, diethylthiocarbamate; TEA, tetraethylammonium; 3-NP, 3-nitropropionic acid; TTFA, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione.

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artery smooth muscle (Seto et al., 2007) which may be responsible for the inhibition of β-adrenoceptor-mediated relaxations that we have observed (Uhiara et al., 2012).

The effects of statins on plasma membrane channels may be indirect through alteration of mitochondrial function. Simvastatin has been shown to cause mitochondrial depolarization, at least in skeletal muscle (Sirvent et al., 2005) and hepatocytes (Abdoli et al., 2013). Similarly fluvastatin has been shown to produce depolarization of the mitochondrial membrane (Zhang et al., 2010). Inhibition of mitochondrial activity could lead to an increase in production of reactive oxygen species (ROS), which can activate Ca^{2+}-activated K^+ channels through generation of short, calcium sparks, leading to vasodilatation (Xi et al., 2005). Inhibition of mitochondrial respiration will also depress intracellular ATP levels and activate ATP-sensitive K^+–channels (K_{ATP}). Indeed, it has been demonstrated that simvastatin increases K_{ATP} channel activity in smooth muscle (Tavackoli et al., 2004; Yang et al., 2007), an action that will cause a relaxation of the smooth muscle. Studies with isolated mitochondria suggest that simvastatin and lovastatin inhibit mitochondrial complexes I, II, III, IV and V, and may even act as respiratory uncouplers. In contrast, pravastatin has no observable effect on any of the complexes (Nadanaciva et al., 2007a; Nadanaciva et al., 2007b).

Multiple mechanisms have been proposed to underlie to the vasodilator effects of statins. These effects may be related to statin-induced inhibition of mitochondrial function. Therefore, the aim of this study was to determine whether simvastatin alters mitochondrial function in vascular smooth muscle cells and whether this plays a role in the vasorelaxation to simvastatin in the porcine coronary artery. The data presented here demonstrate that simvastatin alters mitochondrial membrane potential in porcine coronary segments and this effect on mitochondria may contribute to the vasorelaxation response.

2. Materials and methods

2.1. Isolated tissue bath experiments

Porcine hearts from pigs of both sexes were obtained from a local abattoir and transported back to the laboratory in ice-cold Krebs-Henseleit buffer (NaCl 118, KCl 4.8, $CaCl_2 \cdot H_2O$ 1.3, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$, glucose 11.1 (in mM) gassed with a mixture of 95% O_2 and 5% CO_2) [pH 7.4]. The anterior proximal descending branch of the coronary artery was dissected out, cleaned of fat and connective tissues and set up for isometric tension recording, as previously described (Uhiara et al., 2012). In order to account for variability in responses between animals, internal controls using adjoining segments from each coronary artery were employed in each experiment. Segments were pre-contracted with cumulative additions of U46619 (a thromboxane A_2-mimetic; 20–30 nM) to about 50–70% of a prior response to 60 mM KCl, before addition of a single concentration of simvastatin (30 nM to 10 μM), the sodium salt of simvastatin (Na^+ simvastatin), lovastatin (all 10 μM), or pravastatin (10 μM & 100 μM). The tone was measured for a further 2 h. Control segments contained vehicle only (0.1% v/v DMSO for simvastatin and lovastatin). In separate experiments, the effect of simvastatin after pre-contraction with 60 mM KCl was determined.

In order to determine the role of the endothelium in the statin-induced relaxation, endothelium denudation was achieved by gently rubbing the innermost surface of the artery with a pair of fine forceps. Successful removal of endothelium was confirmed at the end of the experiment by the failure of the tissue to relax to substance P (10 nM). Without denudation relaxation to substance P was 75 ± 10%. With denudation, relaxation to substance P was 0.1 ± 2% (mean + SEM, n = 10).

