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Fluvastatin, a New Inhibitor of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase, Resists Hydroxyl Radical Generation in the Rat Myocardium

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Abstract

The aim of this study was to determine whether fluvastatin, an inhibitor of low-density lipoprotein (LDL) oxidation, can resist Cu (II)-induced hydroxyl radical generation (·OH) in the extracellular fluid of rat myocardium.

Rats were anaesthetized and sodium salicylate in Ringer's solution (0·5 nmol μL^{-1} min^{-1}) was infused through a microdialysis probe to detect the generation of ·OH as reflected by the non-enzymatic formation of 2,3-dihydroxybenzoic acid (2,3-DHBA) in the myocardium. When Cu (II) (0, 10, 25 or 50 μM) was administered to 1 mM tyramine-pretreated rats, a marked elevation in the levels of 2,3-DHBA was found, indicating a positive linear correlation between Cu (II) and the increase in ·OH formation trapped as 2,3-DHBA in the dialysate ($r^2 = 0{\cdot}977$). In the presence of fluvastatin (100 μM), a marked decrease in the levels of 2,3-DHBA was found. Corresponding experiments performed with iron (II) (0, 10, 25 or 50 μM), showed a marked elevation in the levels of 2,3-DHBA, indicating a positive linear correlation between iron (II) and the increase in ·OH formation trapped as 2,3-DHBA in the dialysate ($r^2 = 0{\cdot}986$). However, in the presence of fluvastatin (100 μM) a small decrease in the level of 2,3-DHBA was found.

The results show that iron (II) against LDL oxidation may be insensitive compared with Cu (II). Cu (II)-induced ·OH formation may be reduced by inhibiting LDL with fluvastatin.

The cholesterol lowering capacity of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, fluvastatin, is well documented (Kurokawa et al 1995; Hussein et al 1997; Leonhardt et al 1997). Oxidative modification of low-density lipoprotein (LDL) is a key event in early atherogenesis, which contributes to cholesterol accumulation in the arterial wall and the development of the atherosclerotic lesion (Aviram 1993; Ross 1993). Oxidation by oxidized LDL is implicated in the pathogenesis of atherosclerosis (Witztum & Steinberg 1991). Fluvastatin is known to be a potent inhibitor of LDL oxidation (Hussein et al 1997; Leonhardt et al 1997). LDL is oxidized by transition metal ion, such as copper or iron (Hussein et al 1997; Belinky et al 1998). Transition metals contribute to the formation of cytotoxic free radicals (Witztum & Steinberg 1991). Oxygen free radicals have been implicated in the mediation of myocardial ischaemia/reperfusion injury (Das et al 1986). Superoxide dismutase (SOD) and catalase were found to moderate the reperfusion injury, presumably by scavenging the free radicals generated in the reperfused heart (Das et al 1987). However, the mechanism of the antioxidative effect of fluvastatin is not clear.

To elucidate the mechanism by which fluvastatin exerts its antioxidative effect, we have studied the protective effect of tyramine-induced hydroxyl radical (·OH) generation in the rat heart. We measured ·OH formation in in-vivo hearts, using a flexibly mounted microdialysis technique that we have developed (Obata et al 1994). Oxygen free radicals are very reactive, and the non-enzymatic ·OH adduct of salicylate, 2,3-dihydroxybenzoic acid (DHBA), provides an assay of ·OH formation both in-vitro and in-vivo (Floyd et al 1984; Halliwell et al 1991). Our interest is in the understanding of the mechanisms by which Cu (II) promotes the oxidative modification of LDL.

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Materials and Methods

Animal preparation

Wistar rats of either sex (300–400 g) were anaesthetized by an intraperitoneal injection of chloral hydrate (400 mg kg^{-1}). After intubation, the rat was mechanically ventilated with room air supplemented with oxygen. The chest was opened at the left fifth intercostal space, and the pericardium was removed to expose the left ventricle. At the end of the experiments the rats were killed by an overdose of anaesthetic. All procedures with the experimental rats met the guideline principles stipulated by the Physiological Society of Japan and the Animal Ethics Committee of the Oita Medical University.

Experimental protocol

Fluvastatin was provided by Tanabe Seiyaku Co. Ltd, Japan. Tyramine and sodium salicylate and its metabolites were purchased from Sigma Chemical Co. (St Louis, MO). The formation of 2,3-DHBA by Cu (II) and iron (II) used $CuSO_4$ (Sigma) and ferrous ammonium salt (Sigma), respectively. These drugs were dissolved in Ringer's solution containing 147 mM NaCl, 2·3 mM $CaCl_2$ and 4 mM KCl; pH 7·4. When a perfusion flow of 1 μL min^{-1} was used, the relative recovery of 10^{-6} M standard solution of 2,3-DHBA was 12·0 ± 0·7%. For trapping ·OH radicals in the myocardium, sodium salicylate in Ringer's solution (0·5 nmol μL^{-1} min^{-1}) was perfused by a micro-injection pump (Carnegie Medicine, CMA/100 Stockholm, Sweden), and the basal level of 2,3-DHBA during a definite time period was determined. Samples (1 μL min^{-1}) were collected after 60 min into small collecting tubes containing 60 μL 0·1 M $HClO_4$.

Analytical procedures

The dialysate samples were immediately injected for analysis into a high-performance liquid chromatographic-electrochemical (HPLC-EC) system equipped with a glassy carbon working electrode (Eicom, Kyoto, Japan) and an analytic reverse-phase column on an Eicompak MA-5ODS column (5 μm 4·6 × 150 mm; EICOM). The working electrode was set at a detector potential of 0·75 V. Each litre of mobile phase contained 1·5 g 1-heptansulphonic acid sodium salt (Sigma), 0·1 g Na_2EDTA, 3 mL triethylamine (Wako Pure Chemical Industries, Japan) and 125 mL acetonitrile (Wako) dissolved in H_2O. The pH of the solution was adjusted to 2·8 with 3 mL phosphoric acid (Wako).

Microdialysis technique

Details of the flexibly mounted microdialysis technique and its application to measure biological substances in the interstitial space have been described previously (Obata et al 1994). Heparin sodium (200 units kg^{-1}) was administered intravenously before probe implantation; 100 units kg^{-1} was then given every hour to prevent blood coagulation. We created a suitable microdialysis probe (Figure 1). The tube of the dialysis probe (approximately 15-cm long) was supported loosely at the mid-point on a rotatable stainless steel wire, so that the movement was totally synchronized with the rapid up-and-down movement of the tip caused by the heart beat. The probe was implanted from the epicardial surface into the left ventricular myocardium to a depth of 3 mm and perfused through the inlet tube. Drugs were administered directly through the fine silica tube (150 μm o.d.). The synchronized movement of the tip of the microdialysis probe with the beating ventricle minimized the tissue injury that would otherwise be caused by friction between the probe and the muscle tissue. The tip of a microdialysis probe (3-mm long, 220-μm o.d. with the distal end closed) was made of dialysis membrane (cellulose hollow fibre, 10-μm thick, 50 000 molecular weight cut-off). Two fine silica tubes (150-μm o.d.) were inserted from the open-end into the tip of the microdialysis tube consisting of a cylinder-shape dialysis membrane, serving as an inlet for the perfusate and an outlet for the dialysate, respectively. The inlet tube was connected to a micro-injection pump (Carnegie Medicine, CMA/100 Stockholm, Sweden), and the outlet tube led to the HPLC pump.

Statistical analysis

All values are presented as means ± s.e.m. The significance of difference was determined by using analysis of variance with Fisher's post-hoc test. $P < 0·05$ was regarded as statistically significant.

Results

When tyramine was infused directly to the rat heart through a microdialysis probe, the levels of 2,3-DHBA were monitored in the dialysate from the myocardium. The level of 2,3-DHBA in the heart dialysate from the control following infusion of sodium salicylate (0·5 nmol μL^{-1} min^{-1}) was 0·033 ± 0·007 μM.