To determine the role of mitochondria in simvastatin-induced relaxation, segments were incubated for 60 min with 10 μM rotenone, a complex I inhibitor (Michelakis et al., 2002; Gao and Wolin, 2008; Dong et al., 2009), 3-NP and TTFA, complex II inhibitors (both at 10 μM; Drose, 2013), or 10 μM myxothiazol, an inhibitor of complex III (Buyukafsar et al., 2001; Hashitani et al., 2009). The effect of oligomycin (10 μM; Cho et al., 1997), a Fo/F1 ATP synthase inhibitor was also determined. The role of mitochondrial K_{ATP} channel activity was determined by pre-incubating with the mitochondrial K_{ATP} channel inhibitor 5-hydroxydecanoate (1 mM; Jones et al., 2003). After incubation for 1 h with these inhibitors, segments were pre-contracted with U46619, as described above. After the contraction had reached a plateau, 10 μM simvastatin was added and changes in tone measured over 2 h. In some experiments, to evaluate the role of K^+ channels, the segments were incubated with the non-selective K^+ channel blocker tetraethylammonium (TEA; 10 mM), or the K_{ATP} channel blocker glibenclamide (3 μM), or the mitochondrial K_{ATP} channel inhibitor 5-hydroxydecanoate (1 mM; Jones et al., 2003) for 60 min prior to pre-contraction with U46619. The role of AMP kinase was determined by pre-incubation with the AMP kinase inhibitor dorsomorphin (10 μM; Seto et al., 2013).

The role of reactive oxygen species in the relaxation response to statins was investigated by pre-incubation with a combination of ebselen (10 μM), a scavenger of hydrogen peroxide (Cotgreave et al., 1987), PEG-catalase (300 U ml^{-1}; Gao et al., 2009), which metabolises hydrogen peroxide, and PEG-superoxide dismutase (100 U ml^{-1}, Gao et al., 2009), which metabolises superoxide free radicals. In a separate set of experiments, segments were incubated with diethylthiocarbamate (DETCA), which inhibits superoxide dismutase (10 mM; Omar et al., 1991).

2.2. Effect of statins on calcium-induced contractions of the porcine coronary artery

To assess the effect of simvastatin on the influx of calcium through voltage-gated calcium channels, concentration–response curves for $CaCl_2$ in segments of porcine coronary artery were constructed. Segments were incubated for 1 h in Ca^{2+}-free Krebs-Henseleit buffer with simvastatin (10 μM). Segments were then exposed to 60 mM KCl and then increasing concentrations of $CaCl_2$ (1 μM to 3 mM) added to induce a contraction. In another set of experiments, segments were incubated with rotenone or antimycin A (10 μM), in place of simvastatin, or pre-incubated with 10 μM rotenone in combination with 10 μM simvastatin.

2.3. Effect of statins on isolated mitochondria

In order to determine whether statins have a direct effect on mitochondria, mitochondria were isolated from pig hearts as previously described, with modification (Frezza et al., 2007). Hearts are a rich source of mitochondria, therefore the yield of mitochondria obtained from this tissue is relatively high. Heart tissue was homogenized in ice-cold isolation buffer (Mannitol 210 mM, Sucrose 70 mM, EDTA 1 mM, Trizma 50 mM; 1 g tissue per 10 ml isolation buffer) using an ultraturax homogenizer. Homogenate then was centrifuged at 1000 × *g* for 5 min to remove nuclei and non-homogenized material. The supernatant was then centrifuged at 5000 × *g* for 10 min. The resulting supernatant was discarded and the pellet re-suspended in an equal volume of isolation buffer. This was re-centrifuged at 5000 × *g* for 15 min. The resultant pellets were then re-suspended in isolation buffer and kept on ice. Mitochondria were loaded with 200 nM Rhodamine 123 (Rh123) at 37 °C in respiration buffer (100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM Tris, 10 mM KH_2PO_4, lipid-free BSA 1 mg/ml, pH 7.1), prior to addition of simvastatin (10 μM), or DMSO (0.1% v/v). Changes in fluorescence of Rh123, as an indication of changes in mitochondrial membrane potential, were measured at an excitation wavelength of 503 nm, and emission 527 nm using a fluorometer (Hitachi F-2500).