When fluvastatin (100 μM) was administered to tyramine (1 mM)-pretreated rats, the levels of

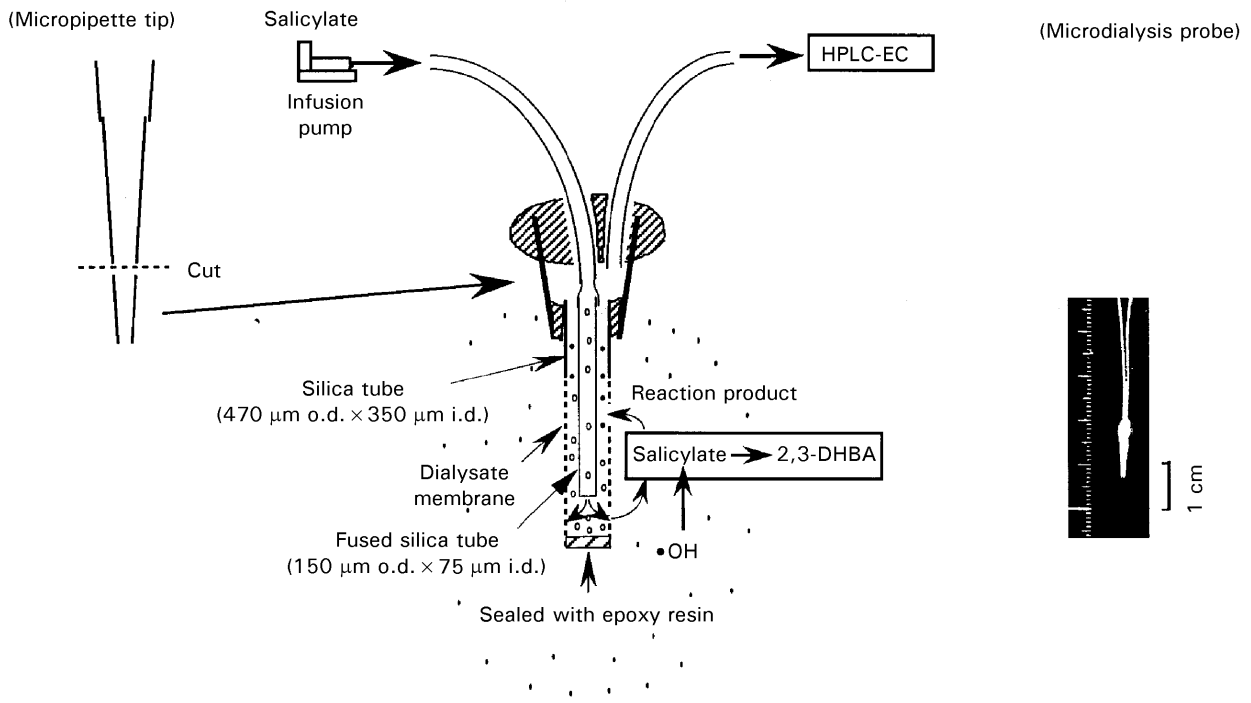

Figure 1. A microdialysis probe was implanted from the epicardial surface into the left ventricular myocardium to a depth of 3 mm and perfused with Ringer's solution by a microinjection pump. The probe (3-mm exposure) was implanted from the epicardial surface into the left ventricular myocardium to a depth of 3 mm. The hydroxyl radical (·OH) reacted with salicylate and generated 2,3-DHBA, which could be measured (pmol) electrochemically by an HPLC-EC procedure.

2,3-DHBA decreased in a time-dependent manner. The levels of 2,3-DHBA at 300–360 min after application of fluvastatin significantly decreased ($n = 6$, $P < 0.05$). To confirm the ·OH generation by Fenton-type reaction, Cu (II) was infused through the microdialysis probe. When Cu (II) (0, 10, 25 or 50 μM) was administered to the tyramine (1 mM)-pretreated animals, a marked elevation in the levels of 2,3-DHBA formation was obtained. This showed a positive linear correlation between Cu (II) and the increase in ·OH formation trapped as 2,3-DHBA ($r^2 = 0.977$) in the dialysate. A marked decrease in the levels of 2,3-DHBA was observed in the presence of fluvastatin (100 μM) (Figure 2). When corresponding experiments were performed with iron (II), a marked elevation in the levels of 2,3-DHBA formation was obtained, indicating a positive linear correlation between iron (II) and the increase in ·OH formation trapped as 2,3-DHBA ($r^2 = 0.986$) in the dialysate. However, a small decrease in the level of 2,3-DHBA products by fluvastatin (100 μM) was observed (Figure 3).

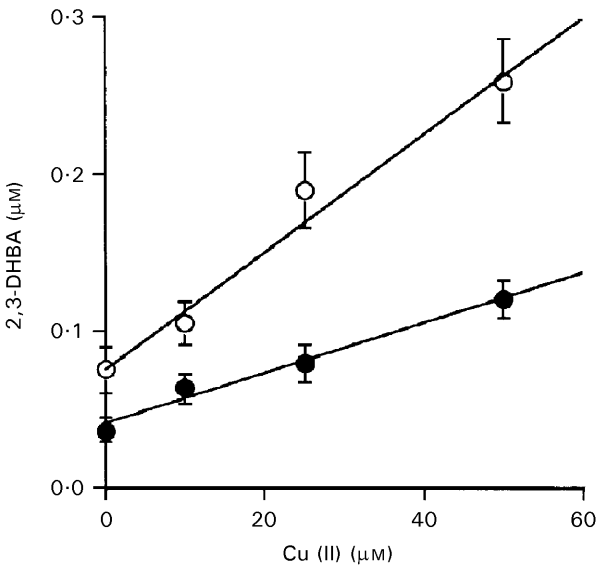

Figure 2. The cumulative dose–response relationship between Cu (II) and ·OH and salicylate in fluvastatin-treated rats. Cu (II) including sodium salicylate (0·5 nmol μL^{-1} min^{-1}) was administered through the dialysis probe. Cu (II) (10, 25 or 50 μM) was infused directly through the dialysis probe in the tyramine (1 mM)-pretreated rats, and the levels of 2,3-DHBA (non-enzymatic ·OH adduct of salicylate produced by tyramine) were measured. The level of 2,3-DHBA in the fluvastatin-treated group (●) was compared with the tyramine-only-treated group (○). Dialysate samples were immediately assayed for 2,3-DHBA by an HPLC-EC procedure. Values are means ± s.e.m., $n = 6$.

Discussion

The results of this study indicate that fluvastatin, an inhibitor of LDL, resisted in the rat myocardium

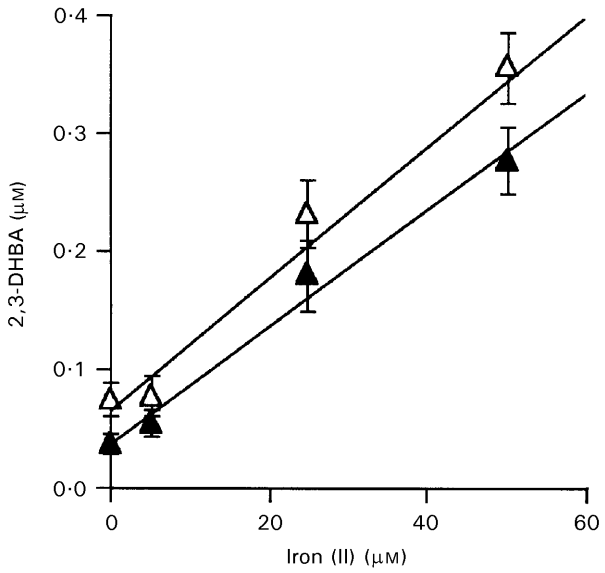

Figure 3. The cumulative dose–response relationship between Cu (II) and ·OH and salicylate in fluvastatin-treated rats. Cu (II) including sodium salicylate (0·5 nmol μL^{-1} min^{-1}) was administered through the dialysis probe. Cu (II) (10, 25 or 50 μM) was infused directly through the dialysis probe in the tyramine (1 mM)-pretreated rats, and the levels of 2,3-DHBA (non-enzymatic ·OH adduct of salicylate produced by tyramine) measured. The level of 2,3-DHBA in the fluvastatin-treated group (▲) was compared with the tyramine-only-treated group (△). Dialysate samples were immediately assayed for 2,3-DHBA by an HPLC-EC procedure. Values are means ± s.e.m., n = 6.

·OH generation. Details of the microdialysis technique requiring the use of a flexibly mounted microdialysis probe in-vivo in rat hearts to measure the biological substance in the interstitial space have been described previously (Obata et al 1994; Obata & Yamanaka 1999). Microdialysis techniques have been used to study neurotransmitter kinetics in the brain (Benveniste 1989), and were recently introduced for in-vivo heart experiments to measure interstitial biological substances, such as catecholamines, ·OH and purine metabolites (Van Wylen et al 1990, 1992; Schulz et al 1995). We measured ·OH in rat hearts by use of a microdialysis technique, which involved the synchronized movement of the tip of the probe with the beating heart to reduce tissue injury (Obata et al 1994). With this technique it was feasible to make stable long-term measurements of ·OH. The concentration profile of the administered compounds in the surrounding interstitial space was unknown; in general, the extracellular concentration of a compound given through the probe would never reach the concentration in the dialysis probe (Benveniste 1989). This is an unavoidable limitation of the microdialysis technique that should be kept in mind when interpreting the experimental data.

Catecholamine stimulation was induced by tyramine, which triggers the release of catecholamine (Downing & Chen 1985). Catecholamine is known to be autoxidized in the presence of oxygen and transition metals (Donaldson et al 1981; Graham 1984; Riederer et al 1989). Exogenous noradrenaline or endogenous release of noradrenaline and/or other catecholamines by tyramine can be autoxidized, which in turn, leads (possibly by an indirect mechanism) to the formation of cytotoxic ·OH free radicals (Obata & Yamanaka 1999). Noradrenaline is known to be autoxidized in the presence of oxygen and transition metals (Graham 1984; Riederer et al 1989). The enzyme xanthine oxidase is also thought to be a source of superoxide anion radical (O_2^-). The O_2^- itself is somewhat poorly reactive in aqueous solution, but does participate in reactions in which iron ions are involved, leading to the generation of more damaging ·OH species. O_2^- has an extremely short half-life and rapidly undergoes dismutation yielding H_2O_2 and then undergoes a Fenton-type reaction in the presence of iron to yield cytotoxic ·OH (Gerlach et al 1994).

When Cu (II) was directly infused in rat myocardium through a microdialysis probe, Cu (II) clearly produced a dose-dependent increase in the level of 2,3-DHBA, as compared with fluvastatin-treated rats. This showed a positive linear correlation between Cu (II) and ·OH formation trapped as 2,3-DHBA in the heart dialysate (Figure 2). When corresponding experiments were performed with iron (II), the results were similar (Figure 3). Cu (II) and iron (II) significantly increased the ·OH formation trapped as 2,3-DHBA in the heart dialysate. However, an increase in 2,3-DHBA level in the absence of Cu (II) or iron (II) treatment was not observed (data not shown). When fluvastatin (100 μM) was administered to tyramine (1 mM)-pretreated rats, the levels of 2,3-DHBA decreased in a time-dependent manner. Oxidative modification of LDL is thought to contribute to the production of oxygen derived-free radicals (Sugawa et al 1997). It is well known that fluvastatin is a more potent inhibitor of LDL oxidation (Hussein et al 1997; Leonhardt et al 1997). When LDL was oxidized to oxidized LDL by Cu (II) (Schnitzer et al 1997), Cu (II) could be reduced further to Cu (I) by LDL oxidation. It is possible that oxidation of Cu (I) to Cu (II) may generate ·OH. Between Cu (I) and Cu (II), H_2O_2 appears to be involved in the production of ·OH (Seacat et al 1997). To confirm the ·OH generation by Fenton-type reaction, Cu (II) was infused through the microdialysis probe. When Cu (II) (0, 10, 25 or 50 μM) was administered to the tyramine (1 mM)-pretreated

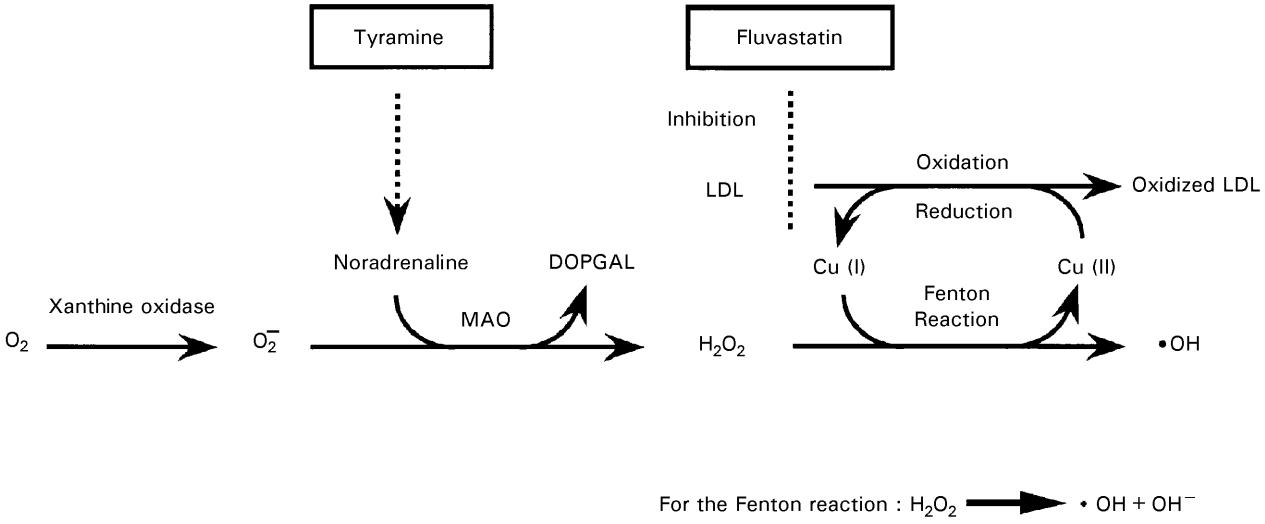

Figure 4. The reaction pathway in rat heart illustrates the formation of hydroxyl radical in the presence of low-density lipoprotein (LDL) and oxygen. MAO, monoamine oxidase; O_2^-, superoxide anion; ·OH, hydroxyl radical; DOPGAL, 3,4-dihydroxyphenylglycolaldehyde.

rats, a marked elevation in the levels of 2,3-DHBA formation was observed, as compared with fluvastatin-treated rats. This showed a positive linear correlation between Cu (II) and produced an increase in ·OH formation trapped as 2,3-DHBA ($r^2 = 0{\cdot}977$) in the dialysate. In the presence of fluvastatin (100 μM), a marked decrease in the levels of 2,3-DHBA was observed (Figure 2). When corresponding experiments were performed with iron (II), a marked elevation in the levels of 2,3-DHBA formation was observed indicating: a positive linear correlation between iron (II) and the increase in ·OH formation trapped as 2,3-DHBA ($r^2 = 0{\cdot}986$) in the dialysate. However, a small decrease in the levels of 2,3-DHBA products by fluvastatin (100 μM) was observed (Figure 3). Therefore, these results suggest that iron (II) against LDL oxidation may be insensitive compared with Cu (II). However, when fluvastatin blocks the LDL oxidation, ·OH may be reduced by the fluvastatin treatment. According to the reaction pathway in Figure 4, these results suggest that Cu (II)-induced ·OH formation may reduce by inhibiting LDL oxidation with fluvastatin. An inhibitory effect on the susceptibility of LDL oxidation can reduce ·OH generation. Fluvastatin can be reduced by LDL oxidation, whereas Cu (II)-induced ·OH formation may be reduced by fluvastatin. This shows that fluvastatin is associated with the generation of ·OH free radicals via the Fenton-type reaction. However, further investigation is necessary to confirm the relation between LDL oxidation and ·OH generation. These findings may be useful in elucidating the actual mechanism of free radical formation in the pathogenesis of neurodegenerative heart disorders, such as myocardial infarction.

Acknowledgements

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